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Electron Microscopy of the Penetration and Colonization of Waterhyacinth by *Acremonium Zonatum*[1]

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ABSTRACT

The penetration and colonization of waterhyacinth [*Eichhornia crassipes* (Mart.) Solms] by *Acremonium zonatum* (Sawada) Gams was examined ultrastructurally to aid in the evaluation of the pathogen as a biocontrol agent for this aquatic weed. Penetration of waterhyacinth leaves by the fungus occurred either through the stomata or directly through the unbroken cuticle. An inward displacement of the cuticle and papillae formation were observed in micrographs of infected cells which is suggestive of mechanical pressure exerted by the fungus during penetration. Electron micrographs indicated that penetration was apparently enhanced by the localized secretion of fungal pectinases and cellulases. Penetration of the host's specialized phenol cells resulted in the apparent death of the invading hyphae and these phenol cells may have limited infection and spread. Cytological changes noted in infected cells included the disappearance of starch granules from the chloroplasts, an increase in size and number of plastoglobuli in the chloroplasts, and an increase in the number of microbodies in the cytoplasma.

Key words: phenol cells, disease resistance, papillae, plastoglobuli, biocontrol, ultrastructure, pathogen.

INTRODUCTION

Acremonium zonatum (Sawada) Gams [= *Cephalosporium zonatum* Sawada], the casual agent of a zonate leafspot disease of waterhyacinth [*Eichhornia crassipes* (Mart.) Solms], was first isolated by Rintz in 1973 (16) and further evaluated as a biocontrol agent by Martyn and Freeman (12). The disease is first evident as small sunken lesions on both upper and lower leaf surfaces and the petiole. Infection is favored by high humidity and under conditions of prolonged high humidity, severe spotting and death of the plants may occur (12). *A. zonatum* produces conidia in muscilagenous heads (16) and is, therefore, not conducive wind dissemination. Under natural conditions, insects appear to be important in both the dissemination of the conidia and inoculation of the plant (7,8). Little is known about the infection process and subsequent tissue colonization of waterhyacinth by *A. zonatum.* Martyn (11) and Martyn *et al.,* (15) described specialized phenol-storing cells in waterhyacinth leaves and implicated them (11) in disease resistance by limiting the spread of the pathogen. The purpose of this investigation was to report the infection process and subsequent colonization of waterhyacinth leaves by *A. zonatum* under greenhouse conditions.

