Ultrastructural Changes in Corn Leaves after Inoculation with Helminthosporium maydis, Race T

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ABSTRACT

Corn seedlings, of a strain having Texas male-sterile cytoplasm, were inoculated with conidia of *Helminthosporium maydis*, race T. Infected leaf tissues were sampled at 6, 12, 24, and 48 hr after inoculation. Electron microscopic examination of these tissues revealed that the first detectable change, rupture of the tonoplast, occurred within 6 hr. By 12 hr, chloroplasts had become spherical, and numerous vacuolelike bodies were present in the plastid stroma. At 24 hr, the plasma membrane had ruptured, chloroplast envelopes had become disorganized, and the mitochondrial matrix appeared to be absent. In many cells, the plastid envelopes had disintegrated, and numerous irregular, osmiophilic bodies were interspersed within the lamellar systems. By 48 hr, the chloroplast lamellae had clumped together and had undergone extensive structural breakdown. Tissue samples taken from 48-hr necrotic lesions revealed the most advanced stages of cell breakdown.

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The high level of virulence exhibited by race T of *Helminthosporium maydis* Nisik. & Miyak. (*Cochliobolus heterostrophus* Drechs.) on corn (*Zea mays* L.) lines having Texas male-sterile cytoplasm has been the subject of numerous investigations. These studies described the gross response of resistant and susceptible hybrids and inbreds to invasion by *H. maydis* (2, 3, 4, 9), and demonstrated the involvement of a host-specific toxin (3, 10, 12). Although there have been no published reports on *H. maydis*-induced ultrastructural changes in host cells, several investigators have proposed a role for the pathotoxin. Hooker et al. (3) reported that detached leaf sections from susceptible corn seedlings floating on a pathotoxin solution developed a water-soaked condition within 3 to 4 days. Such a response indicated an alteration of cell membranes. Miller & Koeppe (6) described the effects of the pathotoxin on isolated mitochondria from susceptible and resistant corn. They suggested that the action of the pathotoxin was related to membrane structure and function.

Another species of *Helminthosporium, H. victoriae*, produces a pathotoxin (victorin) which has been more thoroughly investigated. It has been reported that the plasma membrane is the initial site of action of victorin (5, 7, 8). Luke et al. (5), in describing the ultrastructural effects of victorin on susceptible oat leaves, reported that the toxin caused a breakdown of the chloroplast envelope and disruption of the lamellar system. They also indicated that the membranes of mitochondria were more tolerant of the toxin than were other membrane systems.

Recently, Strobel et al. (11) reported that the host-specific toxin produced by *Helminthosporium sacchari* induced extensive cytological abnormalities in infected sugarcane leaves. Mesophyll chloroplasts were severely disrupted, and the integrity of the cytoplasm was destroyed. It was suggested that the toxin, helminthosporoside, altered the permeability of the lysosome membrane.

Since the study of host-parasite interaction is essential to an understanding of pathogenesis, the present investigation had as its primary objective the delineation of subcellular changes in corn mesophyll cells after inoculation of the leaves of a susceptible line with *H. maydis*, race T.

MATERIALS AND METHODS.—Corn seedlings of a strain having Texas male-sterile cytoplasm (Pioneer 3306 cmsT) were grown in a greenhouse to the three- to four-leaf stage and inoculated as described below. Single spore isolates of *H. maydis*, race T, were obtained in August 1971 from infected corn ear husks and maintained in culture.

Conidia, washed from the surface of the culture medium, were suspended in a 5% sucrose solution and sprayed onto the surface of the first true leaf. Two groups of controls were maintained; one group was sprayed with 5% sucrose solution, the other with distilled water. The plants were then placed in a greenhouse mist chamber (a clear plastic chamber fitted with a Waltham humidifier) maintained at 25 C. After 12 hr in the mist chamber, the plants were transferred to a growth chamber (14-hr photoperiod, 2,200 ft-c, 25-C day, 20-C night). The inoculated leaves were collected at 6, 12, 24, and 48 hr after inoculation. The controls were collected at 6 hr and 48 hr. Inoculated leaves sampled at 6 and 12 hr were examined microscopically in order to insure that the specimens were taken from areas having germinating conidia. Immediately after excision, the leaf samples were processed for electron microscopy.

