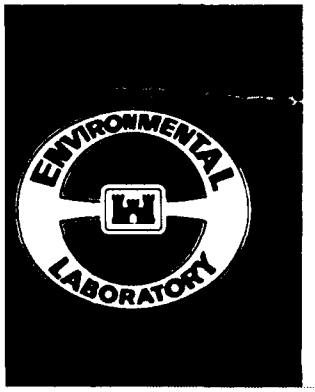

AQUATIC PLANT CONTROL
RESEARCH PROGRAM

TECHNICAL REPORT A-91-6

HISTOLOGY OF INFECTION OF *HYDRILLA VERTICILLATA* BY *MACROPHOMINA PHASEOLINA*

by

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An isolate of *Macrophomina phaseolina* (Tassi) Goid. has demonstrated potential as a biocontrol agent for the noxious submersed aquatic macrophyte *Hydrilla verticillata* (L.f.) Royle in recent studies.

The purpose of this study was to follow the histology of infection of *H. verticillata* by *M. phaseolina* with particular attention to the chronology of attachment, penetration, and colonization during the early stages of pathogenesis.

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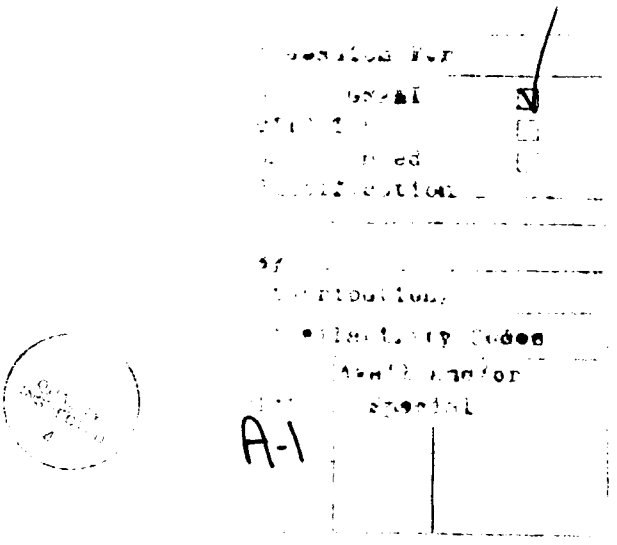

Preface

The work reported herein was conducted as part of the Aquatic Plant Control Research Program (APCRP). The APCRP is sponsored by the Headquarters, US Army Corps of Engineers (HQUSACE), and is assigned to the US Army Engineer Waterways Experiment Station (WES) under the purview of the Environmental Laboratory (EL). Funding was provided under Department of the Army Appropriation 96X3122, Construction General. The APCRP is managed under the Environmental Resources Research and Assistance Programs (ERRAP), Mr. J. L. Decell, Manager. Mr. Robert C. Gunkel was Assistant Manager, ERRAP, for the APCRP. Technical Monitor during this study was Mr. James W. Wolcott, HQUSACE.

This report was prepared by Dr. Gary F. Joye of the WES, employed under an Intergovernmental Personnel Act agreement with the Department of Pathology and Crop Physiology, Louisiana State University, Baton Rouge, LA, and by Dr. Rex Paul of the US Department of Agriculture Agricultural Research Service, Southern Weed Science Laboratory, Stoneville, MS. Dr. Joye was Principal Investigator. The report was edited by Ms. Jessica S. Ruff of the WES Information Technology Laboratory.

The study was conducted under the direct supervision of Dr. Alfred F. Cofrancesco, Jr., Team Leader, Biomanagement Team, and Dr. Edwin A. Theriot, Chief, Aquatic Habitat Group, and under the general supervision of Dr. C. J. Kirby, Chief, Environmental Resources Division, and Dr. John Harrison, Chief, EL.

Commander and Director of WES was COL Larry B. Fulton, EN. Technical Director was Dr. Robert W. Whalin.

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1 Introduction

An isolate of *Macrophomina phaseolina* (Tassi) Goid. has demonstrated potential as a biological control agent for the problematic submersed aquatic macrophyte *Hydrilla verticillata* (L.f.) Royle (Joye 1990). Little is known about the interaction of this pathogen with its aquatic host. Therefore, the objective of this study was to examine the host/pathogen relationship between *M. phaseolina* and hydrilla, with particular attention to attachment, penetration, and colonization during the early stages of pathogenesis.

