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Ph.D.

Ph.D.

INTERACTIONS OF CORN TISSUES WITH HELMINTHOSPORIUM BLIGHT FUNGI

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This is to certify that the

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INTERACTIONS OF CORN TISSUES WITH
HELMINTHOSPORIUM BLIGHT FUNGI

presented by

Jack C. Comstock

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Botany and Plant
Pathology

Robert L. Scheffer
Major professor

Date October 1, 1971

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ABSTRACT

INTERACTIONS OF CORN TISSUES WITH
HELMINTHOSPORIUM BLIGHT FUNGI

By

Jack C. Comstock

The pathogenicity of certain Helminthosporium species depends on the production of host-specific toxins. The role of one of these toxins, that produced by H. carbonum (cause of a leafspot disease of corn), was studied further in Part 1 of this thesis, with two general objectives: (1) to evaluate the role of toxin in colonization of corn tissue by H. carbonum; and (2) to monitor the exchange of materials between H. carbonum and susceptible and resistant corn. Part 2 of the thesis was a study of the host-specific toxin of H. maydis race T, cause of Southern corn leaf blight.

Conidia of H. carbonum germinated, formed appressoria, and penetrated the cuticles equally on corn leaves that were susceptible, intermediate, or resistant to the fungus. After penetration, H. carbonum grew inter- and intracellularly through the mesophyll tissue on susceptible leaves, and reached the lower epidermis by 24 hr after

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inoculation. Extensive fungal growth and conidiophore development were evident by 48 hr after inoculation. Fungal development in the leaf of a cultivar with an intermediate level of resistance was comparable to that in a susceptible cultivar, but much slower. In contrast, the fungus was confined in resistant leaves to either an epidermal cell or an epidermal cell plus one or two adjacent mesophyll cells. Resistance, expressed as restricted growth of the fungus, was evident 16-20 hr after inoculation.

H. victoriae, a pathogen of oats and a non-producer of toxin affecting corn, did not colonize H. carbonum-susceptible, intermediate, or resistant corn leaves. Hyphal growth of H. victoriae in corn leaves was similar to growth of H. carbonum in resistant leaves. However, H. victoriae invaded and colonized H. carbonum-susceptible corn leaves when the conidia were suspended in 2.0 µg H. carbonum-toxin/ml. Furthermore, hyphae of H. victoriae resumed growth in H. carbonum-susceptible leaves when 20 µg toxin/ml was added to the infection droplet at either 24 or 48 hr after inoculation. H. carbonum-resistant tissue was not colonized by H. victoriae in the presence of H. carbonum-toxin.

Electron microprobe data showed that conidia on susceptible and resistant corn leaves lost Mg and K for 16 hr after inoculation. Conidia on susceptible leaves regained Mg and K between 16 and 36 hr after inoculation;

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this resulted in a net increase in Mg, but not in K. In contrast, the Mg and K content in conidia on resistant leaves remained constant or decreased from 16 to 36 hr after inoculation. During infection, conidia on either susceptible or resistant leaves lost Rb, P and S. The largest loss occurred between 8 and 16 hr after inoculation, during the time that germ tubes penetrated the corn leaf cuticle. Conidia on susceptible leaves did not regain either Rb, P or S for 36 hr.

An increased loss of electrolytes from susceptible leaves was evident 20 hr after inoculation with H. carbonum. This indicated that nutrients were available for the fungus during the time of colonization of susceptible leaves. Electrolyte leakage from resistant leaves was not affected until 30 hr after inoculation, and then the effect was slight. H. victoriae did not affect the electrolyte leakage from either H. carbonum-susceptible or -resistant corn leaves.

H. maydis race T produces a host-specific toxin that affects corn having Texas male sterile cytoplasm. This toxin was found to reach maximum accumulation in culture after 12-15 days in modified Fries' medium supplemented with 0.1% yeast extract. Toxin was partially purified by methods similar to those used to purify H. carbonum-toxin. Activity of H. maydis toxin was assayed by its relative ability to inhibit seedling root growth. Toxin preparations purified

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by gel filtration (Sephadex G10) caused 50 per cent inhibition of root growth of resistant and susceptible corn seedlings at 1360 and 16 µg/ml, respectively. Thus, susceptible corn seedlings with Texas male sterile cytoplasm were at least 80 times more sensitive to toxin than were resistant corn seedlings with normal cytoplasm. However, no differences in tolerance were detected among 45 different lines of resistant corn with normal cytoplasm, or among 17 different lines of susceptible corn with TMS cytoplasm. The tolerance of several non-host species (Raphanus sativus, Hordeum vulgare, Sorghum vulgare var. subglabrescens, Cucumis sativus, Lycopersicon esculentum, and Agrostis sp.) was comparable to that of resistant corn. H. maydis-toxin treatment caused susceptible leaves to leak more electrolytes than controls, or toxin-treated resistant leaves.

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By

Jack C. Comstock

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Botany and Plant Pathology

1971

To My Parents

Mr. and Mrs. Charles Comstock

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To My Uncle and Aunt

Mr. and Mrs. George Comstock

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I sincerely thank my major professor, Dr. R. P. Scheffer, for his guidance and interest during this study. His dedicated assistance in preparing this manuscript has been greatly appreciated. I also wish to thank Drs. J. L. Lockwood, L. R. Krupka, and C. J. Pollard for reviewing this manuscript.

I am grateful for the guidance that the late Dr. E. H. Barnes gave me during the first two years of my graduate training.

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PART 1

COLONIZATION OF CORN LEAVES BY *HELMINTHOSPORIUM CARBONUM*, AND ROLE OF TOXIN IN DISEASE DEVELOPMENT

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INTRODUCTION

Helminthosporium carbonum Ullstrup, the causal organism of Helminthosporium leafspot of corn (Zea mays L.), produces a host-specific toxin (HC-toxin) which is necessary for the pathogenicity of the fungus (38). The toxin causes the same physiological changes in the host tissues that the pathogen is known to cause (21). This has allowed the study of the physiology of diseased tissue in the absence of the pathogen. The effects of toxin treatment on respiration, membrane permeability, amino acid incorporation, and carbon dioxide fixation have been examined (37). The role of HC-toxin in the initial colonization of corn leaves has not been studied. It is often implied that because of toxin the mode of colonization by H. carbonum is not typical of plant diseases in general.

The experiments described herein were designed to provide better understanding of pathogenesis by H. carbonum and disease resistance by corn. Four general areas were examined. First, there was a histological study of invasion and colonization of susceptible and resistant host tissue by H. carbonum. The main question was whether or not tissue colonization by H. carbonum is typical of most plant pathogens. Second, the role of HC-toxin in the initial stages

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of colonization was evaluated. Third, the exchange of certain materials between the pathogen and host were monitored during the infection process. An effort was made to correlate the exchange results with the histological results. Exchange results might also be useful in comparing H. carbonum infection with infection by other fungal pathogens of plants. Fourth, possible reasons for the limitation of the pathogen's growth in resistant tissue were evaluated. The roles of inhibitory compounds and nutrition were considered. Two abstracts describing part of this work have been published (4, 5).

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LITERATURE REVIEW

General background.--In 1941 a disease similar to Southern corn leaf blight was reported on some inbred lines of dent corn (46). The disease was first thought to be caused by Helminthosporium maydis Nisik. and Mke., but the fungus was later found to be a new species which was given the name H. carbonum (47). Two races of H. carbonum were recognized on the basis of host specificity (46). In 1965, Scheffer and Ullstrup demonstrated that a host-specific toxin (HC-toxin) was produced by race 1 of H. carbonum (39). HC-toxin has been crystallized but has not been completely characterized. Results of gel filtration experiments indicate that the molecular weight of the toxin is less than 700. The calculated molecular weight based on the suggested empirical formula ($C_{32}H_{50}N_6O_{10}$) is 679. HC-toxin does not react with ninhydrin but after complete acid hydrolysis four different products react with the reagent. These hydrolytic products are alanine (two molecules), proline, 2-amino-2,3 dehydro-3 methylpentanoic acid, and an unidentified hydroxyamino acid (30, and unpublished data of R. B. Pringle). Since the toxin does not react with ninhydrin, it is believed to be a cyclic peptide. The toxin is relatively stable in the unpurified state but becomes unstable

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in the purified state; inactivation occurs under mild alkaline and strong acid conditions (30).

Resistance to H. carbonum is controlled by a single dominant gene located at the Hm/hm locus on chromosome number 1. Corn lines with an intermediate form of resistance have modifying genes that mask the effect of the double recessive hm/hm (susceptible) condition, or have different alleles at the Hm/hm locus (27). Host resistance or susceptibility is correlated with the host's sensitivity to the toxin produced by the pathogen. For example, root growth of resistant, intermediate, and susceptible corn seedlings was inhibited 0, 56, and 83 per cent respectively by a particular HC-toxin preparation at 1.1 µg/ml (37).

There is a complete correlation between the ability of isolates of H. carbonum to produce HC-toxin and the pathogenicity of the isolate to corn. All isolates with this specific pathogenicity produce toxin; all non-pathogenic isolates failed to produce HC-toxin. A single gene was found to control the qualitative ability of H. carbonum to produce HC-toxin (38). H. (Cochliobolus) carbonum is sexually compatible with H. (Cochliobolus) victoriae Meehan & Murphy, a pathogen of oats (Avena sativa L.) which produces an oat-specific toxin (HV-toxin). Matings of the two fungi produced ascospore progenies which had a 1:1:1:1 ratio of corn toxin producers, oat toxin producers, both toxin producers, and no toxin producers. Pathogenicity

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to both corn and oats was correlated with the ability to produce one or both toxins (38).

The same physiological effects are induced by H. carbonum infection and by HC-toxin. Respiration, electrolyte leakage, and capacity to fix CO_2 in the dark are increased by infection and by HC-toxin (37). Biochemical changes induced by HC-toxin also result in increased nitrate reduction (49), growth (22), uptake of NO_3^-, Na^+, Cl^-, 3-0-methylglucose, and leucine (49), as well as increased incorporation of leucine into trichloroacetic acid-insoluble components of the cell (20).

Histological studies.--Histological studies have shown differences in growth of various fungi in resistant and susceptible tissue within a few hr after inoculation. In the susceptible host, pathogens continue to develop until the life cycle is complete. Normally, susceptible tissue is invaded extensively before visible changes occur; this is considered the period of compatibility between the susceptible host and pathogen. In contrast, growth of the pathogen soon stops or is inhibited in resistant host tissue. A period of compatibility is not detected in the highly resistant host, and a relatively short period of compatibility is evident in the host with an intermediate level of tolerance. The highly resistant host does not allow for completion of the pathogen's life cycle and limited tissue invasion occurs.

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Histological studies of Helminthosporium blights of corn showed that the fungus grew less in resistant than in susceptible hosts (14, 16). H. carbonum and H. maydis invaded and were confined to only a few epidermal and mesophyll cells (16). H. carbonum invasion caused flecks in resistant tissue from which the fungus was recovered 60 days after inoculation (16). H. turcicum invaded xylem tissue, and resistance was expressed as an inhibition of fungal growth. In multigenic resistant corn, H. turcicum sporulated much as it did in susceptible tissue. All three fungi were relatively unrestricted in their colonization and sporulation in susceptible tissue.

H. victoriae penetrated epidermal cells of both resistant and susceptible oat leaves; fungal growth was stopped in resistant tissue within 8-12 hr after inoculation, whereas growth continued in susceptible oat leaves. There was no visible host response which could account for retardation of fungal growth in the resistant host (50).

Growth and colonization in susceptible oat tissue by H. victoriae requires a host-specific toxin. The nonpathogenic mutants of H. victoriae can colonize susceptible oats if HV-toxin is added (50).

Other Helminthosporium infections follow somewhat different patterns. For example, H. gramineum, the cause of barley stripe, has a long period of compatibility in the most resistant varieties available. H. gramineum penetrates

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barley seedlings through the coleorhiza and invades at least two layers of resistant cortical cells. As further fungal development in the resistant barley occurs, the cytoplasm becomes granular and the cells collapse. Both the host and fungal cells in the invaded area die, thus preventing further development of the disease (41). Both H. victoriae and H. gramineum colonize and sporulate on susceptible tissue without evident restriction.

The compatibility time for powdery mildew in wheat and barley differed with the various genes affecting resistance (25, 42). Only the fully susceptible (compatible) host allowed the fungus to develop fully and complete its life cycle. With powdery mildew of wheat (Erysiphe graminis f. sp. tritici) the incompatible or resistant reaction was detected as early as 10-12 hr after inoculation, or at the time of haustorial formation. The fungus was stopped and functional haustoria and elongating secondary hyphae did not form (43). Similarly, incompatibility between barley and E. graminis f. sp. hordei was evident at the time that functional elongating secondary hyphae should be formed (25). Other genes for resistance to powdery mildew of wheat and barley gave incompatible reactions at later times.