Fixation of the specimens was carried out in a

mixture of 3% glutaraldehyde, 1.5% acrolein, and 1.5% paraformaldehyde (0.1 M phosphate buffer, pH 7.2) for 4 hr at 4 C. The samples were washed overnight in the buffer solution, then postfixed in 1% OsO_4 (0.1 M phosphate buffer, pH 7.2) for 4 hr at 4 C. Infiltration of the fixatives was aided by evacuation of the specimens. The samples were dehydrated in an acetone series and embedded in Epon 812. Ultrathin sections were cut with a diamond knife and stained with uranyl acetate and lead citrate. The grids were examined in an RCA EMU-3G electron microscope.

Lipid determinations were made on thin-sectioned tissue using the technique described by Eurenius & Jarskar (1). In this procedure, lipid bodies are selectively extracted from Epon-embedded thin sections by a sodium methoxide reagent.

RESULTS.—Examination of control tissues showed that the ultrastructure of sucrose-treated leaves was the same as that of leaves treated with water, and that there was no change in the structure of control cells throughout the course of the experiment. A portion of a representative cell from control (sucrose-treated) tissue is shown in Fig. 1.

Tissue samples taken 6 hr after inoculation showed the first detectable change in cell ultrastructure, breakdown of the tonoplast. This condition appeared as numerous discontinuities in the membrane (Fig. 2). At the same sampling time, the chloroplasts and mitochondria showed no detectable changes in ultrastructure, and the plasma membranes remained intact. With the breakdown of the tonoplast, the cytoplasm was no longer restricted to the periphery of the cells, and began to disperse into the vacuolar space.

Marked changes in the structure of chloroplasts were observed in the 12-hr samples (Fig. 3). The chloroplasts had assumed a spherical shape and large vesicles or vacuolelike bodies appeared within the stroma. The lamellar system retained the spatial configuration of the control chloroplasts, and thus occupied only about one-half the volume of the plastids.

The first symptom visible at the macroscopic level was extensive water-soaking of the leaf. This symptom was first observed in the 24-hr samples. Although there was a certain amount of variation in host cell response, at 24 hr the plasma membranes had broken and chloroplast envelopes had become disorganized (Fig. 4). Although many of the mitochondrial membranes remained structurally intact, at 24 hr the homogeneous matrix within the mitochondria appeared to be absent. A more advanced stage of chloroplast breakdown, also noted in the 24-hr samples, is shown in Fig. 5. The chloroplast envelopes had disintegrated, and numerous, irregular, osmiophilic bodies were interspersed within the lamellar systems.

Forty-eight hr after inoculation, the infected leaves were completely wilted, and numerous necrotic lesions were visible. Samples were taken from wilted tissues and from necrotic lesions. Examination of samples taken from flaccid tissues indicated that collapse of the chloroplast lamellar systems occurred in these areas between 24 and 48 hr. The membranes were clumped together and appeared to be undergoing rapid structural breakdown (Fig. 6).