MATERIALS AND METHODS

Waterhyacinths were collected from natural infestations

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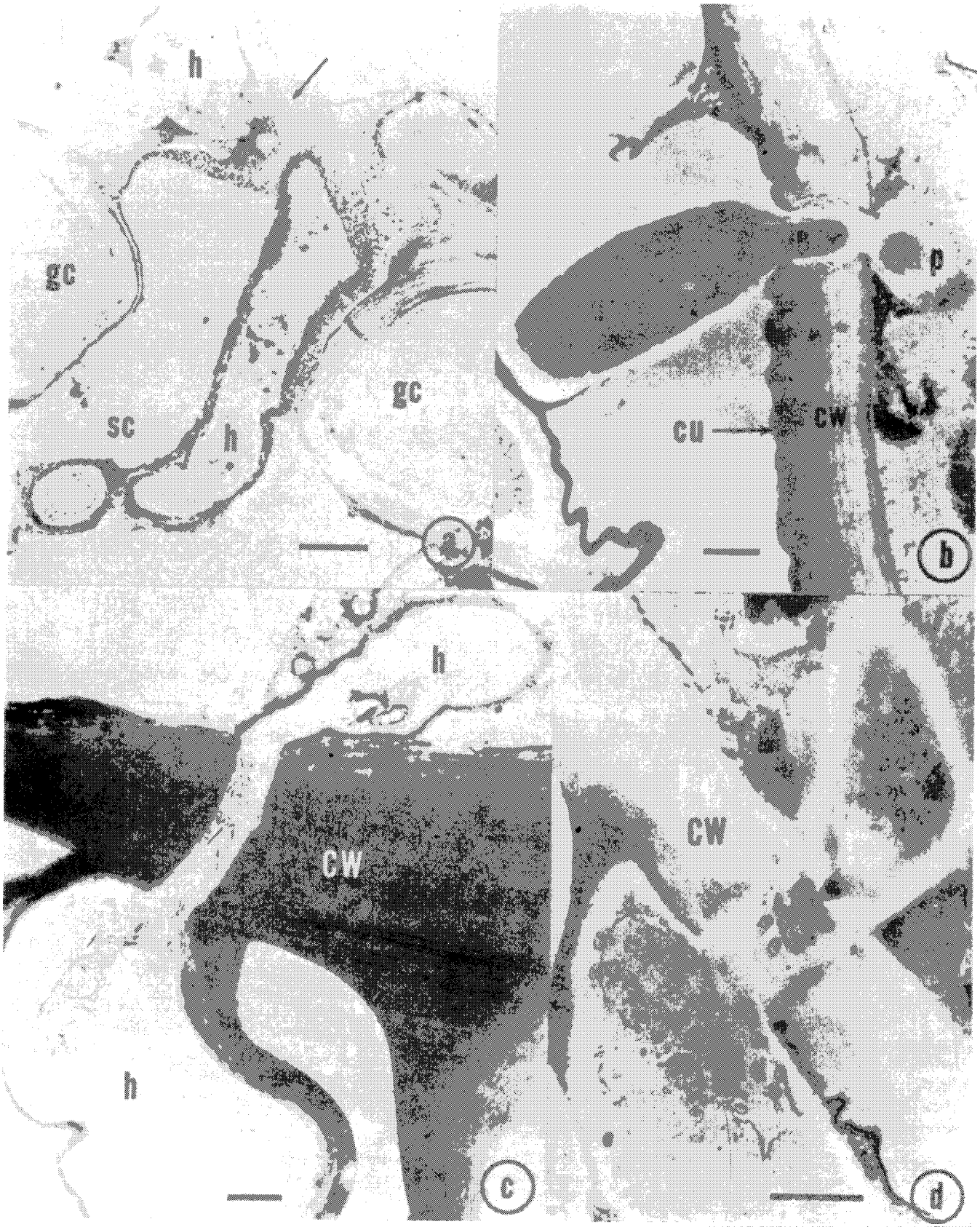

Figure 1. Penetration of waterhyacinth leaf by *Acremonium zonatum*. a) Hypha penetrating through an open stomate. Arrow points to the muscilagenous cementing matrix. h = hypha, gc = guard cell, sc = substomatal cavity. Scale bare = 2μm. b) Hypha penetrating the cuticle and epidermis and the formation of a papilla. h = hypha, ip = infection peg, p = papilla, cu = cuticle, cw = cell wall. Scale bar = 1μm. c) and d) Hyphae penetrating waterhyacinth cell walls. Penetration is through smooth-bordered holes, suggestive of enzymatic softening. h = hypha, cw = cell wall. Scale bar in c = 1μm, d = 0.5μm.