Susceptible potato tissue inoculated with Phytophthora infestans had a compatible host-parasite relationship for at least 2 days. In contrast, tissue having R-genes for

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resistance had a hypersensitive reaction within minutes after penetration. As penetration occurred, cytoplasmic streaming in the resistant cell became very rapid, and then stopped. After streaming stopped, the cytoplasm became granulated, with rapid Brownian movement of the particles. Hyphae of P. infestans elongated in the dead cells for 6-7 hr before growth ceased (45).

Exchange of nutrients between host and pathogen.--Logically, chemical substances must be exchanged between the pathogen and its host. A knowledge of differences in a pathogen's chemical exchange with susceptible and resistant tissue should be helpful in understanding pathogenesis and disease resistance. Unfortunately, much of the research has been concerned only with the exchange of materials within the susceptible host. I will discuss briefly the known chemical exchanges in susceptible and resistant host-parasite interactions.

Germinating spores have been shown to lose materials to their external environment. Puccinia graminis tritici lost over half of its 80 per cent alcohol-soluble material to the germination medium in the first hour (8). Materials were transported from the spore via the developing germ tube, and some materials appeared to be lost from the developing hyphae to the environment. Radioactivity of ^{14}C-labeled uredospores was transferred to wheat and seemed to accumulate in areas around the point of contact with the

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host cell wall (9). ^{32}P was transferred from the mycelium of P. graminis avenae to the actively growing hyphae (2). Furthermore, ^{35}S was transferred from uredospores to non-inoculated leaves of inoculated oat plants (17). Labeled spores of Phytophthora infestans germinated and developed an extensive radioactive mycelium in potato leaves (40). The transfer of substances from the pathogen to its host appears to be a general phenomenon.

Movement of nutrients from host to pathogen must occur. The important question, which has not been answered, concerns the time after penetration when materials are transferred from host to pathogen. Furthermore, the transfer of material to and from the pathogen in the resistant host has not been examined, except in the case of powdery mildews (26, 44). E. graminis f. sp. tritici began to incorporate ^{32}P and ^{35}S from the host approximately 2 hr after penetration or 12 hr after inoculation of susceptible wheat. Small haustoria were detectable at that time. By 16 hr after inoculation there was a 2- to 3-fold increase in radioactivity in the fungus. No differences were detected between the kinetics of transfer of ^{32}P and that of ^{35}S from the susceptible host to the powdery mildew fungus (26). Incorporation of materials by the fungus was more pronounced from the compatible plant than from the incompatible plant. The particular gene-pair for incompatibility determined when the reduction of ^{35}S transfer

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would occur. When incompatibility was expressed at the time of haustorial development 12-18 hr after inoculation, ^{35}S transfer was almost completely inhibited at that time. A genotype characterized as having a delayed incompatibility response with inhibited sporulation, did not inhibit but slightly stimulated ^{35}S transfer (44). Differences in the exchange of materials between susceptible and resistant infections have helped determine the time that resistance was expressed.

In many cases, host tissue is known to become leaky as a result of infection. The leakage of electrolytes from the host provides a possible source of materials for the pathogen. Inoculation of susceptible oat leaves with H. victoriae resulted in increased loss of electrolytes in 6 hr (50). HV-toxin caused increased leakage within 2 min after treatment of susceptible oat leaves with toxin. Electrolyte leakage of resistant tissue was not affected by toxin (35). Similarly, treatment of susceptible sorghum leaves with the host-specific toxin produced by Periconia circinata (Mang.) Sacc. resulted in increased loss of electrolytes within 20 min (10). Treatment with HC-toxin resulted in increased loss of electrolytes from susceptible corn within 8 hr (20).

Phytoalexins and disease resistance.--Phytoalexins, fungal inhibitory compounds produced by the host plant as a result of infection, are said to be responsible for disease

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resistance. Several review articles have been written on the subject (6, 7). Inhibitory compounds were induced in alfalfa leaves following invasion by Stemphylium botryosum, Helminthosporium turcicum and Colletotrichum phomoides (11, 12). S. botryosum is pathogenic to alfalfa whereas H. turcicum and C. phomoides are non-pathogens of alfalfa which penetrate but do not colonize (11). The difference between the fungi that colonize and those unable to colonize alfalfa leaves is thought to be the ability to degrade phytoalexin. S. botryosum is said to be able to colonize alfalfa because it can break down the host-produced inhibitory compounds (12, 13).

A phytoalexin produced in soybean tissue is thought to account for resistance to Phytophthora megasperma var. sojae (19). The phytoalexin accumulated in resistant but not in susceptible tissues. The fungus cannot be isolated from resistant plants 96 hr after inoculation (19). Resistance to P. megasperma var. sojae was created by the addition of phytoalexin to susceptible plants (3). Conversely, the resistant plant was made susceptible by removal of phytoalexin (18). Phytoalexin production was induced in plants susceptible to P. megasperma var. sojae by inoculation with P. cactorum, a non-pathogen of soybeans (19). The inoculation resulted in a localized area which was resistant to P. megasperma var. sojae (29).

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Electron microprobe.--The electron microprobe was used to analyze the relative content of certain elements in fungal spores in part of this research. Detectable limits of the electron microprobe are 10^{-15} g of an element. The electron microprobe can detect all elements above beryllium in the periodic table. In the electron microprobe, an electron beam strikes the specimen and each excited atom emits its own characteristic X-ray spectrum. Localized analysis is accomplished by selecting and measuring the intensity of the characteristic X-rays emitted by the desired element with an X-ray detector (1, 33).

The electron microprobe was first used to analyze elements in plant tissues in 1966. The adaptability of this method to plant studies is apparent from the kinds of data that have been published. K, Ca, Sr, Fe, Si, P, and S were detected in corn leaves (23). The distribution and movement of elements have been determined (33). K and P were found to be localized in the cell walls of corn leaves (23). The K^+ ion concentration was found to increase in guard cells as stomata opened, and to decrease as the stomata closed (36). The distribution and mode of entry of Al into corn roots has been determined (32, 34). Al and P were found to be localized together in the cell, probably as aluminum phosphate, thus causing a deficiency of available phosphorus (32).

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MATERIALS AND METHODS

Fungi.--Helminthosporium carbonum and H. victoriae were used in most experiments. H. carbonum isolate A·LN-31 was an ascospore progeny of a cross between H. carbonum and H. victoriae. A·LN-31 produced both HC- and HV-toxins and was pathogenic to both corn and oats. H. victoriae isolate HV-LN produced HV-toxin and was pathogenic to oats. Alternaria cucumerina used in some experiments was obtained from J. Kuć of Purdue University.

Conidia of H. carbonum and H. victoriae were produced according to Lukens' method (24). Shake cultures were grown aseptically for 3 days in 25 ml Fries' medium (31), supplemented with 0.1% yeast extract, in 125 Erlenmeyer flasks. From this point, aseptic conditions were not maintained. The mycelium was collected on cheesecloth and was comminuted for 2 min in a Waring blender. The fragmented mycelium was washed twice by centrifugation. The mycelium was then suspended in 0.02 M phosphate buffer, pH 6.4. A 2 ml aliquot was placed on a dry filter paper in a 9.0 cm petri dish. Sporulation occurred in 3-5 days under continuous fluorescent illumination. Conidia of A. cucumerina were obtained from cultures on vegetable (V-8) juice

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agar exposed to a 12 hr photoperiod at 24 C. Cultures had abundant spores after 10-14 days growth.

Plants.--Near-isogenic corn hybrids, Pr x K61 and Prl x K61, susceptible and resistant, respectively, to H. carbonum race 1, were used in most experiments. Corn inbred P8 was used in some experiments; this line has an intermediate reaction to HC-toxin and to infection (37). Plants were grown at approximately 23 C, with an 18 hr photoperiod, in vermiculite watered with White's nutrient solution (48). The second true leaves of 10-11 day-old plants were used in all experiments.

Inoculation procedure.--Conidia were suspended in water, in 0.1% glucose-peptone solution, or in toxin solution. Small droplets of spore suspensions were placed on the upper epidermal surface of excised leaves (10 or 20 conidia/mm^2) which were held in a chamber at 100% relative humidity. The leaves were examined at intervals after inoculation. Previous work with H. victoriae (50) and oats showed that comparable results were obtained with excised leaves and with leaves on intact plants.

Sectioning and staining.--For histological studies, leaves were sectioned at various times after inoculation with a Hooker (Lab Line) fresh tissue microtome (15). The microtome used a revolving razor blade to cut sections. A carrot stick supporting a leaf was moved into the path of

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the revolving razor blade and sections 20-60 μ were cut. Leaf sections were collected in water and were transferred with a wooden toothpick to a water droplet on a glass slide. Leaf sections were stained with 0.1% cotton blue in lacto-phenol, immediately rinsed with lacto-phenol to remove excess stain, and mounted under a cover slip in lacto-phenol for microscopic observation. Photographs were taken of the sectioned leaves. For clarity, drawings were made from the photographs. All histological examinations were repeated at least 2 times with different sets of leaves.

Electrolyte leakage.--Corn leaves were rinsed for one hour in glass distilled water to remove electrolytes from surfaces. Leaves were then inoculated with conidial suspensions of either H. carbonum or H. victoriae, with final concentration of conidia on the leaf of either 10 or 20/mm^2. Water droplets placed on the leaves served as controls. Replicate samples of 2 or 3 leaves (0.5 g fresh wt) each were used for each treatment. At various times after inoculation, the leaves were rinsed, cut in 1.0-2.0 cm pieces and placed in pre-washed cheesecloth bags. Each cheesecloth bag was subsequently placed in a 300 ml Erlenmeyer flask containing 50 ml glass distilled water. Flasks were incubated on a reciprocal shaker at 100 strokes/min, and the water was changed 4 times before conductivity measurements were started. Conductivity readings of the leachings were started at 12, 18, 24 or 30 hr after inoculation

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and 3 readings were taken at 2-hr intervals. All experiments were repeated one or more times.

Electron microprobe.--H. carbonum and H. victoriae conidia were produced as indicated earlier, except that Fries' medium was supplemented with the elements under study. Three different elements were monitored simultaneously. Fries' medium was modified to contain 4.0 g RbCl and 2.0 g $MgSO_4 \cdot 7H_2O$/l when Rb, S and Mg were monitored. The medium contained 4.0 g KH_2PO_4 and 2.0 g $MgSO_4 \cdot 7H_2O$/l when K, P and Mg were monitored.

Corn leaves were placed in a moist chamber on porcelain plates with the basal cut end in water. Conidia (10-15/mm^2) were spread on the leaves with a clean camel hair brush. Conidial concentrations were determined by microscopic observation of the leaf surface, using reflected light. Spores were removed from susceptible and resistant leaves at various times after inoculation by gently placing a piece of Permacel invisible mending tape on the leaf surface. When the tape was removed the spores adhered to the tape, but no leaf fragments were seen on the tape. The tapes were attached to glass slides with the spores exposed on the upper surface. Slides containing the spores were frozen to stop further development and stored in a desiccator until elemental analysis was made with the electron microprobe.

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An Applied Research Laboratories electron microprobe was used to monitor the relative concentration of certain elements in conidia. Analyses of conidia on resistant and susceptible leaves were made at 0, 8, 16, 24, 36 and 48 hr after inoculation. In some cases, analyses of conidia were not made at all indicated times. For a particular treatment, the same leaf was sampled at the various times, in order to eliminate variation between leaves. Thus the number of times the experiment was replicated was the number of leaf pairs monitored. A leaf pair was one susceptible and one resistant leaf. Fifteen spores were measured for each time period, using two 15-second determinations per spore. The intensity of X-rays of a certain element was expressed as counts/min. Background counts of the tape alone were subtracted from the average counts of each spore. The microprobe operating conditions used were 16.5 KV and 0.025 μA.

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RESULTS

Histological Studies of Corn Leaf Colonization by H. Carbonum and Other Fungi

Development of H. carbonum in susceptible corn leaves.--Conidia of H. carbonum started to germinate on corn leaves 2-3 hr after inoculation, and by 4-5 hr most had germinated. Appressoria were formed, but because of their small size they were not usually observed (Fig. 1). The fungus penetrated the leaf surface either directly or via stomatal openings (Fig. 2). Some hyphae had penetrated into the epidermal layer by 12 hr after inoculation; all the hyphae from germinated spores had penetrated the leaf cuticle and epidermis by 24 hr. At this time some hyphae had grown completely through the leaf to the lower epidermal layer (Fig. 3), although most of the fungal growth was in the upper half of the leaf. The hyphae continued to grow inter- and intracellularly in the mesophyll tissue, but xylem cells were not invaded. By 48 hr after inoculation the leaf was extensively invaded (Fig. 4), conidiophores were evident on the upper leaf surface (Fig. 4), and tissue was beginning to fall apart. However, individual host cells in infected tissue did not differ visibly from healthy cells

FIG. 1.--Direct penetration of a susceptible corn leaf by H. carbonum, 12 hr after inoculation. Spore, germ tube, appressorium and hyphae are shown.

FIG. 2.--Stomatal penetration of a susceptible corn leaf by H. carbonum, 12 hr after inoculation. Spore, germ tube, and hyphae are shown. Stomatal penetration was much less frequent than direct penetration.