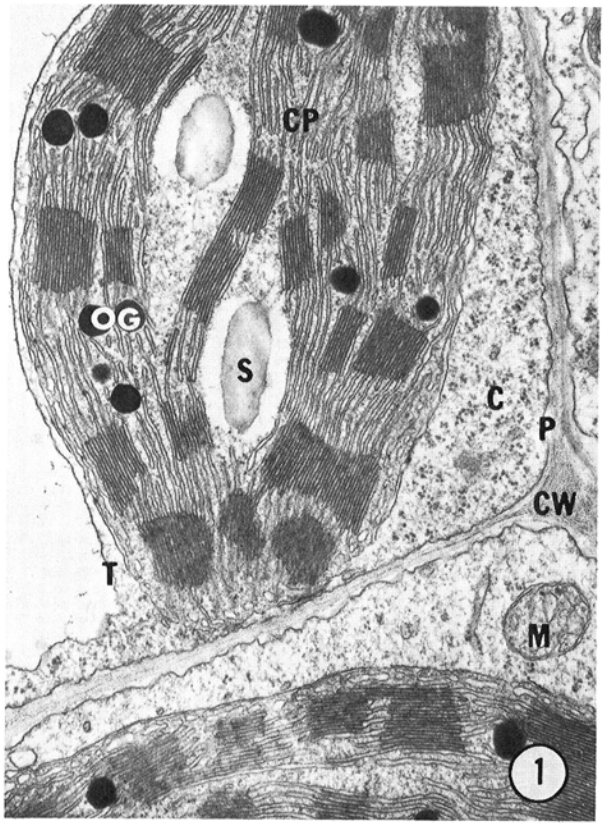
Examination of samples taken from necrotic lesions revealed extensive intercellular ramification of the fungus within the necrotic area. The walls of the mesophyll cells had collapsed, and the cytoplasmic materials had coalesced into dense, irregular masses. These dense masses could be divided into two types. One type consisted of an amorphous coagulum with dark-staining areas scattered throughout (Fig. 7). The second type consisted of membranes, identifiable as chloroplast lamellae, which had not coalesced. These membranes appeared, however, as a negative image; i.e., the membranes were electron-lucent on an electron-opaque background (Fig. 8). Fungal hyphae, which were in close association with these cell remnants, contained relatively large, spherical osmiophilic bodies (Fig. 9).

Lipid determinations were made on both degraded chloroplasts and fungal hyphae. The test was positive for both; i.e., the osmiophilic bodies were removed from chloroplast fragments and from hyphae with sodium methoxide reagent.

DISCUSSION.—The results indicate that, under the conditions of this experiment, race T of *H. maydis* is capable of altering the structural integrity of cell membranes. Although no definitive statements can be made regarding the existence and/or involvement of *H. maydis* toxin, the evidence leads us to suggest that a substance is produced by the fungus that is capable of degrading, by direct or indirect action, the membranes of host cells. Although a host-specific toxin has been demonstrated by other workers (3, 10, 12), many factors other than a toxin must be considered in any interpretation of these data. As Strobel et al. (11) suggested, the release of degradative enzymes in host cells may be an important factor in pathogenesis.

Undoubtedly, the response of the host tissue to fungal invasion involves complex biochemical changes. This complexity is illustrated by the alterations in chloroplast lamellae. The appearance of "negative-stained" lamellae indicates that, in these

