Involvement of Helminthosporium maydis Race T Toxin During Colonization of Maize Leaves

J. C. Comstock and C. A. Martinson

Postdoctoral Research Associate and Associate Professor, Department of Botany and Plant Pathology, Iowa State University, Ames, Iowa 50010. Present address of senior author: Hawaiian Sugar Planters Association, P.O. Box 2450, Honolulu 96804.

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ABSTRACT

A host-specific toxin (Hm-t) produced by *Helminthosporium maydis* race T aided the establishment of *Helminthosporium* sp. on maize leaves containing Texas male-sterile cytoplasm (cms-T). Toxin was detected during the early stages of germination of *H. maydis* race T conidia. Toxin involvement during colonization was determined by monitoring the electrolyte leakage from maize leaves. When Hm-t toxin was supplied at the time of inoculation to spores of race O of *H. maydis* and race 1 of *H. carbonum*, the loss of electrolytes during infection was greater than that induced by either the fungus or toxin alone. Hm-t toxin always caused increased electrolyte leakage from cms-T leaves, but never from normal cytoplasm leaves.

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Helminthosporium maydis Nisikado & Miyake, the causal organism of Southern corn leaf blight, has two morphologically similar, but pathogenically distinct, races (17). Race O of the fungus is equally virulent on maize with either normal (N) or Texas male-sterile cytoplasm (cms-T), while race T is more virulent on cms-T maize (17).

Helminthosporium maydis was first reported to produce a host-specific toxin in 1969, but whether or not the toxin was specific for cms-T maize was not stated (16). In 1970, Hooker et al. (7) described race T of *Helminthosporium maydis* which produced a toxin (Hm-t) specific for maize which contained either cms-T or certain related cytoplasms. Hm-t toxin has been found in race T-infected leaves (12). The ability to produce Hm-t toxin is controlled by a single gene (10). Another compound toxic to both cms-T and N cytoplasm maize is produced by both races of *Helminthosporium maydis,* but its role in disease development has not been determined (11).

Changes in electrolyte leakage from infected plants compared with healthy controls, demonstrate that permeability changes occur during pathogenesis. Susceptible barley infected with *Rhynchosporium secalis* had an increased loss of electrolytes and amino acids before the first symptoms appeared (8). Loss of electrolytes from oat leaves increased 6 hours after inoculation with a pathogenic isolate of *H. victoriae.* There was no increased loss of electrolytes from resistant leaves inoculated with *H. victoriae,* or from leaves inoculated with a nonpathogenic isolate (21). Comparable experiments showed that maize leaves susceptible to *H. carbonum* had a measurable increase in the loss of electrolytes 24 hours after inoculation; the loss of electrolytes from inoculated resistant leaves was less than from inoculated susceptible leaves, and occurred later (4). Increased electrolyte leakage occurred when the pathogen was extensively invading susceptible tissue (4). Tissue susceptible to *H. victoriae* and *H. carbonum* have an increased loss of electrolytes when treated by their

pathogen's host-specific toxin (15). Hm-t toxin produced by *H. maydis* race T causes a selective increased leakage of electrolytes from cms-T tissue (1, 5, 6), but not that of N-cytoplasm tissues. There is no evidence whether Hm-t toxin is present or active during establishment of the pathogen on the host, or whether its activity is discernible at these early stages of pathogenesis.

The objectives of this research were to determine the presence of and importance of Hm-t toxin during the colonization of maize leaves by race O and race T of *H. maydis*. An abstract has been published (2).

MATERIALS AND METHODS.—*Fungi.*—Isolates of *Helminthosporium maydis* race O and *H. carbonum* race 1 were supplied by A. J. Ullstrup, Purdue University, via R. P. Scheffer, Michigan State University. The *H. maydis* race T was isolated from a single lesion on an ear of corn found near Mitchellville, Iowa, in 1969.

Plants.—Leaves were used from 13-day-old maize inbreds B37 cms-T and B37 containing Texas male-sterile and normal cytoplasm, respectively. B37 cms-T is more susceptible than B37 to *H. maydis* race T. Both B37 cms-T and B37 are genetically resistant to *H. carbonum* race 1.