2 Materials and Methods

Preparation and Inoculation Technique

Hydrilla sprigs (10 cm) were taken from outdoor ponds at the US Army Engineers Lewisville Aquatic Ecosystem Research Facility in Lewisville, TX. These were washed in distilled water and placed in 1.5- by 10-cm plastic petri dishes filled with 20 ml of sterile distilled water. Cultures of *M. phaseolina* (isolate FHy18) were grown for 7 days in 250-ml Erlenmeyer flasks with 100 ml of Richard's V8 broth on a shaker at 150 rpm at 25 °C. The fungal cultures were blended in a Waring blender for 15 sec to produce a hyphal suspension for use as inoculum. Plants were inoculated with 10 ml of blended liquid fungal inoculum.

To maintain consistency in the concentration of solutions for transmission electron microscopy (TEM) fixation procedures, inoculations were staggered so that all samples could be fixed and stained from the same batch of TEM solutions. Plants in four plates were inoculated starting with the longest time of exposure to the pathogen, i.e., 288 hr (2 weeks), again at 196 hr (1 week), and then every 8 hr starting at the 72-hr preharvest time to 0 hr. This procedure produced four plant samples inoculated at each of 12 time periods. Tissue samples from uninoculated plants were also fixed and stained for comparison to inoculated tissue samples.

Fixation of Samples for Transmission Electron Microscopy

Samples were fixed in 2-percent glutaraldehyde in 0.05-percent cacodylate for 2 hr, washed in 0.01-percent cacodylate for 30 min, and post-fixed in 1-percent osmium tetroxide for 2 hr. Samples were then washed in distilled water for 10 min, and serially dehydrated for 2 min each in 25-, 50-, and 70-percent acetone; for 30 min in 70-percent acetone with 1 percent weight/volume p-phenylenediamine; and for 2 min each in 70- and 90-percent acetone. Finally, the samples were exposed to three 5-min dehydrations in 100-percent acetone, and then embedded in Spurr's resin.

Thin sections (70 nm) were cut with a diamond knife on a Reichert Ultracut E Ultramicrotome (Cambridge Instruments, Inc., Buffalo, NY). Sections were examined with a Zeiss Em10 CR Transmission Electron Microscope (Carl Zeiss, Inc., Thornwood, NY).