FIG. 3.--Development of H. carbonum in a susceptible corn leaf, 24 hr after inoculation. Two spores, germ tubes, and intracellular hyphae are shown.

FIG. 4.--Development of H. carbonum in a susceptible corn leaf, 48 hr after inoculation. Hyphae in the leaf are inter- and intracellular. The bases of two conidiophores are shown on the upper surface of the leaf.

FIG. 5.--Development of H. carbonum in a corn leaf with an intermediate level of resistance, 24 hr after inoculation.

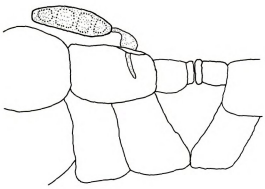


Fig. 1

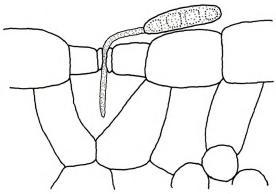


Fig. 2

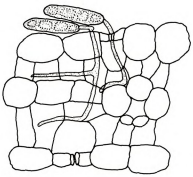


Fig. 3

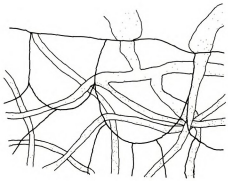
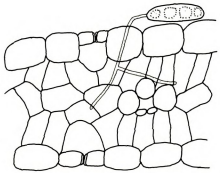


Fig. 4



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in non-inoculated leaves. Inoculation of leaves with either 10 or 20 conidia/mm^2 gave essentially the same amount of invasion per infection site on susceptible leaves (Table 1).

Development of H. carbonum in corn leaves with an intermediate level of resistance.--Conidial germination, appressorial formation, and penetration were similar to those described for the fungus on susceptible corn leaves. Differences between development in intermediate and susceptible tissue became evident after penetration. Fungal growth at 24 hr after inoculation was less extensive and hyphae had not penetrated as far as occurred in susceptible leaves (Fig. 5) at a comparable time. Fungal development in intermediate leaves at 48 hr was comparable to that in susceptible corn at 24 hr (Fig. 3 and Table 2). Relatively few cells were invaded by the fungus, and host cells were not visibly changed. There was no indication that hyphal growth was restricted by host cell wall thickenings. No conidiophores had developed by 48 hr after inoculation.

Development of H. carbonum in resistant corn leaves.--H. carbonum conidia germinated, formed appressoria, and penetrated resistant leaves just as they did with susceptible leaves. By 16-20 hr after inoculation, only one or two resistant epidermal cells were invaded (Fig. 6) whereas 10-20 susceptible cells were invaded. Thus resistance was evident by 16-20 hr after inoculation; it was

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Table 1. Effect of inoculum density and host genotype on colonization of corn leaves by H. carbonum.

Inoculum Conc.	No. of Cells Invaded at 48 Hr.[a]	
	Susceptible	Resistant
10 conidia/mm^2	> 1000	2-4
20 conidia/mm^2	> 1000	4-10

[a]Cells invaded per infection site. Comparable results were obtained in 3 different experiments.

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Table 2. Relative colonization of susceptible, intermediate, and resistant corn leaves by H. carbonum.

Leaf Type	No. of Cells Invaded at[a]		
	12 hr	24 hr	48 hr
Susceptible	1-2	25	> 1000
Intermediate	1-2	8-10	25
Resistant	1-2	2-4	2-4

[a]Cells invaded per infection site. Comparable results were obtained in 3 different experiments.

FIG. 6.--Development of H. carbonum in a resistant corn leaf, 16-20 hr after inoculation. Two spores, germ tubes and intracellular hyphae in two epidermal cells are shown. Inoculum density was 10 conidia/mm^2.

FIG. 7.--Development of H. carbonum in a resistant corn leaf, 48 hr after inoculation. Spore, germ tube and intracellular hyphae are shown. Hyphal growth in this case is limited to an epidermal cell plus and adjacent mesophyll cell. Inoculum density was 10 conidia/mm^2.

FIG. 8.--Development of H. carbonum in a resistant corn leaf, 48 hr after inoculation. Massive hyphal growth is confined to a single epidermal cell. Inoculum density was 10 conidia/mm^2.

FIG. 9.--Development of H. carbonum in a resistant corn leaf, 48 hr after inoculation with 20 conidia/mm^2 leaf surface. Six to 8 host cells are invaded by the fungus.

FIG. 10.--Development of H. victoriae in a resistant corn leaf in the presence of 40.0 µg HC-toxin/ml, 48 hr after inoculation. The hyphae were bulbous in appearance and 8-12 cells were invaded. H. victoriae is pathogenic to oats but not to corn.

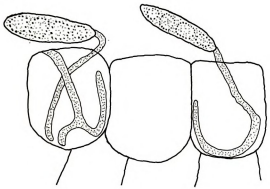


Fig. 6

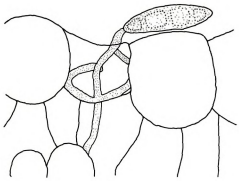


Fig. 7

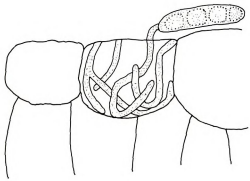


Fig. 8

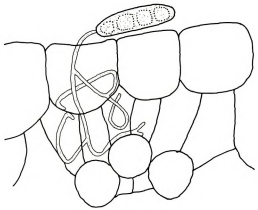
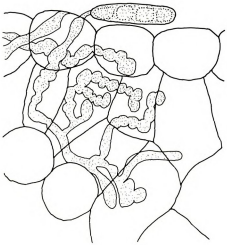


Fig. 9



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expressed as inhibited hyphal growth. There was little or no further invasion of resistant tissue after 16-20 hr. Even at 48 hr after inoculation the fungus was still confined to one or a few cells (Fig. 7). Occasionally, the hyphae continued to grow until the invaded cell or cells appeared to be full of mycelium (Fig. 8). Otherwise, the invaded host cells appeared similar to non-invaded cells. No host cell wall thickenings were observed. Moreover, the confined hyphae in resistant cells did not differ visibly from hyphae in susceptible tissue.

The initial concentration of spores in the inoculum influenced to some extent the number of resistant cells invaded. With 10 conidia/mm^2 leaf surface, only 2-4 cells were invaded. With 20 conidia/mm^2 leaf surface, 4-10 cells were invaded in approximately 25% of the infection sites by 48 hr after inoculation (Fig. 9 and Table 1). Thus the leaf was still resistant to the fungus even when it was inoculated with the higher spore concentration.

Development of H. victoriae in corn leaves.--H. victoriae, a pathogen of oats, made limited growth in corn leaves, and the amount of growth was similar on leaves susceptible, intermediate and resistant to H. carbonum. Conidial germination occurred 4-5 hr after inoculation, penetration occurred by 12 hr, and growth in tissues was restricted by 24 hr after inoculation. Only one or two epidermal cells and/or adjacent mesophyll cells were invaded

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by H. victoriae. Development of the fungus appeared to be similar to that of H. carbonum in resistant corn, as shown in Figure 7. However, the hyphae frequently were thickened and coiled in epidermal cells of corn as illustrated in Figure 8. No conidiophores of H. victoriae were observed on corn leaves.

Effect of HC-toxin on colonization of corn leaves by H. victoriae.--The effect of HC-toxin on tissue colonization was tested by inoculating leaves with H. victoriae, a non-pathogen of corn, in the presence or absence of HC-toxin. Corn lines susceptible, intermediate and resistant to H. carbonum were used. H. victoriae conidia were suspended in HC-toxin or in water prior to inoculation. Several HC-toxin concentrations (0.2, 2.0, 20.0 and 40.0 µg/ml) were tested. Leaf sections were examined at 12, 24 and 48 hr after inoculation.

HC-toxin at 0.2 µg/ml did not affect development of H. victoriae in H. carbonum-susceptible or -resistant corn. The fungus was confined within a few cells, much as H. carbonum is confined in resistant tissue. H. victoriae colonized H. carbonum-susceptible corn in the presence of 2.0 µg HC-toxin/ml (Table 3). The development of the fungus appeared identical to that of H. carbonum on susceptible tissue. The fungus had grown to the lower leaf surface by 24 hr after inoculation. At 48 hr after inoculation, the leaf was overrun with hyphae and a few conidiophores were

Table 3
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Table 3. Effect of HC-toxin at several concentrations on colonization of corn leaves by H. victoriae.

Fungus and Treatment	No. of Cells Invaded at 48 hr.[a]	
	Susceptible	Resistant
H. carbonum (control)	> 1000	2-4
H. victoriae (control)	2-4	2-4
H. victoriae + 0.2 µg HC-toxin/ml	2-4	2-4
H. victoriae + 2.0 µg HC-toxin/ml	> 1000	2-4
H. victoriae + 40.0 µg HC-toxin/ml	> 1000	4-12

[a]Cells invaded per infection site. Comparable results were obtained in 3 different experiments.

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observed. Development of H. victoriae in the presence of HC-toxin at concentrations > 2.0 µg/ml was the same as with 2.0 µg/ml.

Corn tissue which is intermediate in resistance to H. carbonum and to HC-toxin was invaded by H. victoriae when 2.0 µg HC-toxin/ml was present in the inoculation droplet. Development of the fungus in this case was similar to development of the pathogen, H. carbonum, in tissues of the intermediate corn line. Only limited colonization of the intermediate leaf occurred by 48 hr after inoculation as illustrated in Figure 3. Other concentrations of HC-toxin were not tested.

Colonization of resistant corn leaves by H. victoriae was not affected by HC-toxin at 0.2, 2.0, or 40.0 µg/ml. The concentration of HC-toxin used for suspending H. victoriae conidia did, however, have a slight effect on the number of cells invaded (Table 3). The number of cells invaded by H. victoriae in the presence of 2.0 µg HC-toxin/ml did not differ from the controls without toxin. Higher concentrations of HC-toxin (20.0-40.0 µg/ml) allowed H. victoriae to invade a few more cells (Fig. 10 and Table 3). Resistance was still expressed, and the fungus was confined to 4-10 cells, as compared to 2-4 cells in the control without toxin. The hyphae of H. victoriae in H. carbonum-resistant corn cells appeared abnormal in the presence of high concentrations of toxin (Fig. 10). The hyphae varied

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greatly in diameter, were bulbous, and appeared as a string of spherical balls (Fig. 10). This type of hyphae was not observed under any other conditions.

The possibility that inhibitory compounds were responsible for confinement of H. victoriae was investigated. H. carbonum-susceptible and -resistant corn leaves were inoculated with H. victoriae conidia ($10/mm^2$). HC-toxin was then added to the infection site at intervals (0, 24 or 48 hr) after inoculation. When toxin was added to the infection site at 24 or 48 hr after inoculation, the inoculation droplet was first removed and was replaced with a drop containing HC-toxin (2.0 or 20.0 µg/ml). A drop of water without toxin was added to the controls. Microtome sections were cut at 24 and 48 hr after toxin was added, and tissues were observed for fungal development.

H. victoriae failed to resume growth in H. carbonum-susceptible tissue when 2.0 µg HC-toxin/ml was added to the infection drop at either 24 or 48 hr after inoculation. Probably, the toxin was diluted below the critical level by the water in the infection droplet. Toxin-susceptible tissue was colonized by H. victoriae when 20.0 µg HC-toxin/ml was added at 0, 24 or 48 hr after inoculation (Table 4). Forty-eight hr after addition of HC-toxin, all susceptible leaves were similarly colonized and conidiophores were visible, regardless of the time elapsed between inoculation and toxin treatment. If any inhibitory compounds were

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Table 4. Effect of HC-toxin on delayed colonization of corn leaves by H. victoriae.[a]

Time of Toxin Treatment[b]	No. of Cells Invaded at		
	0 hr[c]	24 hr[c]	48 hr[c]
0	0	Ca. 25	> 1000
24	2-4	Ca. 25	> 1000
48	2-4	Ca. 25	> 1000

[a]Cells invaded per infection site. The corn cultivar is susceptible to H. carbonum but not to H. victoriae. Comparable results were obtained in 3 different experiments.

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[c]hr after toxin treatment.

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present, they did not restrict fungal growth after HC-toxin was added. H. victoriae did not colonize H. carbonum-resistant tissue when HC-toxin (20.0 µg/ml) was added at 0, 24 or 48 hr after inoculation.

Effect of nutrients on colonization of corn leaves by H. carbonum and H. victoriae.--H. carbonum conidia were suspended in a 0.1% glucose-peptone solution prior to inoculating corn leaves. Conidia suspended in water served as controls. The amount of fungal growth on the leaf surface was greatly increased when nutrients were supplied. Also, additions of nutrients resulted in two times more fungal penetrations of resistant tissue than occurred without supplemental nutrients. Fungal growth within resistant tissues was increased with nutrient supplements, but growth was still restricted as compared to that in susceptible tissues without supplements. The hyphal growth was confined to the upper half of the resistant leaf by 48 hr after inoculation.