Toxin.—Hm-t toxin was produced according to Comstock and Scheffer (3). *H. maydis* race T was grown for 15 days in stationary culture at 23 C in modified Fries' medium supplemented with 0.1% yeast extract. The fungus was removed by filtration, and the culture filtrate was adjusted to pH 3.5 with 3N HCl. The culture filtrate was reduced in vacuo to 10% its original volume, two volumes of methanol were added, and this was stored overnight at 4 C. Precipitates were removed by filtration, and the volume of the preparation was reduced in vacuo to 4.0% of the original filtrate volume. This stock preparation of Hm-t toxin was used in all experiments.

The stock preparation of Hm-t toxin caused 50% inhibition of inbred W64A cms-T root growth at a dilution of 1:1,000 in a bioassay of inhibition of seedling root growth (3). Hm-t toxin diluted 1:100, was used to suspend conidia before the inoculation of leaves in some treatments.

Inoculation procedures.—Conidia were produced on filter paper according to Lukens' method (13) as modified by Yoder and Scheffer (21). Excised leaves were inoculated by placing drops of conidial suspensions on the upper leaf surface, giving 10-15 spores/mm^2. Droplets of water or toxin were placed on leaves as controls. Leaves were maintained in a moist chamber at 100% relative humidity at 24 ± 1 C.

Electrolyte leakage measurements.—At various times after inoculation, the loss of electrolytes from leaf tissue was determined. Leaf samples weighing 0.5 g were cut into 1.0-2.0 cm lengths and placed in gauze bags. Each bag was placed in a 300-ml Erlenmeyer flask containing 50 ml distilled water. The flasks were agitated on a reciprocal shaker (70 3.5-cm strokes per min). After the leaf tissues were rinsed four times with distilled water, the conductance of the ambient fluid was monitored periodically over a 6-hour period using a Yellow Springs conductivity bridge with a cell constant, K = 1. Each treatment was run in triplicate. Statistical differences between treatments were determined by a *t*-test of the means of two independent samples. Differences

