Ultrastructure of Cells in Toxin-Treated and Helminthosporium sacchari-Infected Sugarcane Leaves

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ABSTRACT

Helminthosporium sacchari is the causal agent of eye spot disease of sugarcane, and produces a host-specific toxin, helminthosporoside. In this study, resistant and susceptible clones of sugarcane were treated with helminthosporoside and with helminthosporoside-^{14}C for varying periods, then examined by electron microscopy and radioautography. Cells from fungal-infected leaves and from leaves of a susceptible clone treated with the toxin varied from showing virtually no alteration of the cytoplasm to its complete disruption. Furthermore, the cytological alterations seen in fungal infected tissues greatly resembled those observed in susceptible tissues treated with helminthosporoside. Abnormalities in the ultrastructure of chloroplasts in susceptible sugarcane were the earliest cytological disturbances that could be attributed to the action of helminthosporoside. In leaves of a resistant clone treated with the toxin, the chloroplasts and other organelles generally were not damaged. Helminthosporoside-^{14}C was recovered from the same area of the leaf that was examined by electron microscopy, indicating that the toxin was present in the area of the leaf showing cytological abnormalities.

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Helminthosporium sacchari (Van Breda de Haan) Butler is the causal agent of eye spot disease of sugarcane which occurs in many of the sugarcane growing areas of the world (6). *Helminthosporium sacchari* produces eye-shaped lesions on susceptible clones of sugarcane. The production of the lesion is followed by the development of a reddish-brown streak or runner area extending from the lesion toward the tip of the leaf. Martin (6) observed that the fungus could be isolated from the lesion, but not from the runner area, suggesting that a toxic substance was involved. Steiner & Byther (12) succeeded in partially purifying the toxin on Sephadex G-15, and noted that at least two toxic substances were present in culture filtrates, as determined by bioassaying the fractions eluting from the column. The most abundant of the two toxins was purified and characterized by Steiner & Strobel (13) and was shown to be 2-hydroxycyclopropyl-α-D-galactopyranoside, and a trivial name, "helminthosporoside", was given to it. Helminthosporoside was capable of producing the reddish-brown runners only on those clones of sugarcane that were susceptible to the fungus. A biological assay was developed by Steiner & Strobel (13) which consisted of making a small puncture in the leaf to which 1 μliter of a solution of helminthosporoside was placed. After 22 hr of incubation in a moist chamber, the length of the runner that developed was related to the amount of toxin applied. The relationship was linear when plotted on a semilog scale. The first visible indication of toxicity to the treated leaf was the development of a light-green area extending 5 to 10 cm up the leaf from the point of injection. This area, surrounded by the dark-green area of the nonaffected portion of the leaf, appeared within 1 hr after injection of the toxin. From 12 to 24 hr after injection, the affected area of the leaf developed into a reddish-brown streak or runner. No visible effects of the toxin on resistant clones were ever observed (13). Thus, these earlier observations suggested that ultrastructural investigations might reveal information concerning the effect of helminthosporoside upon the host organelles, and give some clue concerning the nature of disease resistance.

MATERIALS AND METHODS.—*Toxin production*.—The culture of *H. sacchari* used in this investigation was originally isolated from naturally infected sugarcane in Hawaii. The organism was maintained on sugarcane leaf extract in agar (20). Helminthosporoside was obtained from cultures grown for 18 to 21 days in 1-liter Roux bottles containing 160 ml of modified Fries medium supplemented with 0.1% yeast extract dialysate (5). The cultures were grown at 22 to 24 C under stationary conditions. Helminthosporoside was purified from these cultures according to procedures described elsewhere (13).

Radioactivity experiments.—Strobel & Steiner (16) observed that mevalonic acid lactone-2-^{14}C (5.8 mc/mM) was an excellent precursor of the aglycone portion of the toxin. Helminthosporoside with a specific activity of 3.38 μc/mmole was obtained from cultures incubated with 50 μc of the lactone of mevalonic acid, and used in these experiments. Radioactivity was measured with a Nuclear Chicago liquid scintillation counter, Model 6804. The

scintillation fluid and method for correction to disintegration per minute (dpm) were as indicated by Strobel & Hess (14).

Radioautograms of leaves treated with helminthosporoside and helminthosporoside-^{14}C were made by placing Kodak No-screen X-ray film over the leaf and exposing it for 2 weeks at −15 C.