3 Results

Characteristics of Normal Hydrilla Leaves

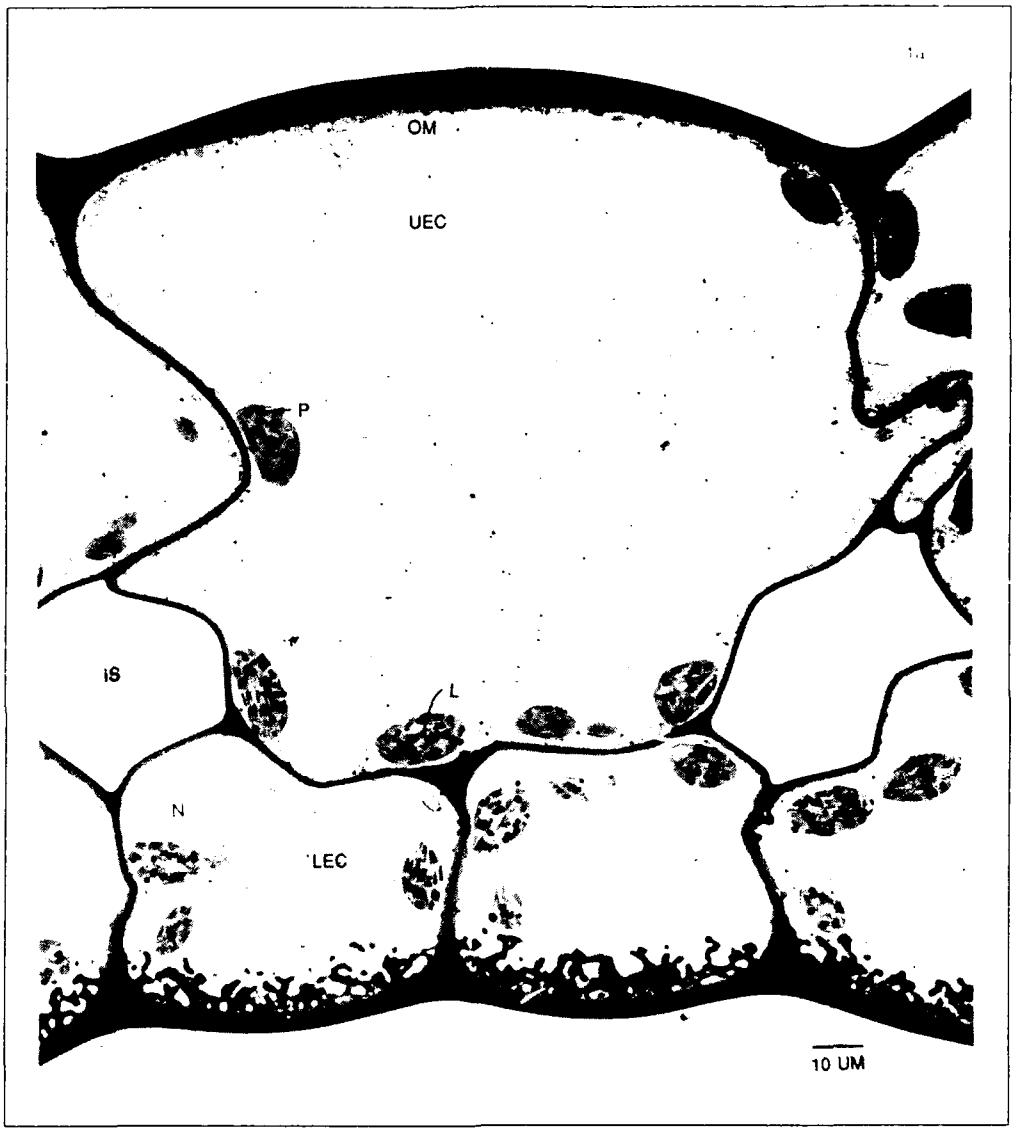
In normal tissue (Figure 1a), the leaves are composed primarily of two cell layers—an upper and lower epidermis. Both epidermal cell layers appear to possess a cuticle. The upper epidermal cells are several times larger than the lower epidermal cells. Within the upper epidermal cells, below and adjacent to the upper cell wall is an osmiophilic layer of unknown composition. Material from this layer appears to permeate the cell wall (Figure 1b). Epidermal cells contain numerous chloroplasts that stain darkly in the presence of osmium tetroxide.

Within lower epidermal cells, a "wall labyrinth" (Pendland 1976) is associated with the outer cell wall (Figure 1c). This area is reported to be of a polysaccharide nature (Martino and Zamboni 1967). Cells with these ingrowths have been described as transfer cells, and the increased surface area of the plasmalemma is thought to allow greater absorption of solutes from the environment (Gunning and Pate 1969, Pendland 1976).

Inoculated Hydrilla Tissue

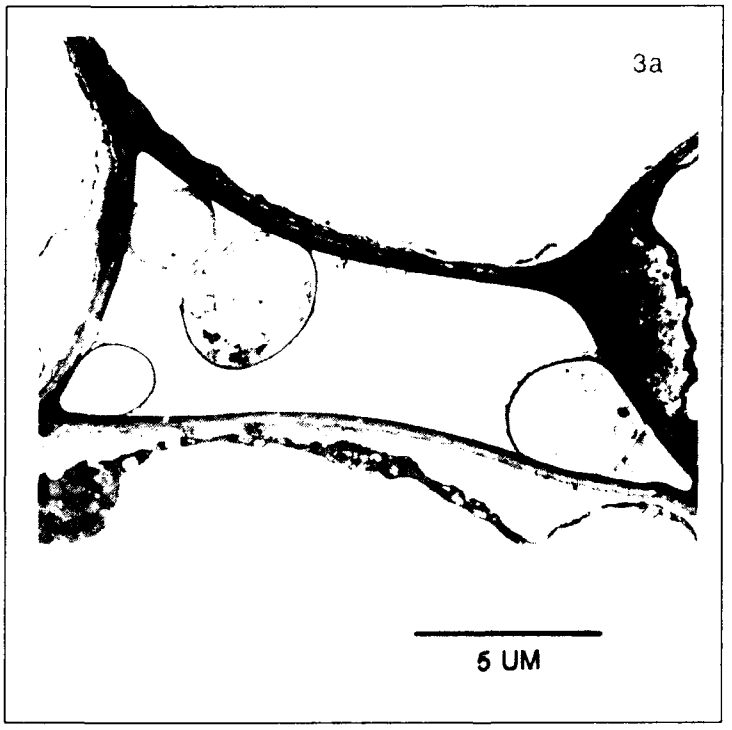
No fungal cell attachment was observed within the first 8 hr after inoculation. However, chloroplasts of the lower epidermal cells appeared to be affected by some agent made evident by differential staining. Affected chloroplasts were distinctly less osmiophilic than unaffected chloroplasts, and the grana of affected chloroplasts were noticeably disorganized when compared with unaffected chloroplasts (Figure 2). The cause of the changes in chloroplasts appeared to be related to some unknown agent that was adsorbed by the lower epidermal cells but not by the upper epidermal cells.