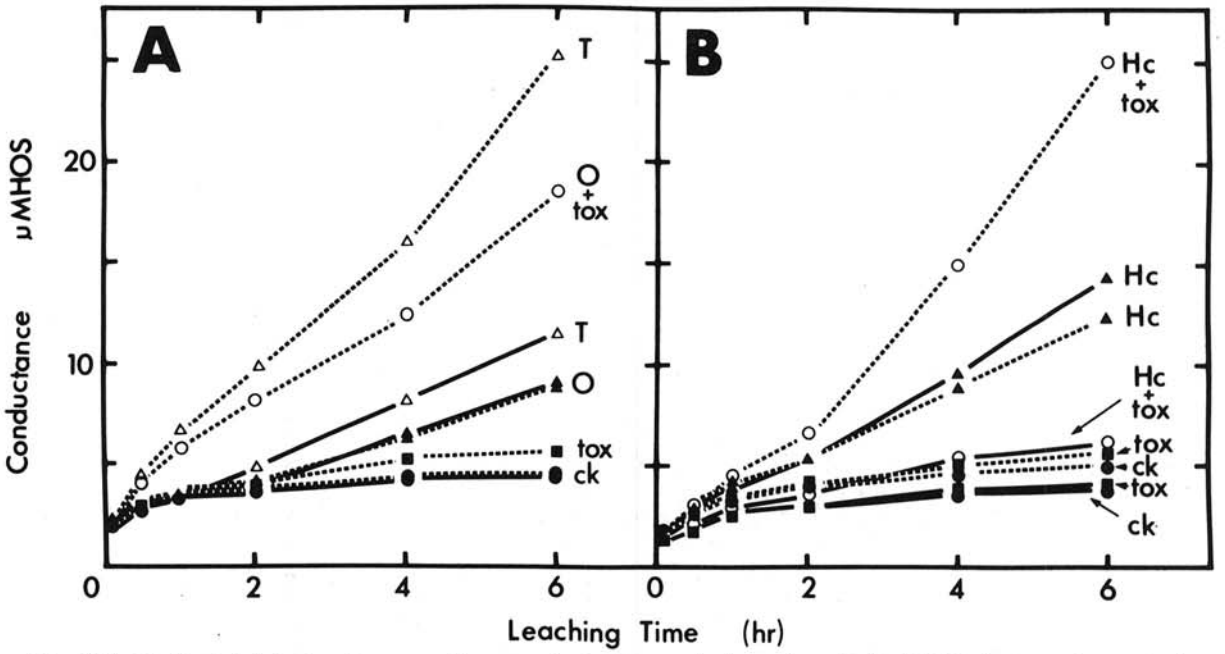

Fig. 1-(A, B). Electrolyte leakage from cms-T or normal cytoplasm maize leaves inoculated with *Helminthosporium* sp. in the presence or absence of *Helminthosporium maydis* race T toxin. A solid line = normal leaves. A dashed line = cms-T leaves. **A)** *H. maydis* race O and race T inoculated leaves. T = race T. O = race O. tox = Toxin present in droplet. ck = no toxin in droplet. Not shown is the electrolyte leakage of normal leaves inoculated with race O with Hm-t toxin present. The leakage was comparable to that of leaves inoculated with race O without Hm-t toxin. **B)** *H. carbonum* race 1 inoculated leaves. Hc = *H. carbonum*. tox = toxin present in droplet. ck = no toxin in droplet.

expressed are significant, $P = 0.05$.

RESULTS.—*Effect of Helminthosporium maydis infection on the loss of electrolytes from maize leaves.*—Leaves of maize infected with *H. maydis* had a significantly greater loss of electrolytes than did healthy controls (Fig. 1-A). Race T of *H. maydis* caused a threefold greater loss of electrolytes than did race O from cms-T leaves during the 6-hour period monitored, 12-18 hours after inoculation. Furthermore, the first significant increased loss of electrolytes from cms-T leaves due to infection occurred sooner after inoculation with race T than with race O, 13 hours vs. 16 hours, respectively. The rate of electrolyte leakage from leaves (as indicated by the slope of the lines in Fig. 1-A) inoculated with race O was similar for N and cms-T lines. Also, the rate of electrolyte leakage from leaves inoculated with race O was comparable to the rate from leaves containing N-cytoplasm inoculated with race T. Both the N-cytoplasm leaves that were resistant to Hm-t toxin, and the cms-T cytoplasm leaves inoculated with race O, had a lower rate of electrolyte leakage than Hm-t toxin-sensitive cms-T leaves inoculated with race T.

Effect of Helminthosporium maydis race T toxin on electrolyte leakage from infected maize leaves.—The role of Hm-t toxin during colonization was tested by comparing the electrolyte leakage from leaves inoculated with race O, with race O with Hm-t toxin present, and with race T. Comparisons were also made with the uninoculated controls where droplets of diluted (1:100) Hm-t toxin and water instead of droplets containing fungal spores were placed on the upper surface of the leaves. The addition of Hm-t toxin to race O conidia significantly increased the loss of electrolytes from cms-T tissue (Fig. 1-A). Hm-t toxin treatment has been reported to cause an increased electrolyte leakage from cms-T tissue (5, 6). When droplets of toxin were placed on cms-T leaves, however, the loss of electrolytes was not significantly increased, indicating a failure of the toxin to penetrate the cuticle. When diluted Hm-t toxin was supplied to race O, the time when increased leakage was detected from cms-T leaves was comparable to the occurrence of increased leakage from race T-inoculated cms-T leaves. The rate of electrolyte leakage from cms-T leaves induced by race O supplemented with Hm-t toxin was comparable to that of race T-infected cms-T leaves. Toxin, however, did not significantly affect the loss of electrolytes from N-cytoplasm leaves inoculated with either race O or race T. Similar results were obtained in experiments when the loss of electrolytes was monitored from 24 to 30 hours after inoculation.

The role of Hm-t toxin also was evaluated by using another pathogen of corn, *H. carbonum* race 1. N and cms-T leaves of B37 (both are genetically resistant to race 1 of *H. carbonum*) were inoculated with the fungus with and without Hm-t toxin being present. Hm-t toxin significantly affected the electrolyte leakage from both *H. carbonum*-infected cms-T and N leaves, and the toxin had a differential effect on the two tissues (Fig. 1-B). When *H. carbonum*-resistant cms-T leaves were inoculated with *H. carbonum*, the addition of Hm-t toxin caused a significant increased rate of electrolyte leakage at 28 hours after inoculation. In contrast, Hm-t toxins caused a significant decrease in electrolyte leakage from *H. carbonum*-infected N leaves. Further clarification is required to explain the decreased electrolyte leakage when Hm-t toxin is supplied to *H. carbonum*-infected N leaves.

Hm-t toxin activity was detected at 18 hours after inoculation in *H. maydis* race T conidial suspensions used to inoculate leaves. Inoculation droplets were collected and filtered through a 0.2-μm (pore size) cellulose acetate filter to remove the fungus. Toxin activity was assayed in these filtrates by induction of leaf chlorosis (19). Toxin presence in inoculation droplets prior to 18 hours after inoculation was not tested.