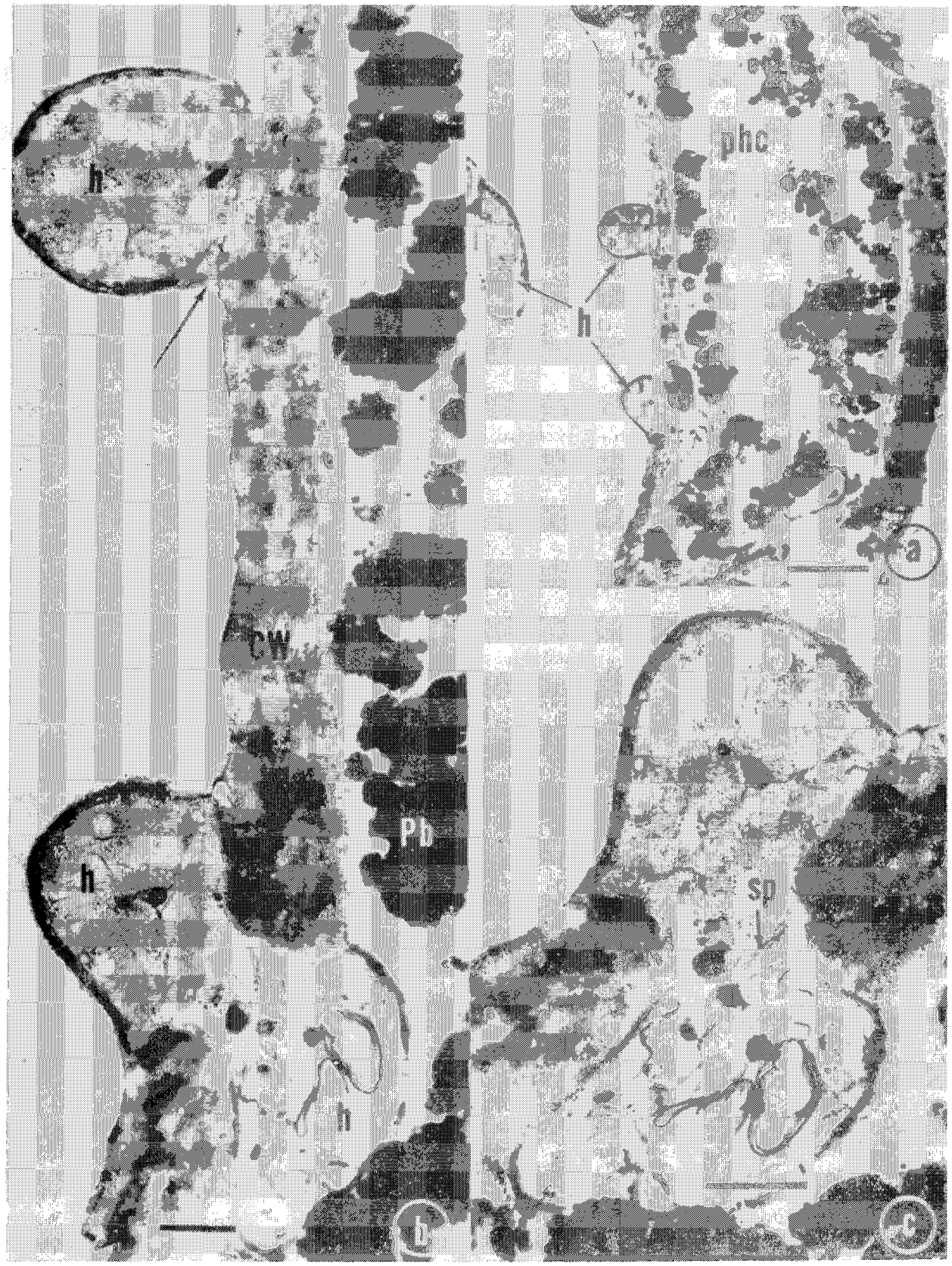

Figure 2. Penetration of a waterhyacinth phenol cell by *Acremonium zonatum*. Attachment of the hyphae to the cell wall is by a muscilagenous matrix (Arrow - b). Hyphae on the inside of the phenol cell are vesiculated, granular, and much less electron-dense than that on the outside of the cell wall. Figures b and c are higher magnifications of Figure a. Phc = phenol cell, h = hypha, cw = cell wall, sp = septum. Scale bar a = 2μm, b and c = 0.5μm.

in Florida and maintained in 37.85-L (10-gal) glass aquaria half-filled with tap water. The plants were trimmed of any necrotic or senescent leaves and inoculated with a water agar-slurry of *A. zonatum* as previously described (12). After inoculation, all plants were maintained in the aquaria fitted with plastic covers to maintain the relative humidity near saturation. One week and 2 weeks postinoculation, leaves displaying characteristic symptoms of disease were excised. Leaf segments from the edge of developing lesions, from areas 3 to 4 cm distant from any lesions, and from the interior of lesions were removed and prepared for electron microscopy as previously described (14). Thin sections were cut on a Sorvall MT-2 ultramicrotome with a diamond knife, poststained in 0.5% uranyl acetate and 1.0% lead citrate and examined with a Hitachi HU-11E electron microscope.