Fig. 1-4. 1) A portion of a mesophyll cell of corn (control) showing chloroplast (CP) with osmiophilic globules (plastoglobuli) (OG) and starch grains (S); cytoplasm (C); plasma membrane (P); tonoplast (T); cell wall (CW); and mitochondrion (M) (× 21,000). **2)** A portion of a mesophyll cell, 6 hr after inoculation, showing broken tonoplast (arrow) (× 17,100). **3)** A spherical chloroplast 12 hr after inoculation. Note plastid envelope (E) and large vesicles (V) (× 13,200). **4)** A portion of a mesophyll cell 24 hr after inoculation. Note broken plasma membrane (P), mitochondrial matrix (M), and disorganization of plastid envelope (E). CP = chloroplast (× 17,100). **Inset)** Detail of a broken plasma membrane (arrow). CW = cell wall (× 60,700).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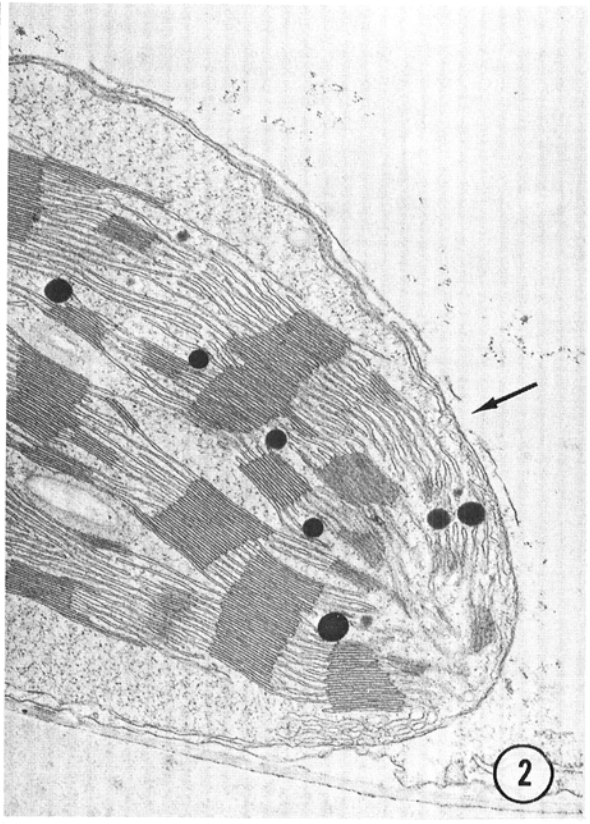
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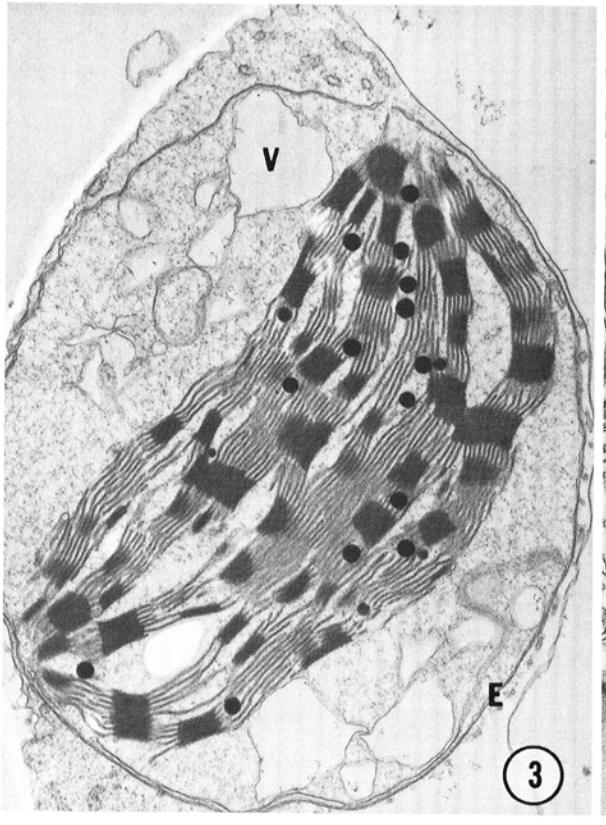
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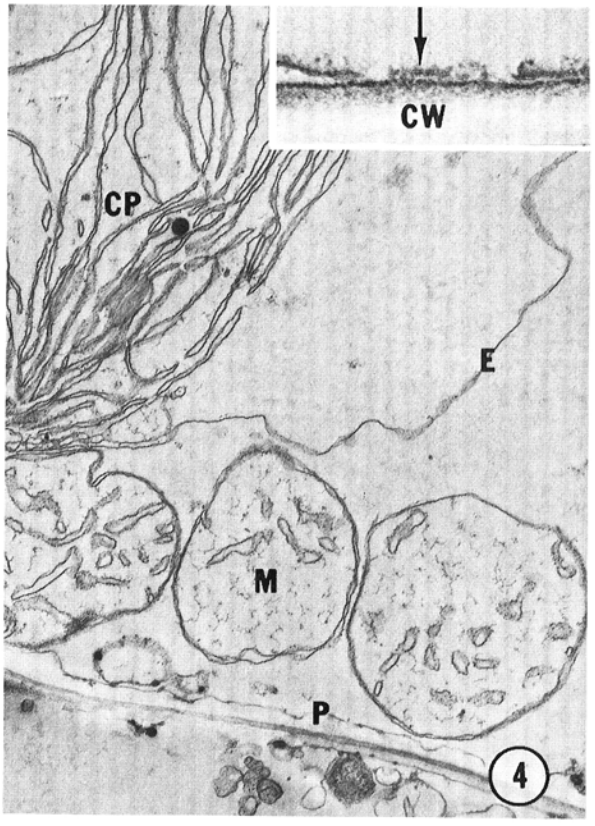