Between 16 and 40 hr after inoculation, fungal cells attached to and penetrated the outer cell wall of the lower epidermis of the host (Figure 3a). Channels observed in the cuticle between the two cells appeared to provide evidence of enzymatic breakdown of the cell wall (Figure 3b). No fungal cells were observed attached to the cell wall of the upper



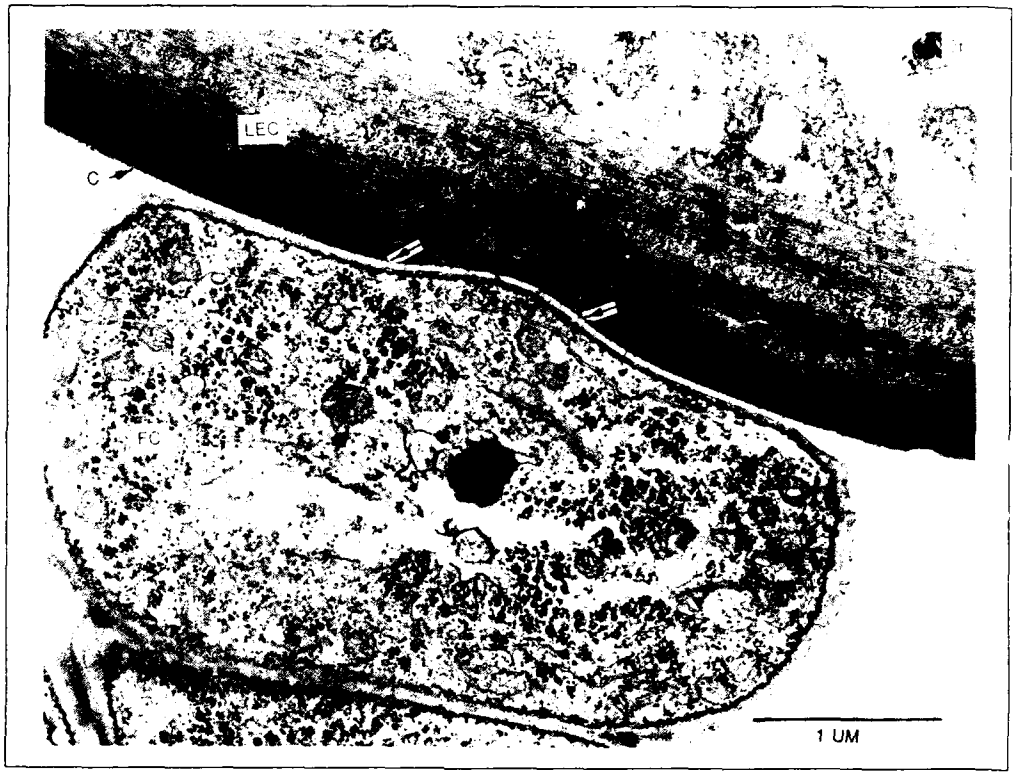
a. View of upper epidermal cells (UEC) and lower epidermal cells (LEC), showing the ultrastructural differences. The UEC are several times larger than the LEC, and they possess an osmiophilic layer (OM) below the outer cell wall. No OM is present in the LEC. (Note: P = plastid, IS = intercellular space, L = lipid, N = nucleus)