RESULTS AND DISCUSSION

Penetration of waterhyacinth by *A. zonatum* occurred either through the stomata (Figure 1a) or directly through the unbroken cuticle (Figure 1b). Penetration directly through the unbroken cuticle, however, was observed only when the relative humidity was maintained near saturation for several days.

The method by which fungi penetrate unbroken surfaces of their hosts is a topic of considerable interest. Pectinolytic and cellulolytic enzymes are implicated in many host-pathogen interactions (2,3). Martyn (11) demonstrated *in vitro* production of a cellulolytic enzyme by *A. zonatum* but did not detect any pectinase activity. In this present study, electron micrographs revealed penetration of the cell wall and middle lamellae by *A. zonatum* without destruction or mechanical disturbance of the surrounding portions (Figure 1b,c,d). Mechanical pressure alone would be expected to show evidence of tearing and distortion of the cell wall around the penetration site. Instead, penetration resulted in smooth-bordered holes in the cell wall which is indicative of a presoftening of the wall constituents prior to penetration. Therefore, if extracellular pectinases and cellulases are secreted by the fungus *in vivo* as an aid in host cell penetration, they are most probably localized at the tip of the invading hyphae.

Direct penetration of the cuticle by mechanical force did apparently occur as evidenced by the inward displacement of the cuticle at the site of penetration (Figure 1b). Similar results were reported with *Colletotrichum graminicola* on maize (16). Additional evidence for direct penetration of waterhyacinth by *A. zonatum* was the formation of papillae (Figure 1b). Papillae are the earliest and most consistent morphological response to pathogens which penetrate their host directly (1). Evidence suggests that mechanical forces applied by the pathogen during penetration provide the stimulus for a papillae formation. A proposed function of papillae is to impede or block fungal penetration, but evidence for such a role is far from conclusive (18).

A proposed role for phenol-storing cells in waterhyacinth is a defense against invading pathogens (11). Electron micrographs of invaded phenol-storing cells supported this theory (Figures 2 and 3). Attachment to and penetration of the phenol cell by *A. zonatum* appeared similar to that

Figure 3. Phenol cell invaded by *Acremonium zonatum*. Hyphae are very granular and electron-translucent, and the phenol bodies have aggregated around the hyphae. Phc = phenol cell, pb = phenol bodies, h = hyphae. Scale bar = 2μm.

in other cell types. The hyphae were cemented to the cell wall by a muscilagenous matrix (Figure 2b, arrow) and penetration resulted in smooth-bordered holes. The hyphae inside the phenol cell however, appeared highly vesiculated and granulated, with no discernable organelles, and they were much less electron-dense than hyphae outside the phenol cell wall (Figure 2a,b,c and Figure 3). Visually, they appeared non-viable. Cody (4) and Cody and Martyn (5) reported that p-coumaric, protocatechuic, chlorogenic and vanillic acids were inhibitory to *A. zonatum in vitro* at 1000 ppm and that those phenolic acids were present in waterhyacinth phenol cells. Additionally, Martyn *et al.* (14) showed that the phenol-oxidizing enzyme polyphenoloxidase (PPO) was present in thylakoids of phenol cell plastids. The