Chromatography.—Descending paper chromatography was conducted on Whatman No. 1 filter paper using the following solvents: (i) 1-butanol-acetic acid-H_2O 4:1:5, v/v; and (ii) 1-propanol-NH_4OH-H_2O 6:3:1, v/v. Sugars were detected by the method of Trevelyan et al. (17), and helminthosporoside was detected by spraying the chromatogram with a chloroform solution saturated with $SbCl_3$ and heated at 90 C for 3 min (13).

Tissues and treatments.—Leaves of one eye spot-susceptible clone, 51 NG 97, of *Saccharum officinarum* L., and one -resistant clone, H50-7209, were used in these studies. Sections of whole leaves 20 cm long were incubated in a moist chamber after making a small puncture with a Hamilton syringe (10 μliters) and placing 1 μliter of an aqueous solution of helminthosporoside (30 to 40 μg) on the wound (13). The leaves were incubated in a moist chamber for 0.25, 1, 3, 6, and 12 hr, and at the end of each time period, a section of leaf tissue (5 X 15 mm) located 5 mm above the point of inoculation was removed with a sharp razor blade and cut into 1- X 2-mm pieces. These pieces were fixed in an aqueous solution containing 3% glutaraldehyde and 3% acrolein buffered pH 7.2 to 7.4 with 0.2 M sodium cacodylate (3). Control tissues consisted of those obtained from leaves that had been injected with distilled water. Tissue from susceptible clones (51 NG 97) infected with *H. sacchari* was obtained 3 weeks after inoculation, and was cut from the reddish-brown area located above the eye spot lesion. After fixation, the tissues were embedded in plastic according to the procedures described by Hess (3). The experiment was repeated at least once, and an additional set of tissues obtained and examined.

RESULTS.—*Ultrastructure of control tissue.*—No apparent cytological differences were noted between the resistant and susceptible sugarcane tissues. The leaves of both 51 NG 97 and H50-7209 contained characteristic nuclei, mitochondria, and chloroplasts. The plasma membrane was commonly adjacent to the cell wall, and the cytoplasmic contents of the cells were preserved well by the fixation procedures used (Fig. 1).

Fungal-infected tissue.—The ultrastructure of cells from the runner area extending from the eye spot lesion varied from no observable cytoplasmic disruption to severe disruption of cellular contents. In general, cellular damage was greater and more extensive in this tissue than in toxin-treated leaves. Laetsch & Price (4) have shown that vascular bundle sheath cells of sugarcane contain starch-storing chloroplasts with parallel lamellae which commonly lack grana. Adjacent mesophyll cells contain chloroplasts which store little starch and possess abundant grana. In fungal infected tissue, both types of chloroplasts became severely disrupted. Furthermore, during advanced stages of tissue decomposition, chloroplasts with parallel lamellae were difficult to distinguish (Fig. 2). In cells having chloroplasts that were greatly damaged, the other organelles were commonly disrupted beyond recognition. However, some cells were so badly disrupted that chloroplast lamellae were difficult to recognize (Fig. 3).

Toxin-treated leaves.—The ultrastructure of susceptible leaves treated with the toxin closely resembled those infected by the fungus. Leaves which were treated for 1 hr or less were not as severely affected as those treated for 12 hr. During the shorter toxin treatments (15 min to 1 hr) the cellular membranes, including those of the organelles, were mostly intact, although the chloroplast lamellae began to separate. Figure 4 shows one of these chloroplasts during the early stages of alteration in a cell that was treated for 1 hr. This phenomenon was not observed in cells of leaves that served as a control. Some chloroplasts in leaves treated for only 1 hr were more severely affected than is evident in Fig. 4, and others were not noticeably affected. More than half the cells examined in leaves in the 12-hr toxin treatment contained disrupted chloroplasts (Fig. 5). Generally, cells containing damaged chloroplasts also possessed extreme disruption of other cytoplasmic contents. In Fig. 5, the chloroplasts are still somewhat intact, although the outer chloroplast membranes are completely disrupted. The cell wall and remnants of the plasma membrane are evident, but the integrity of the cytoplasm has been destroyed.

Generally, no detectable ultrastructural alterations were present in toxin-treated leaves of the resistant host, except occasional chloroplasts which had ruptured membranes, and lamellae that appeared to be separating (Fig. 6). Completely disrupted chloroplasts were never observed.

Location of -^{14}C.—Helminthosporoside-^{14}C in doses of ca. 50 μg (1,600 dpm) in 1 μliter were injected into several leaves of the susceptible and resistant cultivars. Symptoms (light-green areas) appeared on the susceptible clone extending at least 10 cm above the point of injection 1 hr after injection, but the X-ray film did not show radioactivity in this area, probably due to the relatively low specific radioactivity of the toxin.