Figure 1. Normal, uninoculated cells of a hydrilla leaf (Continued)

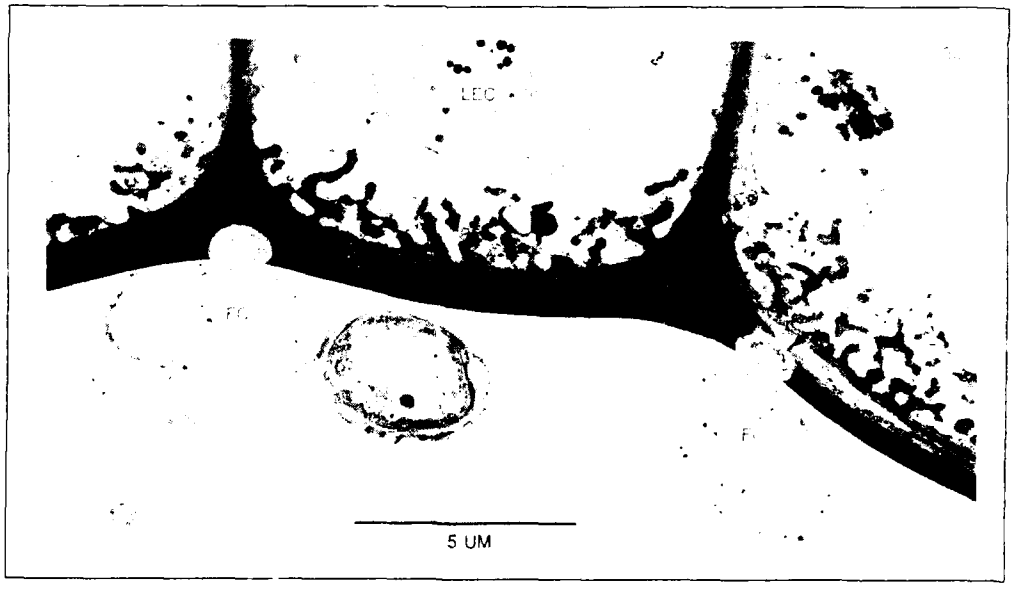


a. Fungal cells of *M. phaseolina* attached to the host cell wall in the intercellular space in a hydrilla leaf

Figure 3. Sequence of damage observed in inoculated hydrilla tissue (Sheet 1 of 4)

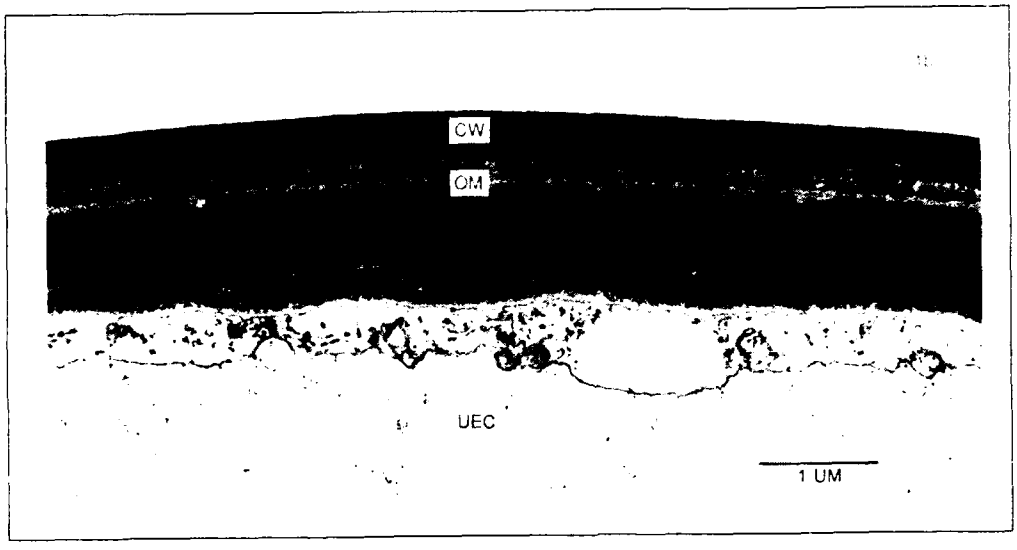


b. Channels (C) form in the cuticle between *M. phaseolina* and a lower epidermal cell wall (LEC) of hydrilla (arrow denote channels). (Note: FC = fungal cell)