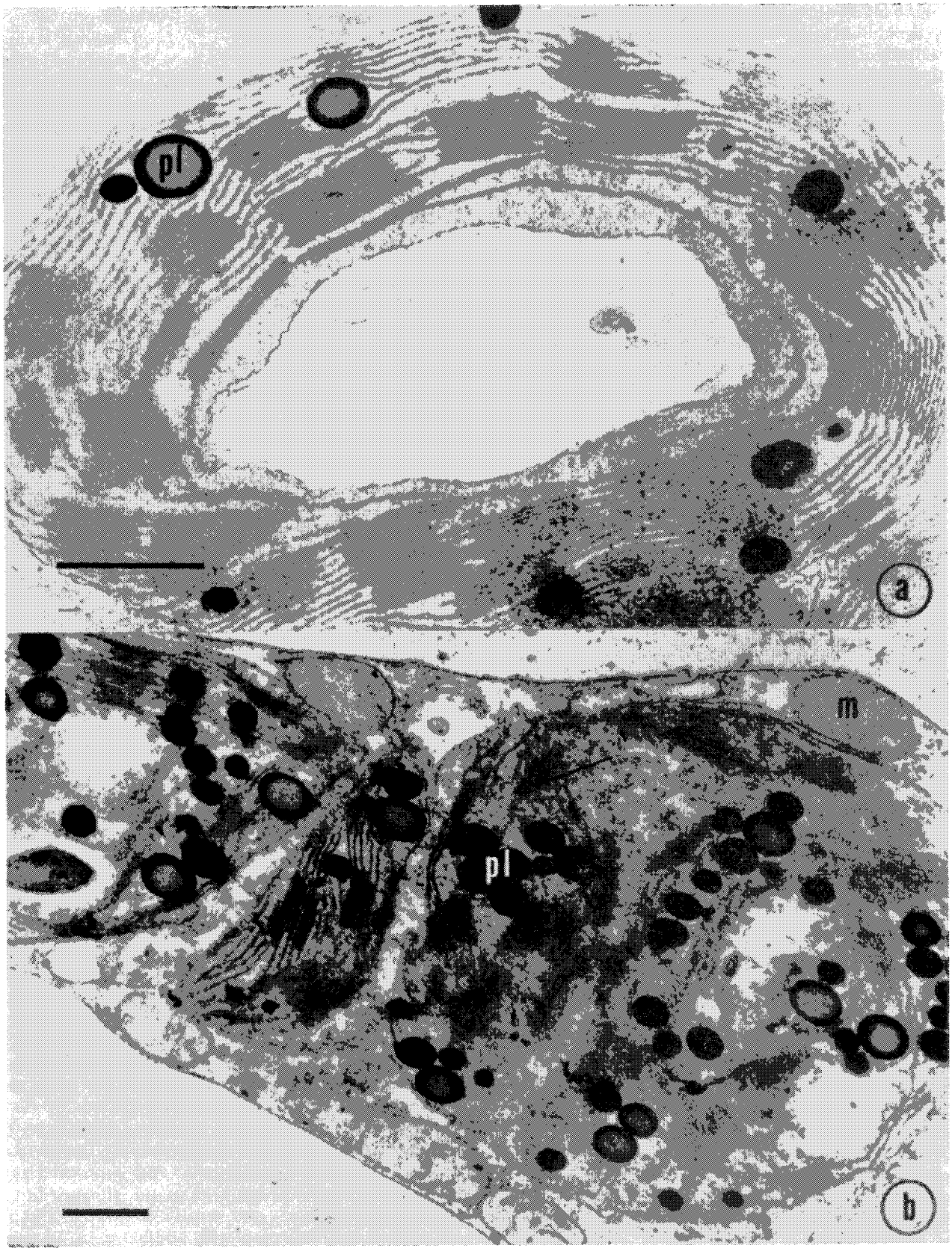

Figure 4. Chloroplasts showing an increase in the number of plastoglobuli after infection by *Acremonium zonatum*. a) Chloroplast of a healthy cell. Pl = plastoglobuli. Scale bar = 0.5μm. b) chloroplasts of an infected plant cell. Pl = plastoglobuli, m = mitochondrion. Scale bar = 0.5μm.

combined effect of the phenolic acids and PPO in the phenol cell could result in a fungitoxic environment. This might partially explain why infection of waterhyacinth by *A. zonatum* results in only 40% diseased leaf area (12).

Several cytological changes in mesophyll cells resulted from *A. zonatum* infection. The most noticeable was an increase in the number of plastoglobuli in chloroplasts (Figure 4a,b). Similar results were reported in spinach plants infected with *Albugo occidentalis* (9). The function of plastoglobuli is not fully understood, but they are believed to be reservoirs of excess lipid (10), and may be a product senescence since they increase in both size and number durduring aging (6). The significance of increased plastoglobuli in infected waterhyacinths is not known at the present time.