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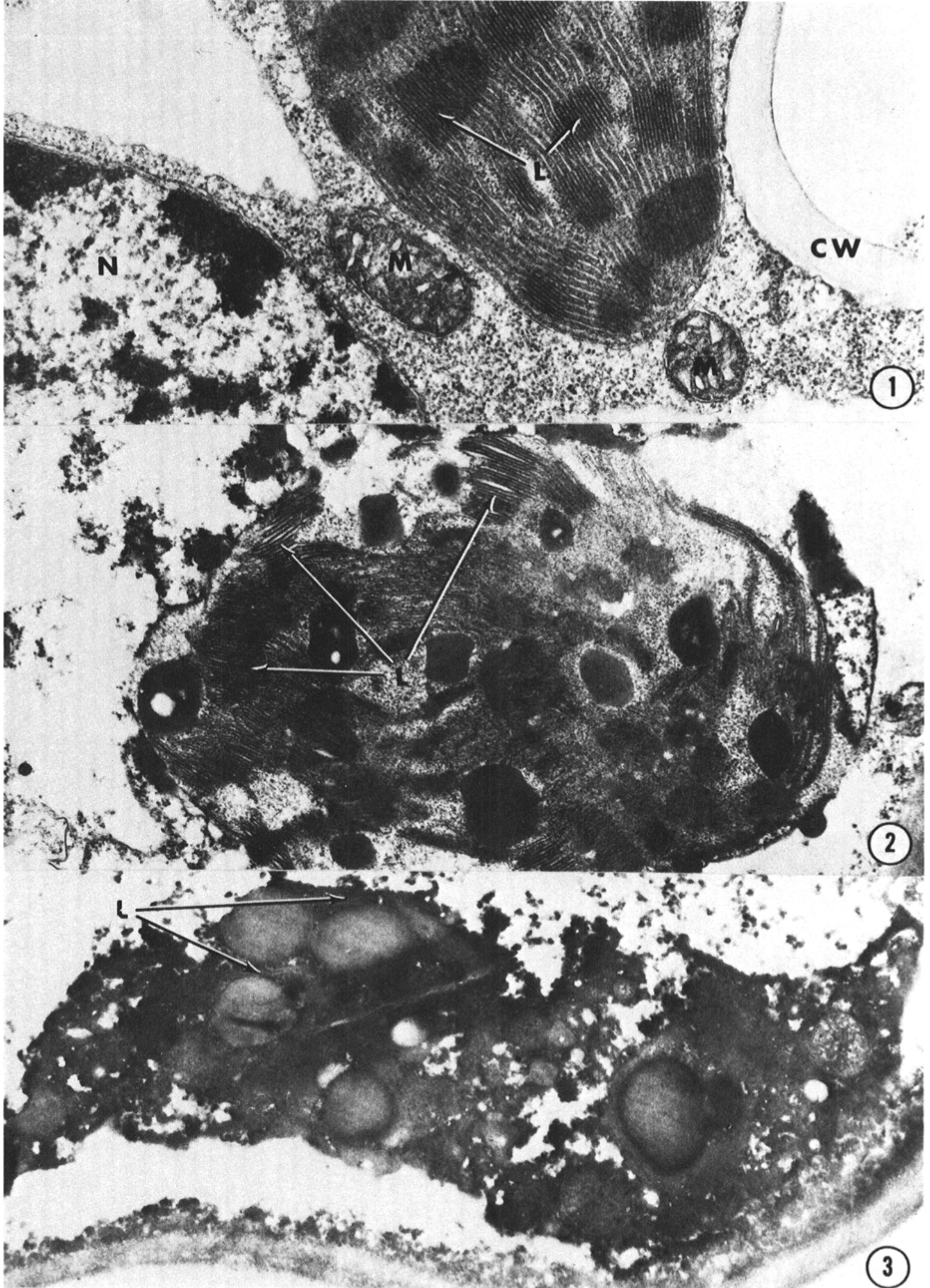
Fig. 1-3. Susceptible sugarcane leaf cells. **1)** Portion of a control leaf cell showing the cell wall (CW), a portion of the nucleus (N), Mitochondria (M), and a chloroplast with lamellae (L) (X 26,500). **2)** Portion of a leaf cell from the runner area that extends upwardly from the lesion of the leaf infected with *Helminthosporium sacchari*, showing a disrupted chloroplast and disrupted lamellae (L) (X 22,500). **3)** Portion of a cell from the runner area of a leaf infected with *H. sacchari* showing a disrupted chloroplast and decomposed lamellae (L) (X 22,500).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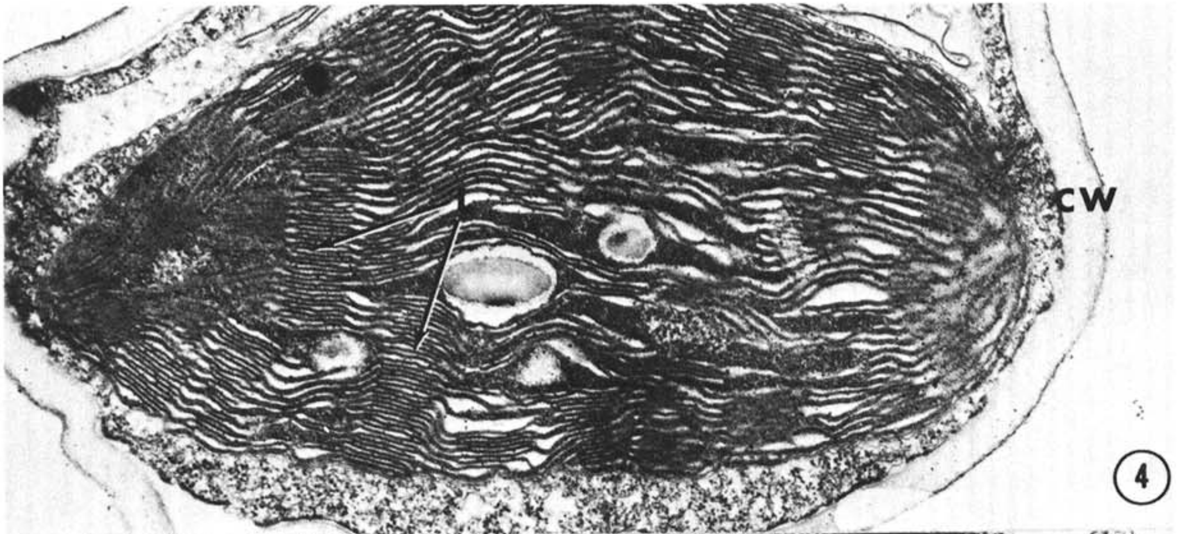
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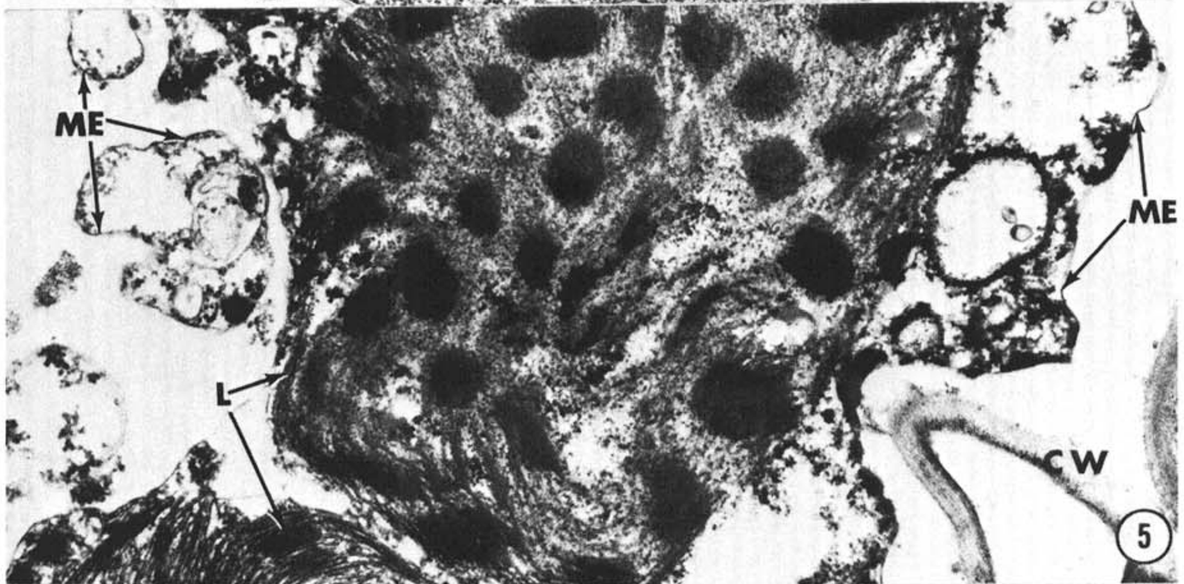
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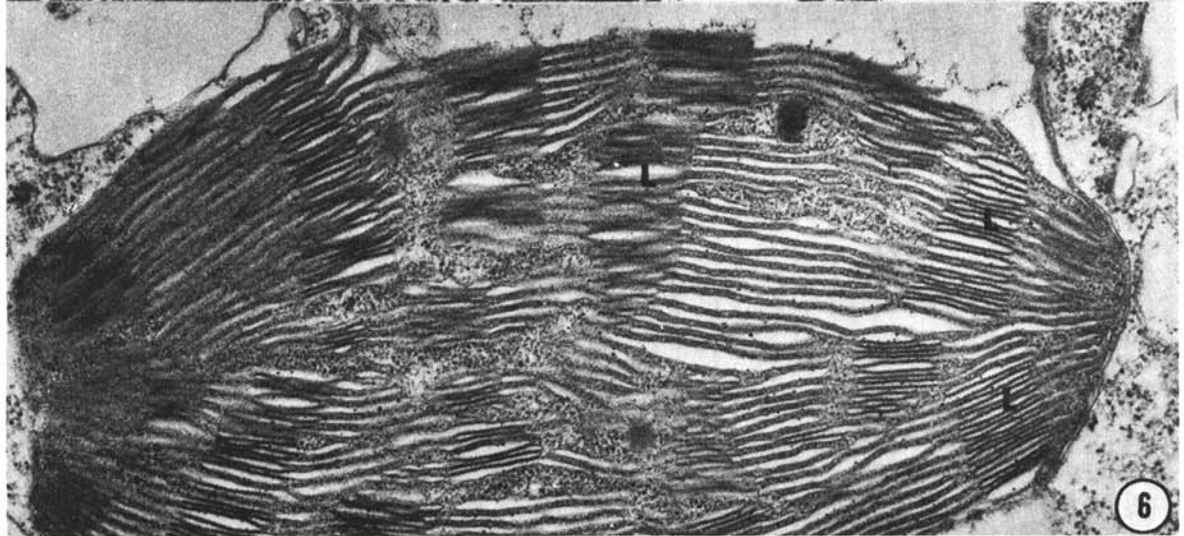
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From the 12-hr treatment, which resulted in symptoms extending to the upper cut end of the leaf, X-ray films showed radioactivity above the point of inoculation in both clones. To determine how much radioactivity was present in tissue above the point of injection at varying periods, tissue sections of the same size and location in the leaf as those taken for electron-microscopic observations were removed and cut into 1- X 2-mm pieces. All these pieces were refluxed in 1.0 ml of H_2O-saturated 1-butanol for 1 hr. The butanol was removed from the flask and