H. victoriae, a pathogen of oats, invaded both H. carbonum-susceptible and -resistant corn leaves when inoculating conidia were suspended in a 0.1% glucose-peptone solution. H. victoriae hyphal invasion was extensive by 48 hr after inoculation but conidiophores were seldom observed. The hyphal growth in the leaves appeared similar to either H. carbonum or H. victoriae plus HC-toxin on susceptible corn leaves. Comparable results were obtained in 3 different experiments.

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Effect of inoculation with H. victoriae on subsequent colonization of corn leaves by H. carbonum.--Corn leaves inoculated with H. victoriae were challenge inoculated 24 or 48 hr later with H. carbonum. Control leaves were inoculated with only H. carbonum conidia. Leaf sections were processed and examined 48 hr after the challenge inoculation with H. carbonum. The experiment was repeated 3 times.

Results showed that H. carbonum invaded susceptible leaves that had been inoculated 24 or 48 hr previously with H. victoriae. H. carbonum growth in such leaves was similar to the growth in leaves not previously inoculated with H. victoriae. Results of this experiment do not support the hypothesis that phytoalexins formed in resistant tissue (6, 7) are responsible for cessation of fungal growth.

There were similar experiments with Alternaria cucumerina. At 24 or 48 hr after inoculation with A. cucumerina, leaves were challenge inoculated with H. carbonum. Results showed that prior inoculation with A. cucumerina had no influence on the development of H. carbonum. However, A. cucumerina failed to penetrate the corn leaf cuticle.

Experiments with the Electron Microprobe

Logically, materials must be transported back and forth between the host plant and its pathogen. If materials are less available to the pathogen on the resistant host, this should be evident at some time after initial invasion. Precise measurement of such movements in vivo have not been

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possible to date. However, the recent development of the electron microprobe offers some hope that meaningful measurements can be made. Accordingly, the microprobe was used to monitor certain elements in the conidia during infection, with the aim of obtaining data on the exchange of materials between H. carbonum and resistant or susceptible plants. An attempt was made to relate exchange of materials between host and pathogen to events in the colonization process.

Relative content of Mg in conidia of H. carbonum on corn leaves, at intervals after inoculation.--The electron microprobe data showed that H. carbonum conidia lost Mg during germination (0 to 8 hr after inoculation) (Fig. 11). Further rapid loss of Mg occurred during the penetration phase (8-16 hr after inoculation). Thus by 16 hr after inoculation conidia had lost over 50 per cent of their Mg (Fig. 11 and Table 5). Conidia on susceptible and resistant leaves lost Mg similarly up to 16 hr after inoculation. The Mg content on different susceptible leaves varied, but beginning at 16 hr it increased (Table 5). The average Mg content in conidia on susceptible leaves at 16 and 36 hr after inoculation was 44% and 81%, respectively, of the original content at 0 hr. By 36 hr, the hyphae had made extensive growth in susceptible leaves. In contrast, the Mg content in conidia on resistant leaves remained constant, or more was lost, between 16-36 hr after inoculation (Table 5). The relative Mg concentration in the conidia on

FIG.

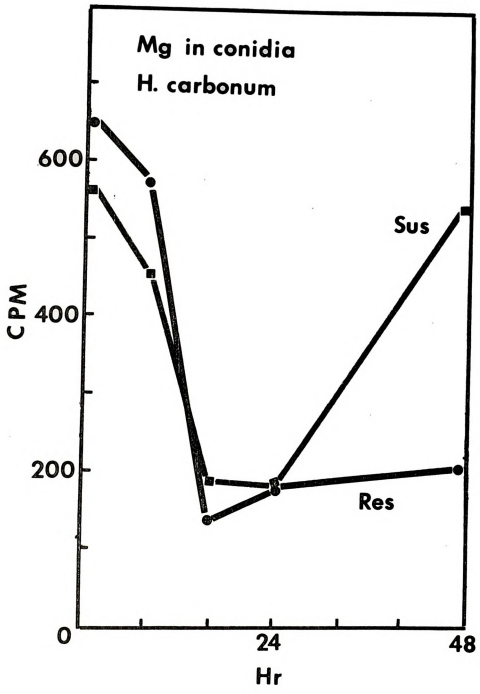

FIG. 11.--Relative content of magnesium in conidia of H. carbonum on resistant and susceptible corn leaves, at time intervals after inoculation. CPM refers to counts per minute as determined with the electron microprobe.

Table 5.
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Leaf pair no.	
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[b]48 hr

Table 5. Relative content of magnesium in conidia of H. carbonum on resistant and susceptible corn leaves, at time intervals after inoculation.

Leaf pair no.	CPM for Mg in Spores[a]					
	On susceptible leaves			On resistant leaves		
	Hr after inoculation			Hr after inoculation		
	0	16	36	0	16	36
1	201	137	208	203	68	18
2	316	163	168	258	41	12
3	271	130	207	296	50	35
4	166	36	140[b]	145	49	52[b]
5	469	170	242	340	155	177
6	393	102	671	386	121	223
7	219	142	179	244	80	34
8	278	158	66	278	173	11
$\bar{x}$	289	130	235	269	92	70

[a]CPM refers to counts per minute as determined with the electron microprobe.

[b]48 hr after inoculation.

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resistant leaves was only 25 per cent of the original value at 36 hr after inoculation, even though the hyphae were confined within 1 or 2 cells. Thus the exchange of Mg between host and pathogen differed on susceptible and resistant tissue at the time resistance was evident in the histological observations.

Relative content of K in conidia of H. carbonum on corn leaves, at intervals after inoculation.--The relative potassium concentration was monitored in H. carbonum conidia on 4 different leaf pairs during infection. The data of a representative leaf pair are shown in Table 6. There was a rapid loss of K from H. carbonum conidia during germination and penetration, from 0 to 16 hr after inoculation. The K content in conidia on resistant and susceptible leaves, respectively, was 30 per cent and 70 per cent of the original K content at 16 hr after inoculation. Essentially all the K was lost from conidia on resistant leaves by 36 hr after inoculation. In contrast, conidia on susceptible leaves still contained 30 per cent of their original K content. The K and Mg data are similar in that the exchange of Mg and K differed between susceptible and resistant tissues at the time resistance was evident.

Relative content of Rb in conidia of H. carbonum on corn leaves, at intervals after inoculation.--H. carbonum conidia labeled with Rb were obtained by growing the fungus

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Table 6. Relative content of potassium in conidia of H. carbonum on resistant and susceptible corn leaves, at time intervals after inoculation.

Plant Type	Hr after Inoculation		
	0	16	36
	CPM[a]	CPM[a]	CPM[a]
Susceptible	4876	3544	1592
Resistant	5188	1592	28

[a]CPM refers to the counts per minute as determined with the electron microprobe.

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in Fries' medium supplemented with RbCl. The Rb content in conidia on 4 different corn leaf pairs was monitored during colonization. Results from a representative leaf pair are shown in Figure 12. H. carbonum conidia lost Rb during the germination process, 0-8 hr after inoculation. During penetration (8 to 16 hr after inoculation), the Rb content in conidia fell from 550 CPM to less than 100 CPM. After 16 hr, the Rb content remained essentially the same. The transfer of Rb from conidia to resistant and to susceptible leaves was comparable throughout the experiment. Since corn leaves did not contain Rb, the element was not available for movement from host to pathogen. Since Rb and K can substitute for each other, the differences between the exchange of Rb and K suggest that K was transferred to the conidia on susceptible but not on resistant leaves. The data with K and Mg indicate that conidia can regain certain elements from the susceptible host at a relatively early time during infection.

Relative content of P and S in conidia of H. carbonum on corn leaves, at intervals after inoculation.--H. carbonum conidia on 4 leaf pairs were simultaneously monitored for P and K. Conidia lost P rapidly during germination and penetration of leaves. Approximately 50-60 per cent of the original P content was lost from the conidia by 16 hr after inoculation (Table 7). The conidia continued to lose P and by 36 hr after inoculation the relative content was very low.

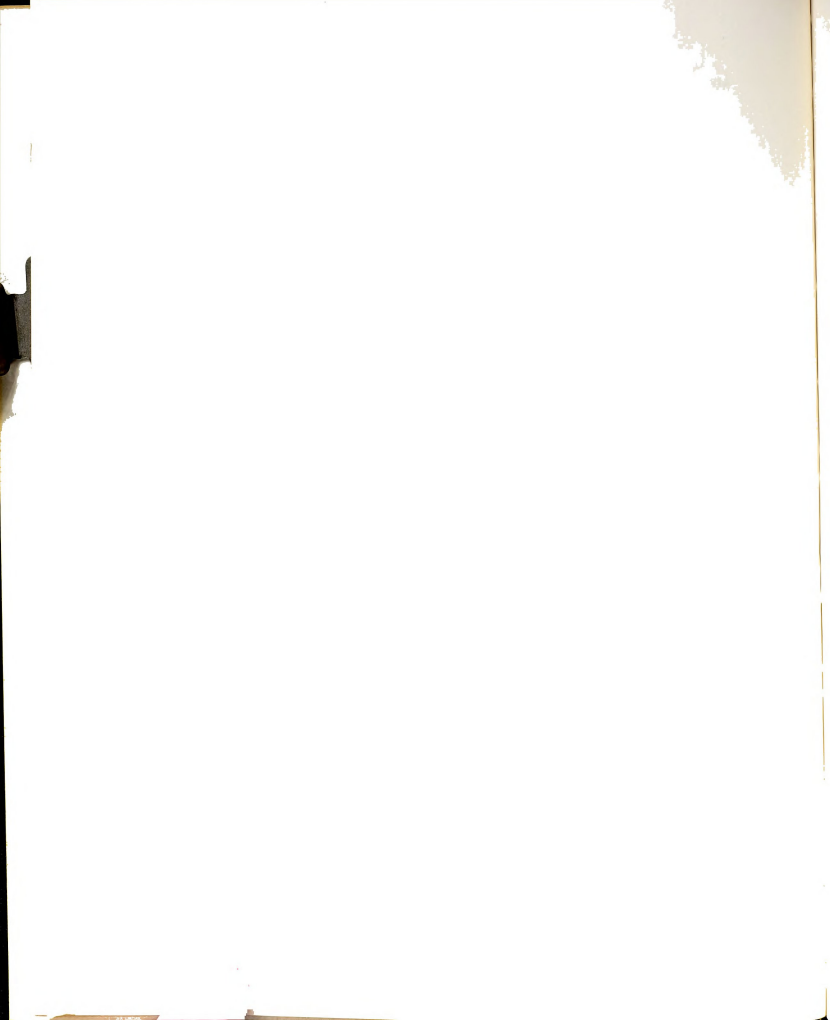

FIG. 12.

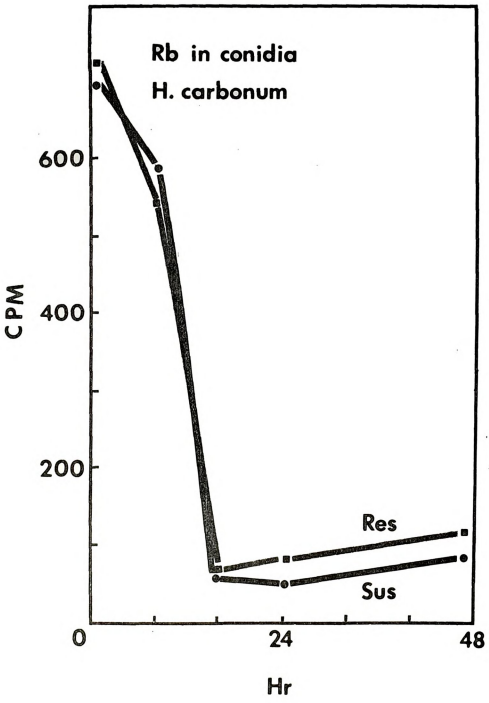

FIG. 12.--Relative content of rubidium in conidia of H. carbonum on resistant and susceptible corn leaves, at time intervals after inoculation. CPM refers to counts per minute as determined with the electron microprobe.

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Table 7. Relative content of phosphorus in conidia of H. carbonum on susceptible and resistant corn leaves, at time intervals after inoculation.

Plant Type	Hr after inoculation		
	0	16	36
	CPM[a]	CPM[a]	CPM[a]
Susceptible	7764	3764	280
Resistant	7492	3404	276

[a]CPM refers to counts per minute as determined with the electron microprobe.