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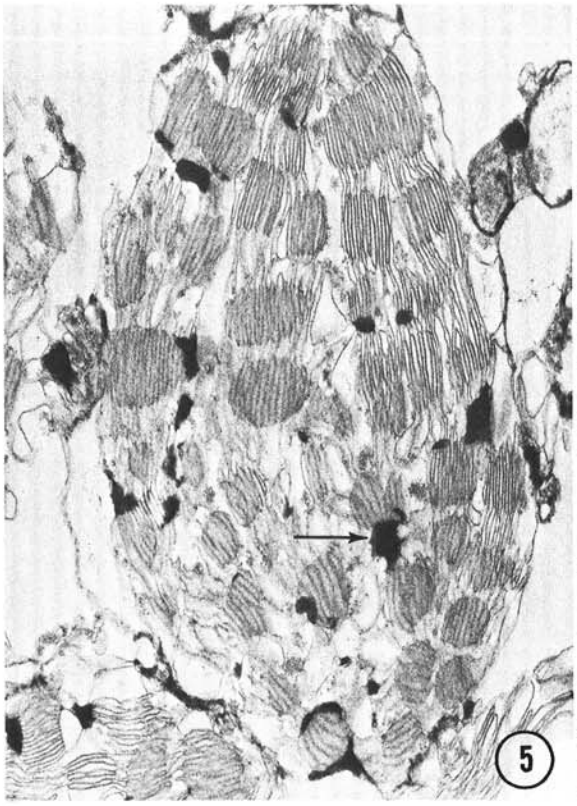
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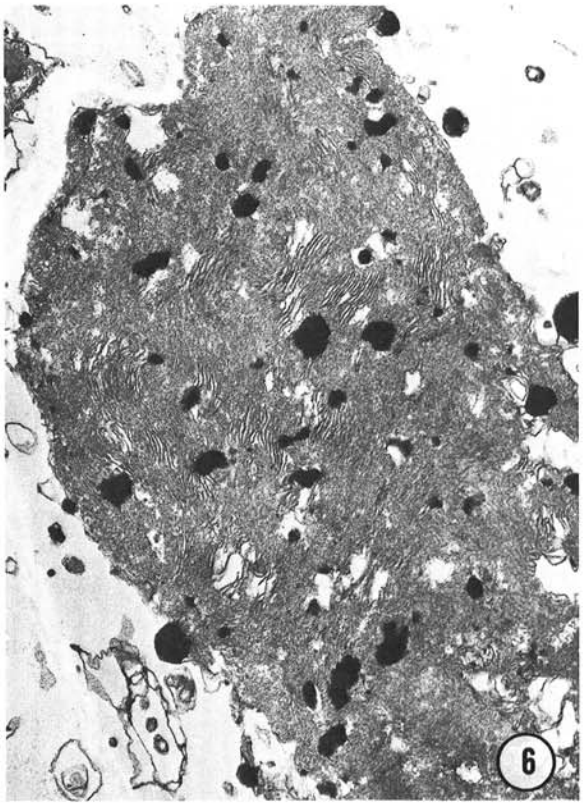
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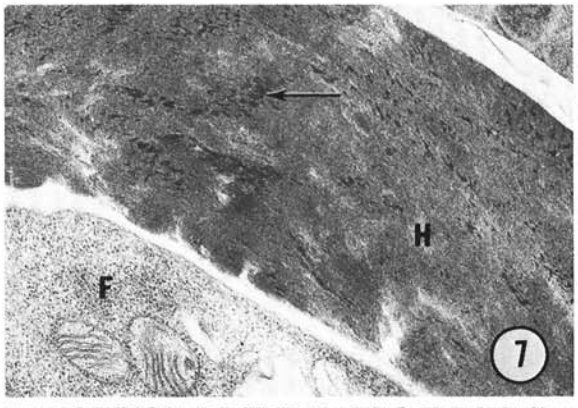
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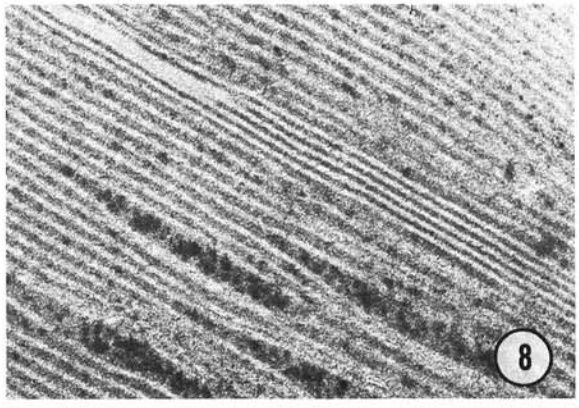
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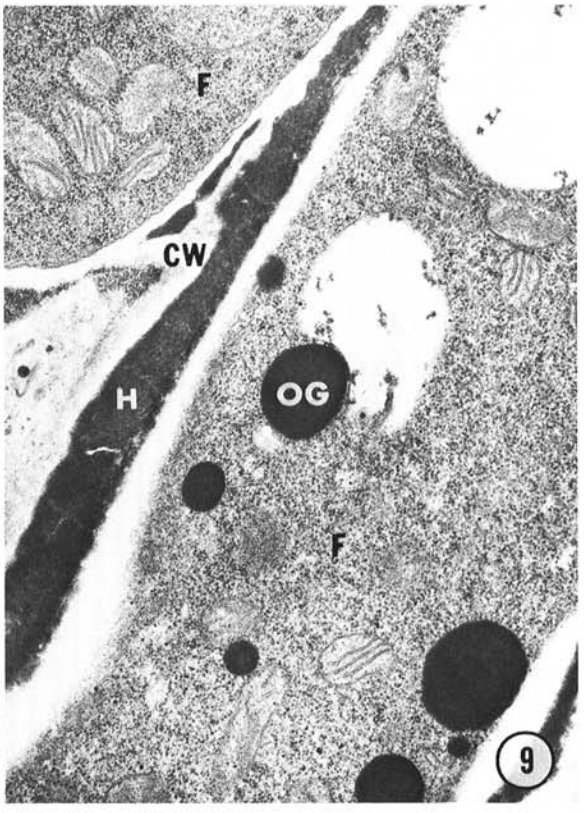
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Fig. 5-9. 5) A degraded chloroplast 24 hr after inoculation. The plastid envelope has disintegrated and numerous, irregular, osmiophilic bodies (arrow) are interspersed within the lamellar system (× 18,200). **6)** A more advanced state of chloroplast breakdown taken from flaccid leaf tissue 48 hr after inoculation. Compare with Fig. 5 (× 18,700). **7)** An advanced stage of host cell degradation taken from a 48-hr necrotic lesion. The host cytoplasm (H) has become a dark, amorphous coagulum with densely staining areas (arrow) scattered throughout. F = fungal hypha (× 19,100). **8)** Negative-image chloroplast membranes from a 48-hr necrotic lesion (× 79,600). **9)** Fungal hyphae (F) from a 48-hr lesion. OG = osmiophilic globule; H = host cytoplasm; CW = host cell wall (× 17,600).

membranes, the components that normally stain (proteins and lipids) are either absent from the membranes or have been altered in such a way as to render them totally nonreactive with the electron stains. Such a change in membrane structure is difficult to interpret, and serves not only as an example of the complexity of host-parasite interaction, but also illustrates the need for caution in the interpretation of electron microscopic data. In this connection, the authors wish to emphasize that this is a descriptive study and that the results should be regarded in terms relative to the methodology employed.

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