evaporated to dryness. The residue was taken up in 1.0 ml of H_2O and 0.1 ml counted by the liquid scintillation technique. Figure 7 shows that radioactivity was present in the same area (5 X 15 mm) of the leaf that was also examined by electron microscopy, and that the amount of radioactivity in this area increased with an increase in the incubation period. No great differences were noted in the amount of radioactivity recovered from the treated leaves of both clones. Aliquots of the residue obtained after the butanol extraction of the 12-hr treatments were chromatographed in solvent a and solvent b to determine which compounds were labeled. After chromatography, the chromatograms were sectioned into 2.5-cm strips, cut into small pieces, and placed into the scintillation solution and counted. Figure 7 shows that the majority of the radioactivity in any given substance was present in a compound cochromatographing with authentic helminthosporoside in solvent b. A chromatogram of the extract from the treated susceptible leaf was identical with the chromatogram shown in Fig. 8. Furthermore, chromatograms of the extracts from the leaves treated for 12 hr with helminthosporoside-^{14}C, developed in solvent a, also showed that the majority of the radioactivity chromatographed with an R_F identical with authentic helminthosporoside. In addition, the compound having the same R_F as authentic helminthosporoside in solvent b, when eluted from chromatograms and injected into leaves of the suceptible clone, gave typical symptoms produced by the toxin.