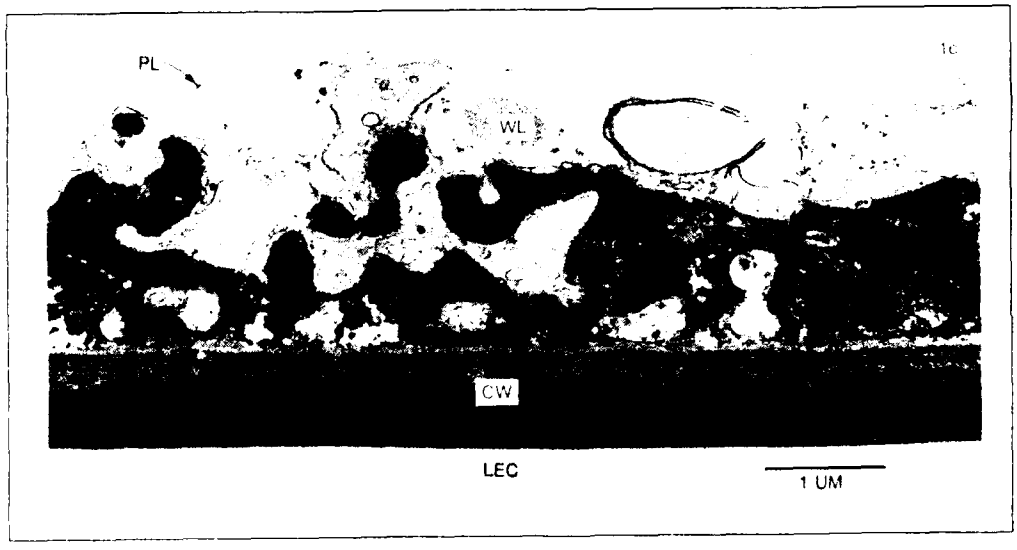


c. Early penetration of *M. phaseolina* into the lower epidermal cell (LEC) of hydrilla

Figure 3. (Sheet 2 of 4)



b. Closeup view of an osmiophilic layer (OM) of an upper epidermal cell (UEC) below and adjacent to the cell wall (CW)



c. Closeup view of a wall labyrinth (WL) of a lower epidermal cell (LEC). (Note: PL = plasmalemma, CW = cell wall)

Figure 1. (Concluded)