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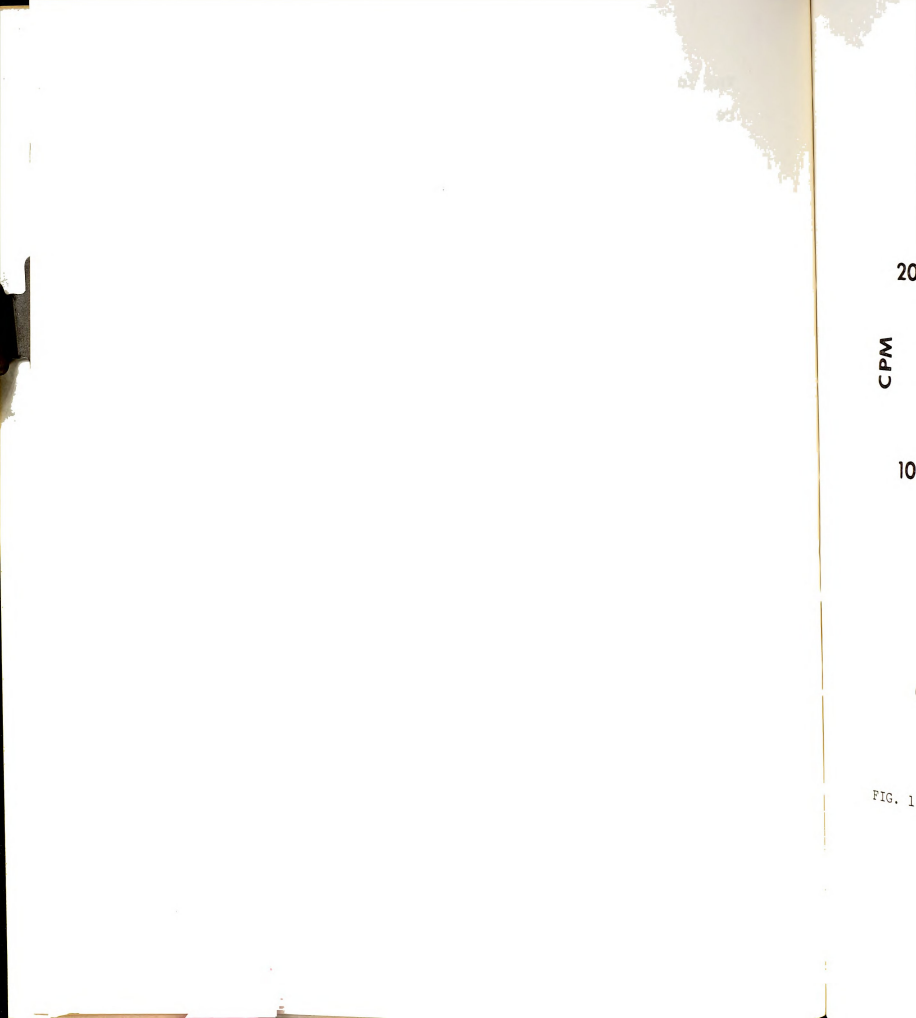
The loss of P from conidia was the same on susceptible and resistant leaves. P was not regained by conidia on susceptible leaves, thus differing from the cases of Mg and K.

H. carbonum conidia on 4 leaf pairs were monitored for S. A representative curve of the results is shown in Figure 13. The concentration of sulfur in the conidia was low; only 250 CPM were detected at the time of inoculation. The conidia lost S from 0 to 16 hr after inoculation. After 16 hr, the relative concentration of sulfur remained the same until the end of the experiment. No differences were detected in S exchange between conidia on susceptible and on resistant leaves.

H. carbonum conidia lost at least part of all the elements that were monitored during the germination and penetration phases. The elements were assumed to be either translocated to the actively growing hyphae or were simply lost from the fungus to the host tissue. The elements fell into two different categories: Mg and K which appeared to be regained by the germinated conidia on susceptible but not on resistant leaves; and P and S which appeared to be lost and not regained by the conidia on both resistant and susceptible corn leaves.

Relative content of various elements in conidia of H. victoriae on corn leaves, at intervals after inoculation.-- H. victoriae conidia on one leaf pair was monitored for Mg, Rb and S contents. The loss of the 3 elements from H.



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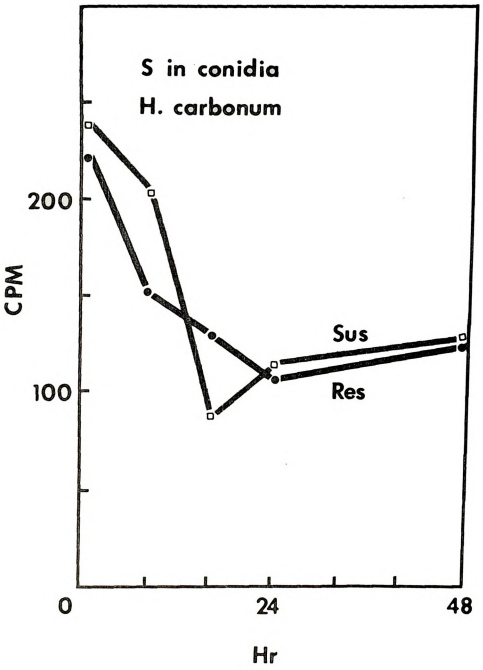

FIG. 13.--Relative content of sulfur in conidia of H. carbonum on resistant and susceptible corn leaves, at time intervals after inoculation. CPM refers to counts per minute as determined with the electron microprobe.

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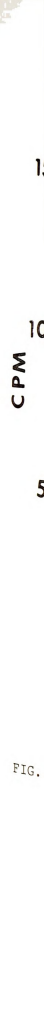
victoriae conidia on corn leaves during infection was similar to the losses from H. carbonum conidia on resistant tissue. Mg was lost from the conidia of H. victoriae during penetration (Fig. 14). Mg was not regained by the conidia on either H. carbonum-susceptible or -resistant corn leaves.

Effect of Infection on Loss of Electrolytes from Corn Leaves

HC-toxin treatment is known from previous work to cause a delayed loss of electrolytes from susceptible corn leaves (20). Therefore, measurements were made on loss of electrolytes from corn leaves inoculated with H. carbonum and H. victoriae. Inoculated leaves were maintained in a moist chamber (100% relative humidity). Two hr before leaching was to begin, leaves were cut into pieces, placed in cheesecloth bags, and rinsed with glass distilled water. Conductivity readings were started on different samples at 12, 18, 24, 30, and 36 hr. At each starting time, conductivity of the ambient solution was measured 3 times at 2-hr intervals. Results showed no detectable loss of electrolytes from susceptible corn leaves until 20 hr after inoculation with H. carbonum (Fig. 15). Increased leakage at this time was only evident in leaves inoculated with 20 H. carbonum conidia/mm^2 leaf surface. Electrolyte leakage from infected susceptible leaves was 4 times the amount from non-inoculated control leaves by 24 hr after



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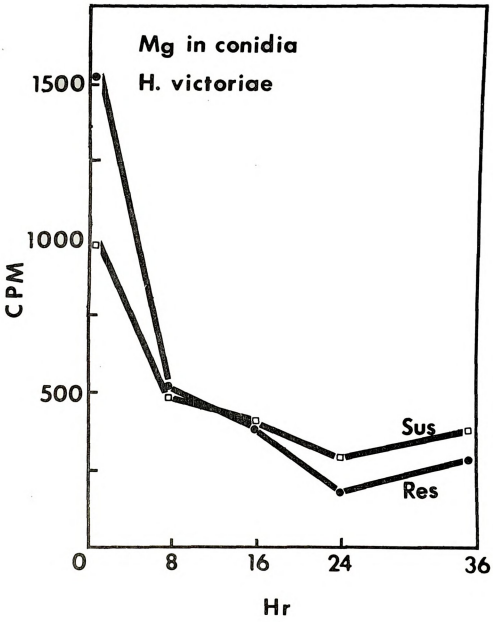

FIG. 14.--Relative content of magnesium in conidia of H. victoriae on H. carbonum-resistant and -susceptible corn leaves, at time intervals after inoculation. CPM refers to counts per minute as determined with the electron microprobe.

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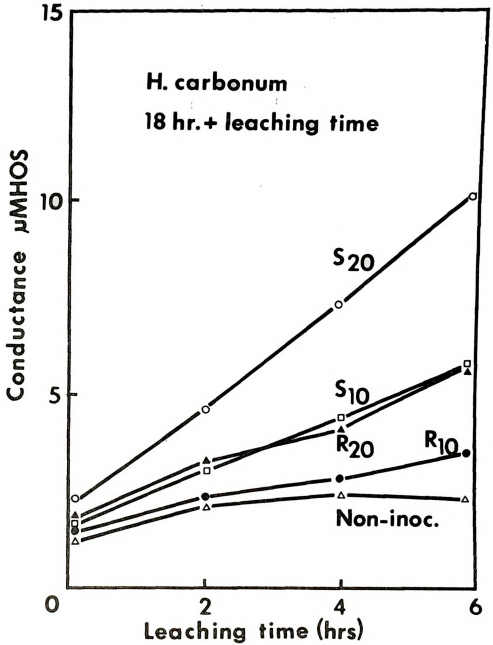

FIG. 15.--Effect of H. carbonum infection on loss of electrolytes from susceptible and resistant corn leaves, 18 hr after inoculation. The leaching times are in addition to an 18 hr incubation time prior to the start of conductivity measurements. S_{10} and S_{20} = susceptible leaves inoculated with 10 and 20 conidia/mm^2 leaf surface; R_{10} and R_{20} = resistant leaves inoculated with 10 and 20 conidia/mm^2 leaf surface; non-inoc. = non-inoculated susceptible and resistant controls.

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inoculation (Fig. 15). Susceptible leaves inoculated with 10 conidia/mm^2 leaf surface had an increased electrolyte leakage which was first detected 26-27 hr after inoculation (Fig. 16).

Resistant leaves inoculated with H. carbonum had slightly more leakage of electrolytes than did control leaves by 30 hr after inoculation with 20 H. carbonum conidia/mm^2 leaf surface. Less response occurred when resistant leaves were inoculated with 10 conidia/mm^2 leaf surface.

H. victoriae did not induce leakage of electrolytes from H. carbonum-susceptible or -resistant corn leaves. Even at 36 hr after inoculation with 20 conidia/mm^2, the leakage of electrolytes did not differ from that of non-inoculated controls.

Conductance, μMHOS

FIG.

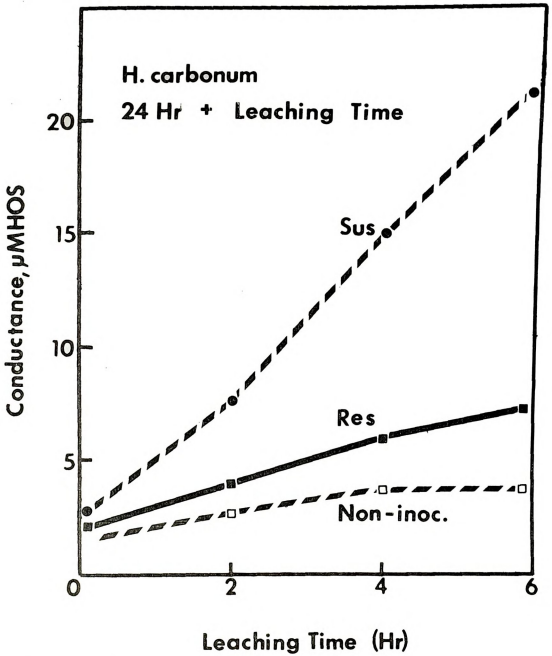

FIG. 16.--Effect of H. carbonum infection on loss of electrolytes from susceptible and resistant corn leaves, 24 hr after inoculation. All conductivity measurements were started 24 hr after inoculation. Sus = susceptible leaves inoculated with 10 conidia/mm^2 leaf surface; Res = resistant leaves inoculated with 10 conidia/mm^2 leaf surface; non-inoc. = non-inoculated susceptible and resistant controls.

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DISCUSSION

The data presented here, along with genetic (38) and other kinds of evidence, show that HC-toxin is required for colonization of susceptible corn by H. carbonum. H. victoriae, a pathogen of oats, invaded and colonized H. carbonum-susceptible corn leaves when HC-toxin was present; the non-pathogen of corn was not capable of colonizing corn leaves without supplemental HC-toxin. All isolates of H. carbonum which produced HC-toxin were pathogenic and all isolates which did not produce the toxin were non-pathogenic to corn leaves (38). Leaves resistant to HC-toxin were not colonized by either H. carbonum or by H. victoriae in the presence of HC-toxin. Leaves intermediate in resistance to H. carbonum were invaded to a limited degree by H. victoriae when sufficient HC-toxin was present in the infection drop. Resistance to the fungus was determined by the hosts' resistance to the host-specific toxin.

A basic question concerns the role of HC-toxin in colonization of susceptible tissue by H. carbonum. The question can be answered in part by determining what occurs during colonization, what limits invasion by a non-pathogen, and the critical times in the development of infection that may require the presence of toxin. These points are

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discussed below in relation to the role of HC-toxin in pathogenesis.

HC-toxin was not required for penetration of corn leaves by H. victoriae, and presumably by H. carbonum. Furthermore, H. victoriae colonized susceptible tissue when HC-toxin was added to the site of infection 24 or 48 hr after inoculation. Without HC-toxin, the growth of H. victoriae ceased by 16-20 hr after inoculation. Therefore, HC-toxin was required for some process that occurred after penetration was complete.