Since Hm-t toxin activity was detectable during the early stages of tissue colonization, the time when toxin was first detectable from *H. maydis* race T conidia was determined. Conidia produced on filter paper were removed from the dry filter papers by means of a cyclone spore collector suspended over the culture. This greatly reduced any possibility of toxin contamination. After suspending the conidia in water at a concentration of 16,000 spores/ml, samples were taken periodically and assayed for toxin using the induction of leaf chlorosis bioassay (19). Within 5-10 minutes after suspending conidia, Hm-t toxin was detectable in the bathing water.

DISCUSSION.—Several physiological functions in the cms-T cytoplasm maize plant are known to be selectively affected by Hm-t toxin. The increased rate of electrolyte leakage from cms-T tissues that is induced by Hm-t toxin (6) is probably the most sensitive assay that can be applied to monitor toxin activity during pathogenesis. Lim and Hooker (12) found Hm-t toxin in leaves 12 days after inoculation and Karr et al. (9) found their toxins I and II of the Hm-t toxin complex to be present 48 hours after inoculation. Through ultrastructural studies of the developing lesion, White et al. (20) observed a rupture of the tonoplast in cms-T maize leaves 6 hours after inoculation; this tonoplast rupture may have been due to toxin activity, however, one must not disregard the enzymatic activities and mechanical disruption associated with pathogenesis. The data reported herein indicate that Hm-t toxin was present very early during colonization of the leaf tissue. Hm-t toxin was detectable within 5 to 10 minutes after *H. maydis* race T conidia were suspended in water. The droplets of conidia used to inoculate maize leaves contained toxin at 18 hours after inoculation. Thus, Hm-t toxin was available to induce the increased rate of electrolyte leakage observed 13 hours after inoculation of cms-T leaves with *H. maydis* race T.

The Hm-t toxin did not increase the rate of electrolyte leakage from N-cytoplasm maize leaves. The isolate of *H. maydis* race T was slightly more aggressive on N-cytoplasm tissues than the isolate of race O, because race T induced a higher rate of electrolyte loss than did race O. The increased aggressiveness of race T cannot be ascribed to Hm-t toxin production, because when race O spores were suspended in dilute Hm-t toxin instead of water, there was no increase in the rate of electrolyte leakage from N leaves.

The addition of Hm-t toxin with spores of *H. maydis* race O and *H. carbonum* race 1 caused a significantly higher rate of electrolyte leakage from cms-T leaves than when these fungi were present without Hm-t toxin. Thus, Hm-t toxin is obviously active during the early stages of cms-T tissue colonization. Toxin droplets alone did not

significantly increase electrolyte leakage, so the fungi provided a mechanism or route for cuticular and possibly deeper tissue penetration of Hm-t toxin.

Several lines of evidence indicate that Hm-t toxin caused membrane permeability changes in cms-T tissues (5, 6, 14, and 18); all point to a loss of plasma-membrane integrity or function. Hm-t toxin exposure caused an increase in electrolyte leakage (5, 6) and carbohydrate leakage (6) from roots and leaves, inhibited the normal transfer of K^+ from subsidiary cells to guard cells (14), induced a rapid partial depolarization of the electrochemical membrane potential of root cells (14), and selectively inhibited K^+ stimulated ATPase activity in cms-T maize (18). The linkage of these phenomena and whether or not Hm-t toxin induces all these changes during pathogenesis needs further clarification.

The host-specific toxins produced by *H. victoriae* and *H. carbonum* race 1 are required for pathogenicity of the organism (15). All isolates of *H. victoriae* and *H. carbonum* that are pathogenic produce their particular host-specific toxin; all nonpathogenic isolates do not. Furthermore, when pathogenic isolates of these two fungi lose their ability to produce toxin in culture, they lose their pathogenicity (15). Hm-t toxin is not required for pathogenicity of *H. maydis* race T, but it is responsible for the increased virulence towards cms-T maize. Race O of *H. maydis* does not produce Hm-t toxin, is pathogenic, and infects maize to a limited extent. When Hm-t toxin was supplied with race O, the fungus caused more damage as indicated by an increased loss of electrolytes. Lim and Hooker (10) demonstrated that Hm-t toxin production and increased virulence for cms-T maize are highly associated, and controlled by a single gene.

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