DISCUSSION.—Ultrastructural studies on toxin-treated resistant and susceptible clones of sugarcane revealed that one effect of helminthosporoside is on cellular membranes (Fig. 4-6), either directly or indirectly. This is not unexpected, as others have made similar observations with host-specific toxins (8, 10, 11, 18, 19), toxic bacterial glycopeptides (9, 15), and in bacterial-host relationships (1, 2). The effects of helminthosporoside on the leaf tissue are rapid, and extremely small amounts (5 X 10^{-12} moles) are sufficient to produce symptoms on the leaf (13). Steiner & Strobel (13) suggested that

Fig. 7-8. 7) The total amount of radioactivity in the sample area (5 X 15 mm) on resistant and susceptible sugarcane leaves as a function of time. Each treatment consisted of the placement of 1 μliter of toxin-^{14}C containing 1,600 disintegrations per minute (dpm) on a small wound made in the leaf tissue. At the end of each time period the sample area was removed, cut into small pieces, and extracted with butanol. **8)** A histogram of the radioactivity detected on a chromatogram developed in 1-propanol, NH_4OH, H_2O 6:3:1, v/v, of 25% of the extract obtained from the susceptible sugarcane leaf treated with helminthosporoside-^{14}C (1,600 dpm). The diagram illustrates the location of authentic galactose (G) and authentic helminthosporoside (H). The scale in cm should be multiplied X 4 to give the actual size of the original chromatogram.