Susceptible and resistant tissue differed in their exchange of materials with H. carbonum. H. carbonum conidia on susceptible leaves had a net gain in Mg 24 to 48 hr after inoculation. Indirect evidence indicated that K was transported to the conidia from the susceptible tissue although there was not a net increase in K. The movement of materials from the susceptible host to the fungus was first detected between 16 and 24 hr after inoculation. An increased loss of electrolytes from susceptible leaves was evident at 24 hr after inoculation, or shortly after the critical time when toxin is thought to be necessary for further development of the fungus. The loss of electrolytes indicated materials should be available to the fungus for growth and metabolism. In contrast, conidia on resistant leaves failed to regain Mg and K and continued to lose P and S. Furthermore,

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electrolyte leakage from resistant leaves was slight and was not detectable until 30 hr after inoculation.

Hyphal growth by H. victoriae was confined to one or a few cells in H. carbonum-susceptible corn, prior to addition of HC-toxin. Host-produced phytoalexins have been postulated as the factors that restrict fungal growth in tissues (6, 7). If this was the case in corn, the inhibitory compounds should have continued to retard the growth of H. victoriae after HC-toxin was added. Since this did not happen, phytoalexins did not appear to limit fungal growth in corn leaves. Furthermore, phytoalexins were shown not to be responsible for disease resistance in the H. victoriae-oat system (28), and HV-toxin did not affect the formation or concentration of phytoalexin in oats. Phytoalexins were produced too late to retard the fungus and production occurred in both susceptible and resistant oats. Obviously some change in characteristics of the host cell occurred in response to toxin, making the tissue susceptible to H. victoriae.

It may be argued that H. carbonum can live as a saprophyte, because HC-toxin can kill susceptible corn cells in advance of colonization. However, susceptible tissue is actually stimulated by low concentrations of HC-toxin. Growth (22), respiration (21), ion-uptake (49) and other processes of the susceptible cell are stimulated by HC-toxin (49). The data indicate that toxin triggers changes in host

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metabolism during infection. Tissue death appears to be the end result of infection or treatment with toxin, and not a prerequisite for invasion. Furthermore, the histological evidence is that H. carbonum does not kill cells in advance of the invading hyphae.

The host is the only source of nutrients for a parasite such as H. carbonum after the spore reserves are exhausted. An increase in hyphal mass, logically, means an incorporation of materials from the host. H. carbonum hyphal growth is extensive in susceptible leaves, indicating a high amount of incorporation of host nutrients. In contrast, the development of H. carbonum in resistant tissue is limited, indicating a lack of incorporation of host nutrients. HC-toxin appears to have a role in making nutrients available, since H. victoriae (as pathogen of oats) colonizes susceptible corn leaves in the presence of HC-toxin. Thus nutrient deficiency for the invading fungus might be considered a contributing factor for failure of a non-pathogen to colonize corn tissue.

Increased electrolyte leakage is a general phenomenon during infection. H. victoriae and H. carbonum infections both cause an increased loss of electrolytes in their susceptible oat and corn leaf tissue. A non-pathogenic isolate of H. victoriae does not cause an increased leakage of electrolytes from susceptible oat leaves (50). Likewise, H. victoriae, a non-producer of HC-toxin, does not cause an

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increased leakage of electrolytes from H. carbonum-susceptible corn leaves. The pathogen appears to require the appropriate host-specific toxin to cause an increased loss of electrolytes from susceptible tissue.

Exogenously-supplied nutrients stimulated development of H. victoriae in corn leaves. H. victoriae was able to colonize corn leaves (resistant and susceptible to H. carbonum) when conidia were suspended in a 0.1% glucose-peptone solution. The data support the idea that nutrients can limit fungal growth in the resistant host. Furthermore, the data indicate that HC-toxin causes nutrients to be released from susceptible tissue. Tissue insensitive to HC-toxin was not affected by H. carbonum or H. victoriae in the presence of HC-toxin, suggesting no new nutrients are made available to the fungus.

Certain other observations do not appear to be compatible with the hypothesis that lack of available nutrients limits growth in resistant tissue. H. carbonum conidia germinated and invaded susceptible corn whether or not 0.1% glucose-peptone was present at the site of penetration. On resistant tissue, more surface growth occurred and there were more penetrations and more extensive invasion of leaves when H. carbonum was supplemented with these nutrients. Still, the hyphal invasion was not as extensive in resistant as in susceptible tissue and the fungus was restricted in growth. The nutrients functioned to increase the inoculum

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potential and caused some increase in the extent of invasion of resistant tissue by H. carbonum. Possibly, the nutrient supplement was inadequate for H. carbonum, or the fungus grew beyond the area supplied by the exogenous nutrients. Equally possible is that the host cells were damaged by the peptone and were not as able to prevent fungal invasion.

Results with Puccinia graminis f. sp. avenae do not support the hypothesis that nutrient deficiency prevents growth in resistant tissue (2). When susceptible leaves serve as a food base, the fungus is able to grow through a thin piece of agar and infect another susceptible leaf. However, the fungus was unable to infect a resistant leaf whether or not a susceptible leaf served as a food base. Nutrients must have been transported through the hyphal bridge in the agar from the susceptible leaf to the fungus as it attempted to invade the resistant leaf. A deficiency of nutrients did not appear to be responsible for the restriction of fungal growth in resistant tissue.

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Part 2

SOME CHARACTERISTICS OF A HOST-SPECIFIC TOXIN FROM *HELMINTHOSPORIUM MAYDIS* RACE T

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INTRODUCTION

Preliminary evidence indicates that Helminthosporium maydis race T, the causal organism of Southern corn leaf blight, produces a host-specific toxin (7). Southern corn leaf blight has many similarities with Victoria blight of oats and Helminthosporium leafspot of corn, both of which have causal fungi which produce host-specific toxins. All 3 of these diseases were first detected in new lines resulting from breeding programs, or became prevalent as a result of the introduction of new cultivars. All three causal fungi produce blight-type symptoms, with necrosis spreading from the site of infection. Resistant and susceptible plants are distinctly different in reactions to the causal fungi. H. maydis, for example, causes large spreading lesions on susceptible leaves; on resistant leaves, it causes small lesions with brown or tan centers, surrounded by reddish-brown zones with chlorotic margins (7).

The first objective of this research was to confirm whether or not a host-specific toxin is produced by H. maydis race T. The second objective was to develop methods of producing and partially purifying the toxin (HM-toxin). The third objective was to determine some effects of the toxin on plant tissue. Finally, toxin was to be evaluated for its

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possible use in breeding for disease resistance. The work of Scheffer and his associates with H. victoriae, H. carbonum, and Periconia circinata-toxins served as a guide (15). An abstract describing part of the work has been published (1).

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LITERATURE REVIEW

In 1925, Drechsler first described a leaf disease of corn caused by a Helminthosporium sp. that differed from H. turcicum Pass. (2, 3). The organism, later named Helminthosporium maydis Nisik. and Miyake, is the incitant of Southern corn leaf blight (11). In the original reports, the leaf symptoms were described as small lesions with a tan or buff necrotic center surrounded by a red-brown margin (2, 3).

A new race of H. maydis was first reported in 1961 when Philippine workers reported differences in susceptibility of corn with normal and Texas male sterile cytoplasm (9). The new race of H. maydis was first detected in the United States in 1969 (19). Thus, it became evident that H. maydis was composed of at least 2 physiologic races: race O which was equally virulent on corn with either normal or Texas male sterile cytoplasm (TMS), and the new race T which was extremely virulent on corn with TMS or P (Brazilian type) cytoplasm (21). Corn with normal cytoplasm, and corn with S or C cytoplasm for male sterility were resistant to H. maydis race T (7). Most of the corn planted in the United States in 1970 carried TMS cytoplasm. During the summer of 1970, the corn belt suffered extensively from an

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epiphytotic of Southern corn leaf blight, which caused an estimated 12% reduction in the nation's corn yield (23).

Most attempts to demonstrate host-specific toxins have either failed or met with limited success. Relatively large quantities of host-specific toxins have been obtained from cultures of H. victoriae, H. carbonum and Periconia circinata, but special conditions and media were required (15). H. maydis race O was reported to produce only trace amounts of toxin (20).

Mitochondria from susceptible (TMS) corn were found to be extremely sensitive to the toxin from H. maydis race T. Mitochondria from resistant corn were not affected (10, and unpublished data of M. A. Bednarski). The toxin caused susceptible mitochondria to swell at an accelerated rate in a KCl medium. The respiratory rate of the susceptible mitochondria was either increased or decreased as a result of toxin treatment, depending upon the substrate. Furthermore, phosphorylation and P:O ratios were reduced for susceptible but not for resistant mitochondria as a result of toxin treatment (10). The chloroplasts of both susceptible and resistant corn were not affected by toxin treatment (unpublished data of M. A. Bednarski). Possible effects of toxin on other organelles have not been reported to date. The data show that mitochondria contain a sensitive site for HM-toxin.

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There have been limited practical applications of host-specific toxins in plant breeding programs. Wheeler (24) exposed freshly-germinated oat seedlings of susceptible cultivars to H. victoriae toxin. There were very few survivors, which were either toxin-resistant mutants or individuals that escaped toxin treatment. Several hundred resistant oat mutants have been detected by toxin treatment in massive screening programs. These included natural mutants and mutants induced by ionizing radiation and chemical mutagens. Other workers (18) have shown that Periconia circinata-toxin can be used in breeding sorghum for resistance to P. circinata. Resistance to this fungus is also controlled by a single gene locus. Since the tendency for natural mutations was high at this locus, massive screening programs using PC-toxin have been successful (18). Finally, the host-selective toxin of H. sacchari has been used successfully to detect and screen for resistance to the fungus in sugarcane. The host-specific toxin of H. sacchari was injected into leaves; induction of a streak indicated susceptibility to toxin and to the fungus (22), whereas little or no necrosis was induced in H. sacchari-resistant sugarcane.

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MATERIALS AND METHODS

Toxin bioassay.--Inhibition of seedling root growth was used as a toxin bioassay; the procedure was similar to the bioassay for HC-toxin (16). The per cent inhibition of seedling root growth was determined at various dilutions of culture filtrates or toxin preparations. The dilution end-point was defined as that dilution of the toxin preparation which gave 50% inhibition of seedling root growth. Corresponding near-isogenic corn lines with TMS (W64ATMS or WF9TMS) or normal (W64A or WF9) cytoplasm were used. They were respectively, susceptible and resistant to H. maydis race T (21). Toxin was also tested against several non-host plants (Raphanus sativus, Hordeum vulgare, Sorghum vulgare var. subglabrescens, Cucumis sativus, Lycopersicon esculentum, and Agrostis sp.) for ability to inhibit root growth.

Toxin preparation.--Partial purification procedures for H. maydis toxin, similar to methods used in isolation of HC-toxin (13), are outlined in Figure 17. H. maydis race T was grown in Roux bottles containing 200 ml modified Fries' medium supplemented with 0.1% yeast extract (12). Twelve day-old cultures were filtered to remove

FIG.

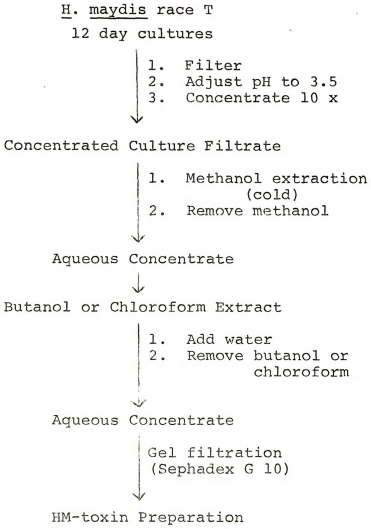

FIG. 17. Procedure for partial purification of HM-toxin.

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mycelia. Culture filtrates were concentrated under vacuum to less than 1/10 their original volume. Two volumes of methanol were added to the concentrated culture filtrates and stored overnight at 4 C. A white precipitate was removed by filtration through Whatman no. 1 filter paper. Methanol was removed under vacuum. The remaining aqueous preparation was extracted 4-5 times with equal volumes of butanol. In some cases, chloroform rather than butanol was used for this step. The butanol or chloroform extracts were combined; water was added, and the preparation was concentrated in vacuo. This procedure was repeated until all butanol or chloroform was removed. The aqueous preparation was stored at -20 C for later use or for further purification.

The aqueous preparation was further purified by successive gel filtrations through two columns of Sephadex G10. Sephadex beads were soaked overnight and washed in several changes of water. The first column contained 11 g hydrated beads and measured 1.5 x 15 cm; the second column contained 17 g beads and measured 1.5 x 24 cm. The water level of the first column was lowered to the top of the bed and 3 ml of an aqueous toxin preparation was carefully added to the top of the bed. The toxin sample was drained into the bed and the column was developed with water at a constant flow rate of 1 ml water/1.5 min. The first 5 ml of effluent was discarded and the next 15 ml, containing the toxin, was concentrated under vacuum to 3 ml and

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chromatographed through the second column. The second column was developed with water with a constant flow rate of 1 ml/8.3 min. Six ml fractions were collected and tested for toxin activity by the seedling root growth bioassay.

Other procedures used in experiments with HM-toxin were described in Part 1 of this thesis.