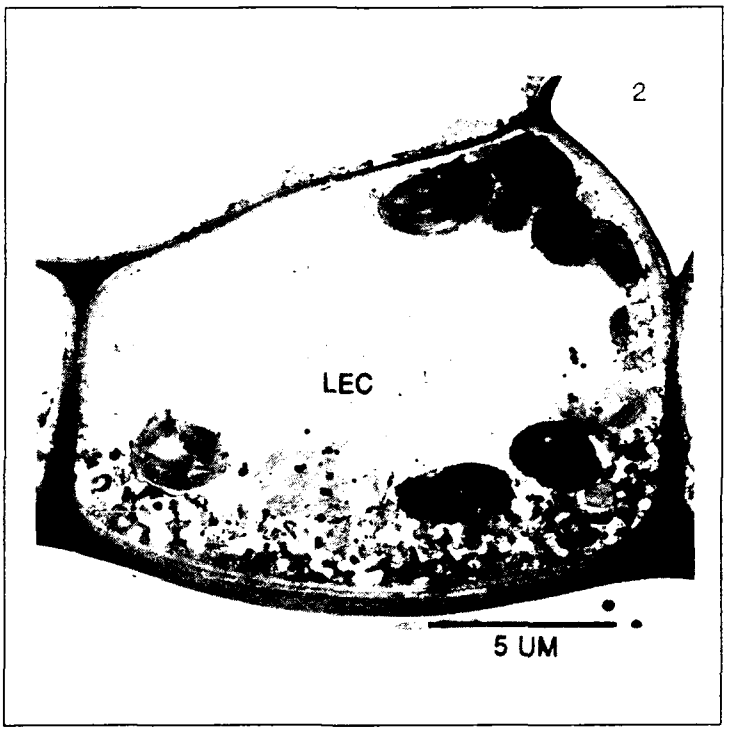
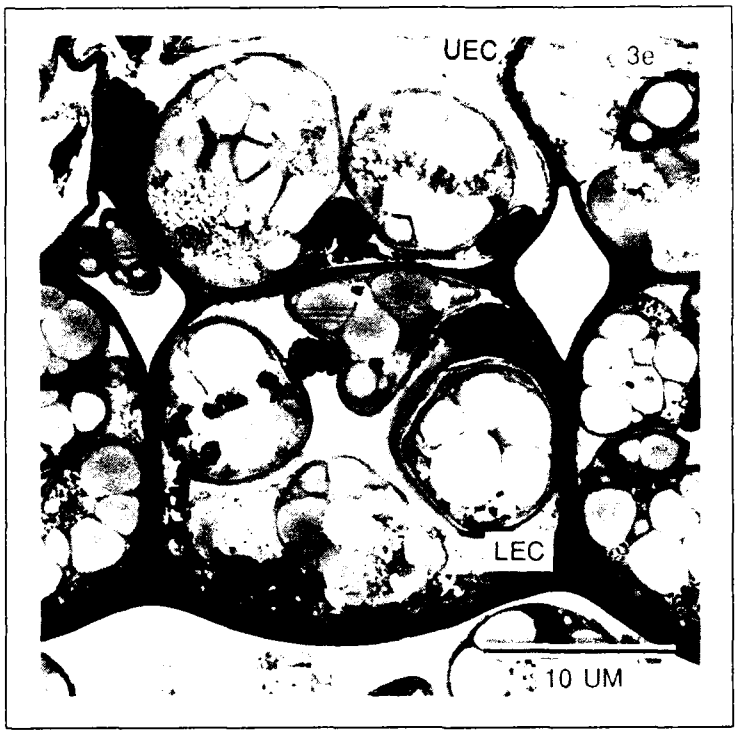


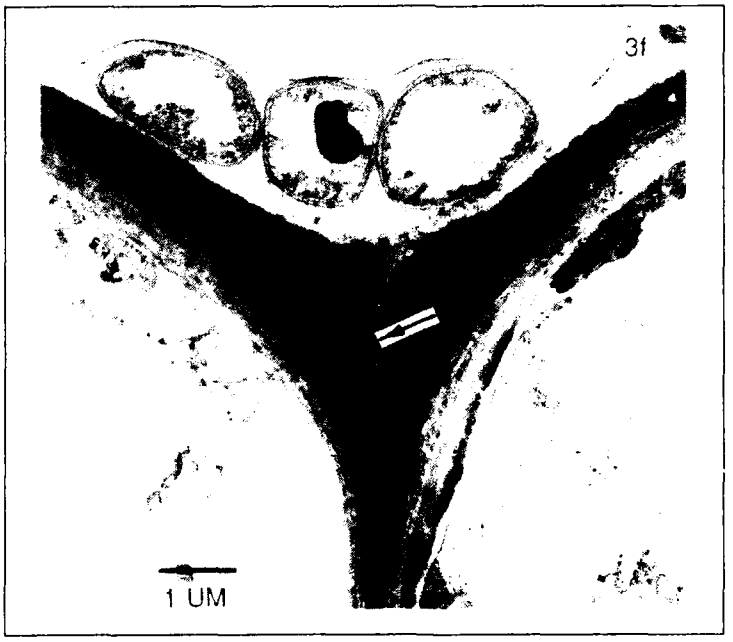
Figure 2. Differential staining of chloroplasts after exposure to osmium tetroxide in a lower epidermal cell (LEC) of hydrilla, indicating probable reaction to toxins produced by *M. phaseolina*. Affected plastids stain less darkly than unaffected plastids

d. Penetration of *M. phaseolina* into a lower epidermal cell (LEC) of hydrilla (HA = hypha, CW = cell wall of host cell, S = fungal septum). Evidence of enzymatic breakdown of the host cell wall in indicated by the absence of any sign of bending, as a result of mechanical pressure from the fungus (arrow)