Fig. 4-6. Sugarcane leaf cells. **4)** Portion of a susceptible leaf cell which was treated for 1 hr with helminthosporoside showing the cell wall (CW) and altered lamellae (L) in a partially disrupted chloroplast (X 22,500). **5)** Portion of a susceptible leaf cell treated 12 hr with helminthosporoside, showing disrupted chloroplasts with altered lamellae (L), the cell wall (CW), and remnants of membrane (ME) (X 24,000). **6)** Portion of a resistant leaf cell treated 12 hr with helminthosporoside showing a chloroplast with disrupted membranes and separating lamellae (L) (X 26,500).

helminthosporoside may affect the lysosome membrane of the cell, resulting in its rupture leading to the release of degradative enzymes. Matile (7) has shown that proteases, deoxyribonuclease, ribonuclease, β- and α-glucosidases, and phosphatases are among other degradative enzymes present in the plant lysosome. If released from the lysosome by toxin action, these enzymes could degrade structures in the toxin-affected cell as well as those in adjacent cells, accounting for the extreme cellular disruption that is observed in toxin-treated susceptible tissue. Although no direct evidence is presented in this report that supports the lysosome disruption theory, some indirect evidence relates to this point. Namely, external symptoms in toxin-treated leaves become evident in 0.5 hr after inoculation, yet the first substantial ultrastructural change occurs in chloroplasts after 1 hr. Although some changes appear to occur in cellular membranes 15 to 30 min after inoculation, they are slight and inconsistently observed. It seems conceivable that the permeability of the lysosome membrane could be altered to permit the efflux of degradative enzymes without alterations being noticeable at the ultrastructural level. Alternatively, the toxin may have a direct effect on a number of cellular membranes, including those of the chloroplast (Fig. 4, 5). This notion is in keeping with the observation that an occasional chloroplast in resistant tissue is apparently affected by helminthosporoside. Ultimately, however, it would appear that the reddish-brown runner area produced on susceptible clones is a result of browning and oxidative reactions occurring with the release of enzymes that act on substrates freely distributed in the cell.

That the intact toxin was actually present in the same area of the leaf which was examined ultrastructually was determined by extraction and chromatography of leaves treated with helminthosporoside-^{14}C (Fig. 7). Not enough labeling had reached this area for chromatographic studies to be made at incubation times of less than 12 hr. Interestingly enough, no differences were observed in the labeling patterns on the chromatograms made of the extracts of the toxin-treated susceptible and resistant clones. From this it might be suggested that toxin degradation by the sugarcane leaf is not an immediate mechanism of disease resistance. Other areas of radioactivity appeared on chromatograms (Fig. 8), which does not preclude the possibility that some modification of the toxin does occur in the plant or during chromatography.

Helminthosporoside produces symptoms (runners) on susceptible leaves that are identical to those produced by the pathogen (12, 13). Some evidence for the presence of the helminthosporoside in naturally infected leaves has been obtained (G. A. Strobel & G. W. Steiner, *unpublished data*). Thus, a possible explanation for the more severe cytological effects observed in the runner areas of the fungal infected leaves is that these cells were exposed to the toxin for a longer period (Fig. 2, 3). The results of this study further implicate the importance of toxin production by this fungus to the production of symptoms of this disease.

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