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RESULTS

Toxin production by several isolates of H. maydis race T.--Thirty-seven different isolates of Helminthosporium, presumably H. maydis, were screened for production of host-selective toxin. Thirty-four isolates were obtained from infected corn leaves from southwestern Michigan and from the Michigan State University campus. The remaining 2 isolates, previously identified as H. maydis race T (Indiana T and Kentucky T), were obtained from Dr. A. J. Ullstrup. An authentic race of H. maydis race O was also obtained from Dr. Ullstrup. The isolates were grown in 125 ml Erlenmeyer flasks containing 25 ml Fries' medium supplemented with 0.1% yeast extract (12). Filtrates from 3 flasks of 18-day-old cultures were combined and assayed for their ability to inhibit seedling root growth by W64ATMS (susceptible) and WF9 (resistant) corn.

The 37 isolates varied in their production of host-specific toxin (Table 8). No host-selective toxin was detected in culture filtrates from 14 isolates. The root growth of susceptible and resistant corn seedlings was equally inhibited. However, the culture filtrates of the remaining 23 isolates had host-selective toxicity. There was a 5-fold difference between susceptible and resistant

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Table 8. Relative toxicity and specificity of culture filtrates of several isolates of H. maydis.

No. of isolates	Dilution factor[a] for corn lines		Relative specificity of filtrates[b]
	W64ATMS	WF9	
10[c]	12	12	1
3	24	24	1
1	60	60	1
11	24	12	2
2	60	24	2.5
9[d]	60	12	5
1	300	24	12.5

[a]Values indicate maximum dilution factor of crude culture filtrate that gave 50 per cent inhibition of seedling root growth.

[b]Values indicate the quotient resulting from dividing the dilution factors of W64ATMS (susceptible) and WF9 (resistant).

[c]Including a known isolate of H. maydis race O.

[d]Including the isolate "Kentucky T."

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corn seedlings in sensitivity to the culture filtrates of 22 isolates. These isolates were low producers of toxin under the conditions used. One isolate, Indiana T, obviously gave a relatively high yield of host-specific toxin because resistant corn seedlings tolerated a 12.5-fold higher concentration of filtrate than did susceptible seedlings. Therefore, this isolate was used to produce HM-toxin for further experiments. No host-specific toxicity was evident in culture filtrates of the isolate of H. maydis race O.

Effect of medium and age of culture on toxin production by H. maydis race T.--Toxin production by H. maydis race T was determined using the following media: Czapek's (14), Hooker's (7), Richard's (14), Fries' plus 0.1% yeast extract (12), and Fries' plus 2.0% yeast extract (20). The fungus was grown in 25 ml medium/125 Erlenmeyer flask in still culture at 24 C, with one exception. Cultures in Hooker's medium contained 100 ml/300 ml Erlenmeyer flask, and were incubated on a reciprocal shaker. Culture filtrates from 3 flasks were combined and bioassayed for selective toxicity at 12, 15, and 18 days after inoculation. The highest HM-toxin concentration occurred in Fries' medium plus 0.1% yeast extract at 12-15 days after inoculation (Table 9). The experiment was not repeated.

Cultures were harvested at various ages to determine when peak toxin accumulation occurred in Fries' medium plus 0.1% yeast extract (12). The fungus was grown in still

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Table 9. Effect of culture medium on production of HM-toxin.

Medium	Dilution factor[a] at:		
	12 days	15 days	18 days
Czapek's	50	200	25
Richard's	50	50	50
Hooker's (shake culture)	25	25	12.5
Fries' + 0.1% yeast extract	400	400	200
Fries' + 2.0% yeast extract	100	100	100

[a]All values are maximum dilution factors of filtrates that gave 50% inhibition of susceptible seedling root growth. Twelve, 15, and 18 days refers to age of the cultures at time of harvest.

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culture (25 ml medium/125 ml Erlenmeyer flask) at 24° C. Culture filtrates from 3 flasks were combined for each assay. Harvests were made at 3-day intervals from 6 to 30 days after inoculation. Again, peak toxin activity was obtained in 12- to 15-day-old cultures. These filtrates gave 50 per cent inhibition of susceptible corn root growth when diluted 300 times, whereas resistant corn tolerated only a 12-fold dilution. The experiment was repeated.

Characteristics of HM-toxin.--Toxin was shown to be soluble in methanol, N-butanol, chloroform, and water (Figure 17), but not in diethyl ether. These solubilities were determined by extracting an aqueous toxin preparation 3 times, using equal volumes of organic solvent and aqueous extract each time. The organic solvent extracts were combined, water was added, and the organic solvent removed under vacuum. Seedling bioassays were used to compare the relative toxicity of each extract with the toxicity present in the original solution.

A 0.1 ml sample of the final aqueous toxin preparation was spotted on Whatman no. 1 paper and chromatographed by the descending method. Propanol-acetic acid-water (200:3:100 V/V) and butanol-acetic acid-water (4:1:5, upper layer), were used as solvent systems. The solvent front migrated approximately 12 inches. The paper was air dried and cut into one-inch segments. Individual segments were

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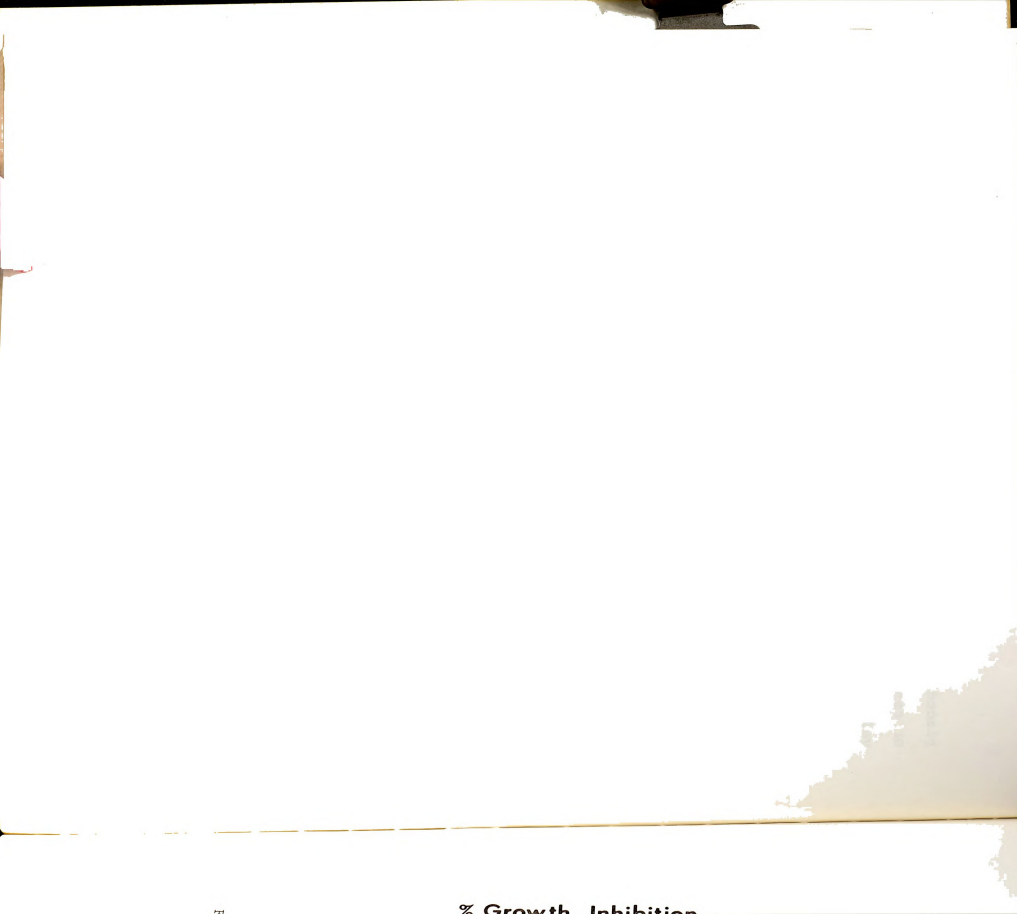
placed in petri dishes containing pre-germinated susceptible or resistant corn seeds. The assay showed that toxin R_f value was 0.85-0.90 when the chromatogram was developed with either propanol-acetic acid-water or with butanol-acetic acid-water.

Most activity was recovered in the third and fourth 6 ml fractions from a Sephadex column, prepared as described in materials and methods (Fig. 18). Fraction 4 was used in certain experiments that required a more highly purified toxin preparation.

HM-toxin was prepared as shown in Figure 17 (up to the Sephadex filtration step). Samples were then adjusted to pH 3.5, 7.0 and 10.5 and autoclaved for 15 min at 121 C. A non-autoclaved sample served as a control. Toxicity of samples was determined by the standard seedling root growth bioassay. Toxin inactivation by heat was found to vary with pH of the solution (Table 10). Toxin was relatively stable under acidic conditions. Some loss of toxicity occurred under neutral or alkaline conditions. Essentially complete toxin inactivation occurred in the pH 10.5 solution with autoclaving. The experiment was repeated. Another experiment showed that little or no activity was lost by preparations at pH 3.5 or 7.0 at room temperature in 48 hr.

Sensitivity of Texas male sterile and normal cytoplasm corn lines to HM-toxin.--Several different corn inbred lines and hybrids were bioassayed to determine whether or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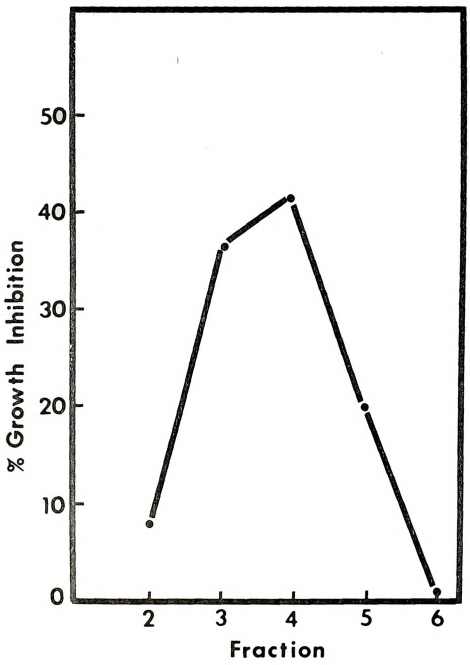

FIG. 18.--Activity profile of HM-toxin from a G10 Sephadex column (1.5 x 24 cm). Each 6 ml fraction was assayed for ability to selectively inhibit root growth of susceptible seedlings.

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Table 10. Effect of heat and pH on stability of HM-toxin.

Treatment	Dilution factor[a] for corn type:	
	TMS	Normal
Non-autoclaved control	1000	50
Autoclaved[b] at pH 3.5	800	25
Autoclaved at pH 7.0	400	10
Autoclaved at pH 10.5	25	< 10

[a]All values are toxin preparation dilution end points that gave 50 per cent inhibition of seedling root growth.

[b]Autoclaved 15 min at 121 C.

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not all TMS corn is equally sensitive to toxin, and all normal cytoplasm corn is equally resistant. There were several sets of assays because of the large number of corn lines to be tested. Each set of assays contained two reference lines of corn: W64ATMS (susceptible) and W64A (resistant). Toxin was prepared as shown in Figure 17, except that Sephadex chromatography was omitted. The same toxin preparation was used in all assays for the sake of standardization. Results showed that all corn lines with TMS cytoplasm were equally sensitive to HM-toxin. All corn seedlings with normal cytoplasm had a much higher, and approximately equal tolerance for HM-toxin (Tables 11 and 12).

In some cases, the host-specific toxicity of the aqueous preparation may have been masked in seedling bioassays by non-selective toxicity by impurities. Therefore, toxin was purified further by column chromatography for use in seedling bioassays. Fraction 4 from the Sephadex column (Fig. 18) gave 50 per cent inhibition of root growth of susceptible corn seedlings with an 800-fold dilution, whereas it inhibited resistant corn at a 10-fold dilution. The dry weights of the toxin preparation at 800- and 10-fold dilutions were 16 µg/ml and 1360 µg/ml, respectively. Thus, there was an 80-fold difference in sensitivity of susceptible and resistant corn seedlings to the most highly purified toxin used to date. Comparable results were obtained in two additional experiments. Further purification might

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(W64A x
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NY821
(MS68 x
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SC22-2-
B37-HT
B37
R-B37-H
$-B37-H
R-B37-H

W10
MS68
MS142
MS142 BC
MS 152 x
B37-TMS-
SD10 BC6
SD10 x B
SD10 x B

Table 11. Corn lines tested for sensitivity to HM-toxin.