Figure 3. (Sheet 3 of 4)



e. Epidermal cells of hydrilla colonized by *M. phaseolina*. (Note: UEC = upper epidermal cell, LEC = lower epidermal cell)



f. Middle lamella (arrow) of epidermal cells of hydrilla splitting apart after colonization of host tissue

Figure 3. (Sheet 4 of 4)

epidermis. Although fungal cells were observed in host cells within 40 hr after inoculation, penetration into the host cytoplasm was not observed until 72 hr after inoculation (Figures 3c and 3d). Fungal cells were also observed between the cuticle and the cell wall between 40 and 72 hr.

Colonization of hydrilla foliage continued until the host cells were completely filled with fungal cells (Figure 3e). In later stages of infection, the middle lamella was weakened, and separation of host cell walls (Figure 3f) eventually led to complete collapse of the host.

4 Discussion

Macrophomina phaseolina was able to attach to, penetrate, and colonize foliar tissue of hydrilla within 72 hr after inoculation. Plants were asymptomatic until approximately 196 hr after inoculation. By 288 hr, hydrilla leaves were completely colonized by the pathogen. Cellular integrity was destroyed, resulting in the collapse of the entire plant. The host tissue turned dark brown and became flaccid. Disease symptoms were similar to those observed in previous greenhouse and field experiments (Joye 1990).

In the present study, attachment of the fungus to hydrilla tissue may have been preceded by activity from toxins and/or enzymes, as suggested by the differences in staining and grana organization of plastids between inoculated and uninoculated tissue and between the upper and lower epidermal cells. Chloroplasts of hydrilla treated with naturally occurring growth inhibitors became similarly less osmiophilic and distorted (Dooris, Dooris, and Martin 1988). Similar phenomena have been observed in other plants (Goodman, Kiraly, and Wood 1986). Toxins produced by other isolates of *M. phaseolina* have been characterized, and these may also be inhibitory to normal cell function in hydrilla (Chan and Sackston 1973, Deshpanda and Wadje 1978, Bhattacharya et al. 1987).

Observations on penetration of the host by *M. phaseolina* suggest that both cellulases and pectinases may be involved in the infection process. At the point of contact between the host and pathogen, the cell wall appeared to dissolve prior to actual penetration of the pathogen. There was no sign of inward bending of the host cell wall resulting from mechanical pressure, which indicates that enzymes were involved in the infection process. However, there was considerable distortion of cellular tissue prior to penetration.

A similar response has been observed with other isolates of this pathogen on other hosts (Ammon, Wyllie, and Brown 1974; Madkour and Aly 1981). Preliminary results suggest that filtrates of this pathogen may be detrimental to hydrilla (G. F. Joye, unpublished data). Isolates of *M. phaseolina* are well known for production of pectinases and cellulases that aid in the infection process (Goel and Mehrotra 1974, Deshpanda and

Wadje 1978, Gangopadhyay and Wyllie 1978, Madkour and Aly 1981, Srivastava and Dhawan 1982).

Inhibition of penetration through the upper epidermal cell wall may be attributable to the osmiophilic layer below the cell wall. Although this layer was not characterized, we suggest that this layer is phenolic in nature. Tannins have been reported in members of the family Hydrocharitaceae, to which hydrilla belongs (Ancibor 1979). Phenolics are known to inhibit growth of this pathogen (Gangopadhyay and Wyllie 1978). The apparent inhibition of fungal penetration through the upper epidermal cells was probably a passive response from the host, since the osmiophilic layer was present in both uninoculated and inoculated tissue.

A comparison of the interaction of *M. phaseolina* and hydrilla with interactions of this pathogen and terrestrial plants revealed one significant difference. Although sclerotia formation by this pathogen is preceded by colonization and is considered "indicative of host cell death" for terrestrial plants (Short, Wyllie, and Ammon 1978), sclerotia formation is apparently not a good indicator of this pathogen in aquatic plants, at least not for hydrilla. The absence of sclerotia may be an indication that survival in an aquatic environment is accomplished simply as hyphal colonization of organic debris. However, this isolate produces sclerotia in colonized hydrilla if the infected plant is not submersed (G. F. Joye, unpublished data).

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