Other cytological changes observed in infected leaves were the disappearance of starch from the chloroplasts and an increase in the number of microbodies in the cytoplasma. Decreased starch is common in many foliar diseases (18); however, the significance of the increase in microbodies during infection is not known.

During the final stages of disease, the integrity of chloroplasts and other cellular organelles was lost, plastoglobuli coalesced, and plasmolysis and oxidation of the cellular components occurred resulting in the formation of dark brown melanin pigments typical of cell necrosis.

LITERATURE CITED

1. Aist, J. R. 1976. Papillae and related would plugs of plant cells. Annu. Rev. Phytopathol. 14:145-163.
2. Albersheim, P., T. M. Jones, and P. D. English. 1969. Biochemistry of the cell wall in relation to infective processes. Annu. Rev. Phytopathol. 7:171-194.
3. Bateman, D. F. and R. L. Millar. 1966. Pectic enzymes in tissue degradation. Annu. Rev. Phytopathol. 4:119-146.
4. Cody, Y. S. 1969. Separation of intact phenol cells from waterhyacinth leaves characterization of their phenolic content and the effect of some phenolic acids on two potential biological control agents. M. S. Thesis, Texas A&M University, College Station. 55 pp.
5. Cody, Y. S. and R. D. Martyn. 1979. Separation of phenol cells from waterhyacinth and the effect of some phenolic acids on the growth of two potential biocontrol agents (Abstr). Phytopathology 69:1025.
6. Dodge, J. D. 1970. Changes in chloroplast fine structure during the autumnal senescence of *Betula* leaves. Ann. Bot. 34:817-824.
7. Freeman, T. E., R. Charudattan, K. E. Conway, R. E. Cullen, R. D. Martyn, D. E. McKinney, M. T. Olexa and D. F. Reese. 1981. Biological control of aquatic plants with pathogenic fungi. Tech. Rept. A-81-1. USACE -WES. 47 pp + appendicies.
8. Freeman, T. E., R. Charudattan, K. E. Conway, F. W. Zettler and R. D. Martyn. 1976. Biological control of water weeds with plant pathogens. Fla. Water Resources Res. Center, Proj. Comp. Rept. Pub. #36 42 p.
9. Goode, M. J., E. M. Martin and K. S. Kim. 1976. An ultrastructural study of spinach white rust caused by *Albugo occidentalis*. (Abstr.) Proc. Am. Phytopathol. Soc. 3:336.
10. Greenwood, A. D., R. M. Leech and J. P. Williams. 1963. The osmiophilic globules of chloroplasts. I. Osmiophilic globules as a normal component of chloroplasts and their isolation and composition in *Vicia faba*. Biochem. Biophys. Acta. 78:148-162.
11. Martyn, R. D. 1977. Disease resistance mechanisms in waterhyacinths and their significance in biocontrol programs with phytopathogens. Ph. D. Disseration, University of Florida, Gainesville. 294 pp.
12. Martyn, R. D. and T. E. Freeman. 1978. Evaluation of *Acremonium zonatum* as a potential biocontrol agent of waterhyacinth. Plant Dis. Rep. 62:604-608.
13. Martyn, R. D., D. A. Samuelson, and T. E. Freeman. 1979. Ultrastructural localization of polyphenoloxidase activity in leaves of healthy and diseased waterhyacinth. Phytopathology 69:1278-1287.
14. Martyn, R. D., D. A. Samuelson, and T. E. Freeman. 1983. Phenol-storing cells in waterhyacinth leaves. J. Aquat. Plant Manage. 21:49-53.
15. Politis, D. J. and H. Wheeler. 1973. Ultrastructural study of penetration of maize leaves by *Collectotrichum graminicola*. Physiol. Plant Pathol. 3:465-471.
16. Rintz, R. E. 1973. A zonal leaf spot of waterhyacinth caused by *Cephalosporium zonatum*. Hyacinth Contr. J. 11:41-44.
17. Wheeler, H. 1975. Plant Pathogenesis. Springer-Verlag, N.Y. 106 p.