Corn lines	
Normal cytoplasm	
593-2-1-3-2	R-B37-HT.A x SD10
W10	R-B37 HT.B x SD10
MS68	SD10 x R-B37-HT.A
MS142	SD10 x R-B37-HT.B
SD10	A632-HT
MS68 x SD10	A619-HT
MS103	8670
W64A x MS103	A632-HT x 8670
MS68 x MS152	A619-HT x 8670
(W64A x W103)(MS68 x MS153)	R-53-HTA Sib
MS153	R-53-HTB Sib
(W64A x MS103) x MS153	R53 Sib
(W64A x MS103) x MS68	OH51 Sib
NY821	RW64A-HTA x ROH43-HTA
(MS68 x MS152) x NY821	RW64A-HTB x R-OH43-HTA
(SC22)-2-4-2	RW64A-HTA x R-OH43-HTB
(MS153 x SD10) x SC22	RW64A-HT.A Sib
SC22-2-4-4	R-OH43-HT.B
B37-HT	RW64A-HT.B Sib
B37	R-W64A-HTB
R-B37-HT.A	R-OH43-HT.B
$-B37-HT.B	ROH43-HTB Sib
R-B37-HT.B	
Male sterile cytoplasm	
W10	SD10 x R-B37-HT.A
MS68	SD10 x R-B37-HT.B
MS142	R-B37-HT.B
MS142 BC4 Sib 3	B37-TMSHT x RB37-HT.B
MS 152 x SD10	B37-TMS
B37-TMS-HT	A632-TMS-HT
SD10 BC6	A619-HT
SD10 x B37-HT	R-B37-HT.B x SD10
SD10 x B37	

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Table 12. Summary table of sensitivity of corn lines to HM-toxin.

Corn lines with:	No. of lines	Toxin dilution[a] factor
Normal cytoplasm	45	50
Male Sterile cytoplasm	17	1000

[a]Maximum dilution which gave 50 per cent inhibition of seedling root growth.

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give a preparation with still more selectivity, and higher activity on a dry wt basis.

Sensitivity of non-host species to HM-toxin.--Several plant species that are not hosts of H. maydis race T were tested for sensitivity to toxin. Fraction no. 4 from the Sephadex column was used as the toxin preparation in the seedling root growth bioassays of the several plant species. Results showed that all non-host species were equally as insensitive to HM-toxin as was resistant (normal cytoplasm) corn (Table 13). Susceptible (TMS) corn seedlings were the most sensitive to toxin of all plants tested. The experiment was repeated 3 times. A more highly purified preparation might reveal a still higher degree of specificity.

Effect of anaerobic conditions on HM-toxin uptake and/or toxin activity.--HM-toxin was tested to determine whether or not it requires oxygen for activity and/or uptake, as is the case with H. carbonum toxin (8). Susceptible (TMS) corn seeds were germinated for 36 hr in White's solution. Seeds were then transferred to 90 mm petri dishes containing either 10 ml toxin solution or 10 ml water for controls. Some of the toxin-treated and control dishes were left on the laboratory bench in the presence of air. Other toxin-treated and control dishes were placed in a desiccator; nitrogen was passed through for 30-45 min before it was sealed. After a 12-hr exposure to HM-toxin all corn

Table 13. Relative sensitivity of host and non-host plants to HM-toxin.

Plants	Dilution factor[a]
Zea mays (resistant)	< 12.5
Zea mays (susceptible)	800
Raphanus sativus	12.5
Hordeum vulgare	< 12.5
Sorghum vulgare var. *subglabrescens*	< 12.5
Cucumis sativus	12.5
Lycopersicon esculentum	12.5
Agrostis sp.	12.5

[a]Maximum toxin dilution which gave 50% inhibition of root growth.

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seedlings were rinsed 10 times with distilled water, placed in petri dishes containing fresh White's solution, and incubated at room temperature (approximately 22 C.) for 3 days. The root growth of untreated control seedlings under anaerobic conditions was less than that of similar seedlings under aerobic conditions (Table 14). Therefore, it was assumed that the oxygen was removed from the desiccator by this treatment. The root growth of susceptible (TMS) corn seedlings was inhibited equally by toxin exposure under either aerobic or anaerobic conditions (Table 14). The results indicated aerobic respiration is not required for HM-toxin uptake and/or activity. HM-toxin resembles H. victoriae toxin but differs from H. carbonum toxin in this respect.

Effect of HM-toxin on electrolyte leakage from corn leaves.--All the host-specific toxins examined thus far caused an increased leakage of electrolytes from susceptible tissue (15). Therefore, the effect of HM-toxin on the electrolyte leakage of susceptible and resistant corn leaves was tested. Fraction 4 from a Sephadex column was used as the toxin preparation. Control leaves were treated with glass-distilled water. Two replicate treatments were used. Leaves from 3-week-old susceptible (W64ATMS x 040) and resistant (W64A x 040) corn plants were cut into 0.5 to 1.0 cm pieces. One g leaf tissue (fresh wt) was placed in pre-washed cheesecloth bags and individual bags were placed in

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Table 14. Effect of anaerobic conditions on HM-toxin uptake and/or activity.

Treatment[a]	Experiment No.	
	1	2
	mm	mm
Air ck	130[b]	114
Air tox	61	59
N_2 ck	110	100
N_2 tox	54	52

[a]Seedlings were treated with HM-toxin for 12 hr.

[b]Average root length of 20 susceptible seedlings. Roots were measured 3 days after removal from treating solutions.

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300 ml Erlenmeyer flasks containing 50 ml toxin solution or water. Leaf tissue was vacuum infiltrated, incubated for 2 hr on a reciprocal shaker, and rinsed 3 times with 50 ml glass distilled water for a total time of 1.5 hr. Conductivity readings of the ambient solutions were taken with a model RC 16Bl Industrial Instruments conductivity bridge. Readings were started 4 hr after the tissue was first placed in toxin (3.5 hr pretreatment plus 0.5 hr leaching). Results showed that toxin-treated susceptible leaves had more loss of electrolytes than control leaves 4-6 hr after first exposure to toxin (Fig. 19). Resistant leaves treated with HM-toxin did not differ from control leaves in electrolyte leakages. Similar results were obtained using a slightly longer toxin exposure time (4 hr). More precise procedures might show earlier effects of toxin on loss of electrolytes.

Toxin production by H. maydis race O.--Culture filtrates from an isolate of H. maydis race O, obtained from Dr. A. J. Ullstrup, were tested for toxicity to corn seedling roots. Twelve-day culture filtrates were partially purified as described in the materials and methods section, except for the Sephadex column chromatography step (Figure 17). During purification, the volume of the culture filtrate was reduced 70-fold.

The seedling root growth of both corn lines was inhibited 50 per cent by the toxin preparation at a 160-fold

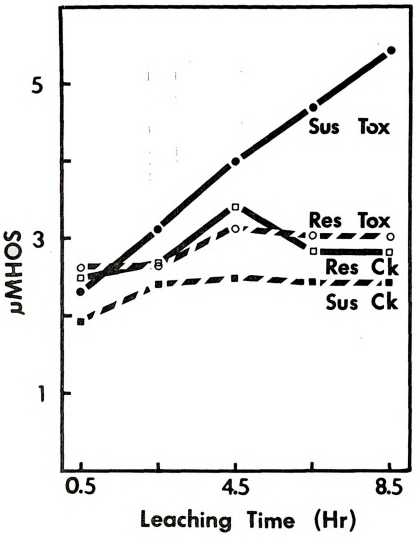

FIG. 19.--Effect of HM-toxin on loss of electrolytes from susceptible and resistant corn leaves. Leaves were vacuum infiltrated for 15 min and incubated for 2 hr with HM-toxin. Tissue was washed and conductivity readings were started 4 hr after first exposure to toxin.

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dilution. Therefore, the H. maydis race O preparation was as inhibitory to root growth of seedlings that are susceptible (WF9 TMS) to H. maydis race T as to seedlings that are resistant (WF9). H. maydis race O field-susceptible (W64A) and resistant (OHO7) corn lines have been reported (unpublished data of A. L. Hooker). The toxin preparation gave 50 per cent inhibition of both field-susceptible and resistant corn seedlings root growth when diluted 200-fold. Either no host-specific toxin was produced, or it was not detectable by the assay procedure, or selective toxicity was masked by the non-selective toxic materials present. The experiment was not repeated, and toxicity to leaves was not tested.

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DISCUSSION

Host-specific toxins are known to be produced by eight pathogens that cause different plant diseases (17). Detection and isolation of the known host-specific toxins have been complicated for several reasons. Toxin detection has required quick bioassay procedures that use both susceptible and resistant host varieties, because of toxin lability. Toxin production in culture has required special media and environmental conditions. Even under ideal cultural conditions, relatively low toxin yields have been obtained. Furthermore, detection and isolation of host-specific products have been complicated by the presence of other toxic products in the culture filtrates. Toxin instability has made purification extremely difficult.

The data presented in this thesis confirmed a preliminary report (7) that H. maydis race T produces a host-specific toxin. Host sensitivity to HM-toxin and susceptibility to H. maydis race T were correlated. Resistant corn with normal cytoplasm and plant species that are not hosts of H. maydis were equally insensitive to the partially purified toxin preparation. Susceptible corn with Texas male sterile cytoplasm was at least 80 times more sensitive to the same toxin preparation. Nevertheless, this difference

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between sensitivity of susceptible and resistant seedlings was less for HM-toxin than for the other host-specific toxins. Oat, corn and sorghum cultivars resistant to H. victoriae, H. carbonum, and Periconia circinata-toxins tolerated respectively, > 400,000, 100, and > 26,000 times more toxin than did susceptible cultivars (15).

Methods of producing HM-toxin were similar to those used for the other host-specific toxins (15, 22). Highest production of HM-toxin occurred in 12 to 15 day-old still cultures grown in Fries' medium plus 0.1% yeast extract. Isolation procedures for HM-toxin were similar to those used for the other host-specific toxins. Culture filtrates were extracted with methanol and butanol or chloroform, followed by gel filtration with Sephadex.

The characteristics of HM-toxin determined to date are similar to those of other host-specific toxins. HM-toxin is a labile, dialyzable molecule. Like the others, HM-toxin is more stable in acidic solutions than in neutral or alkaline solutions. Compared to the other toxins, the HM-toxin preparation was relatively low in activity, on a dry wt basis. However, the best preparation obtained so far probably still contained many impurities.

H. victoriae and P. circinata-toxins have been postulated to act initially on the plasma membrane. The hypothesis is supported by several lines of evidence (15), including the data showing immediate loss of electroltyes

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from toxin-treated susceptible tissue (15). P. circinata-toxin also induces electrolyte leakage from susceptible tissue, in this case within 20 min after initial exposure (4). The effect of H. victoriae-toxin on electrolyte leakage from susceptible leaves is reduced by prior treatment with certain carbonyl and sulfhydryl-binding reagents (4), with uranyl ions (6), and with toxin breakdown products (4). These results indicate a competition of these substances with toxin for receptor sites. Several possible sites of H. victoriae and P. circinata-toxin action were eliminated when it was shown that neither toxin affects isolated mitochondria (4, 15); H. victoriae-toxin does not affect chloroplasts (15). The evidence is not conclusive that the plasma membrane is the initial site for action of these toxins, but all data to date fit the hypothesis.

An important site of action for H. carbonum-toxin appears to be the plasma membrane. The first detectable effect was stimulation of uptake of certain ions and organic substances by the cell (15, 25). Uptake of these materials requires metabolic energy, and is affected by the characteristics of the plasma membrane. Toxin did not affect isolated mitochondria or chloroplasts from susceptible plants (25).

HM-toxin acts selectively on the mitochondria of susceptible and non resistant corn. HM-toxin treatment causes an immediate uncoupling of phosphorylation and from oxidation

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irreversible swelling of mitochondria from susceptible corn leaves (10, and unpublished data of M. A. Bednarski). Chloroplasts are not affected by HM-toxin (unpublished data of M. A. Bednarski). Other possible sites of action have not been detected.

Various data indicate that metabolic energy is involved in the uptake and/or activity of H. carbonum-toxin. The temperature at which susceptible seedlings were exposed to toxin affected activity and/or uptake. Higher temperatures during the time of exposure to toxin resulted in more drastic inhibition of later root growth. Furthermore, oxygen was shown to be required during the time of exposure to toxin; when seedlings were exposed to toxin under anaerobic conditions, no toxin-induced inhibition of growth occurred later (8). In contrast, oxygen does not appear to be required during the time of exposure to H. victoriae and HM-toxins for later toxicity to be expressed. These results do not seem to fit a hypothesis that the only site of HM-toxin activity is in the mitochondrion, although there is no doubt that there is such a site of toxin sensitivity. It would seem that movement of toxin across the plasmalemma to the mitochondrion should require energy of metabolism. It is possible that other cellular sites are affected, and that toxin reaches the mitochondrion after it has affected other sites.

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The host-specific toxins of H. victoriae (24), Periconia circinata (18), and H. sacchari (22) have been used in breeding programs for detecting disease resistance. Resistance to these toxins is correlated with resistance to the fungus. If HM-toxin is to be of value to the plant breeder, resistance to the toxin must also be correlated with resistance to H. maydis race T. HM-toxin can easily distinguish between corn lines with Texas male sterile (susceptible) or normal (resistant) cytoplasm. However, different TMS and normal corn lines have been reported to differ in sensitivity to HM-toxin, using a mature leaf assay (5). No differences in sensitivity among TMS corn lines or among normal corn lines were detected in my bioassays with seedlings roots. Possibly, use of the seedling bioassay will not detect slight differences whereas a mature leaf bioassay might be more sensitive. The possible correlation between resistance to the fungus and resistance to the toxin needs further study before we can conclude that HM-toxin will be of value to the plant breeder.

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