

An ultrastructural investigation of pathological alterations induced by *Macrophomina phaseolina* (Tassi) Goid in seedlings of soybean, *Glycine max* (L.) Merrill

VERNON AMMON, THOMAS D. WYLLIE and MERTON F. BROWN, JR

University of Missouri, Columbia, Mo. 65291, U.S.A.

(Accepted for publication, June 1973)

An ultrastructural investigation of seedling soybean root tissue infected by the charcoal rot fungus indicated that during the initial stages of pathogenesis, the fungus appears to be restricted mostly to the intercellular spaces and that penetration of interior cell walls occurred as a result of both mechanical pressure and chemical softening. Subsequently, the middle lamella disintegrates, the plasmalemma is separated from the cell wall, the cytoplasm becomes highly vesiculated and the mitochondria disorganized. In later stages of pathogenesis, the fungus becomes intracellular and recognizable cytoplasmic organelles disappear.

INTRODUCTION

Macrophomina phaseolina is a root-inhabiting, soil-invading, facultative parasite which causes a seedling blight and rot of roots, stems and fruit of over 284 species of plants [10]. To date, no investigations of the infection processes of this fungus on any plant host have been reported. Our objective was to observe the host-parasite interface at various times during pathogenesis using transmission electron microscopy. We describe penetration of soybean root cells by the charcoal rot fungus, the colonization of tissues, and the changes within host cells associated with penetration and colonization.

MATERIALS AND METHODS

Soybean seeds (cv. Amsoy) were surface sterilized in 1% sodium hypochlorite for 5 min followed by three successive rinses in sterile distilled water. Infected soybean root tissue was acquired in two ways. First by germinating soybean seeds on a Petri dish culture of *M. phaseolina*. In a second method, used to localize infection to a selected area on the primary root, soybean seeds were germinated in a moist chamber between folds of moistened cheese cloth supported by a wire rack in the dark at 23 °C. Small cones of aluminum foil, with the tips cut off to form an aperture large enough to accomodate the diameter of the root, were positioned near the root cap region or at the region of elongation. Cooled Czapek Dox agar, pipetted into the cones, was seeded with mycelium of *M. phaseolina*. After 3 to 7 days incubation, selected pieces of infected and control roots were fixed in 5% glutaraldehyde in 0.1 M-sodium cacodylate buffer at pH 7.2 for 4 h at 4 °C. After an overnight rinse in 0.1 M-sucrose cacodylate buffer, the samples were post-fixed in 2% osmium tetroxide in 0.1 M-cacodylate buffer for 4 h at 4 °C followed by four successive 10-min rinses in 0.1

M-cacodylate buffer. After dehydration in graded series of ethyl alcohol, the tissues were infiltrated and embedded in ERL 4206 [8]. Sections were post stained in uranyl acetate for 15 min followed by lead citrate [7] for 5 min.

RESULTS

The most conspicuous structural features of uninfected cortical cells from the elongating region of soybean roots are shown in Plates 1(a), 1(b) and 1(c). Large, triangular intercellular spaces are frequently observed [Plate 1(a)]. The laminate structure of the relatively thin but prominent cell walls is discernible in Plate 1(b). The middle lamella appears moderately electron dense and can be seen only at the corners of intercellular spaces in healthy tissue [Plate 1(b)]. The plasmalemma is electron dense, continuous and close to the cell wall [Plate 1(c)]. Mitochondria are numerous and evenly distributed within the cytoplasm. The endoplasmic reticulum is oriented parallel to, and is characteristically positioned near, the cell wall. Membrane-bound vacuoles may also be seen within the cytoplasm [Plate 1(c)].

Host cellular alterations associated with infection

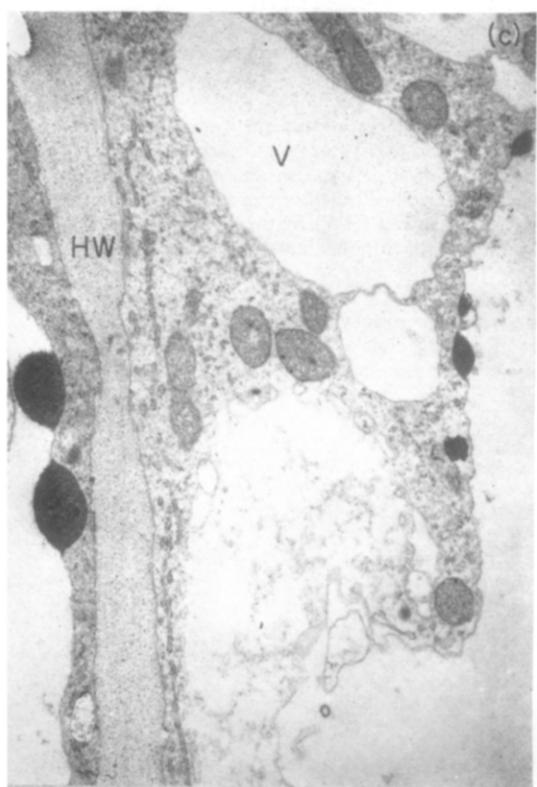
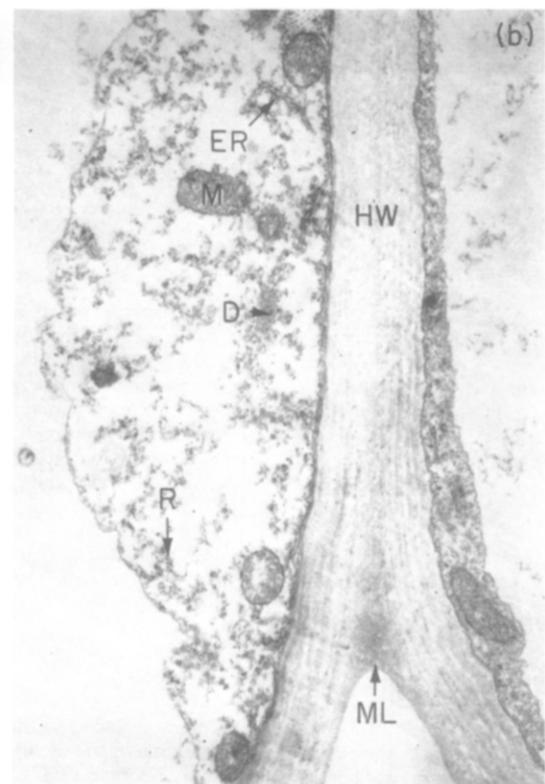
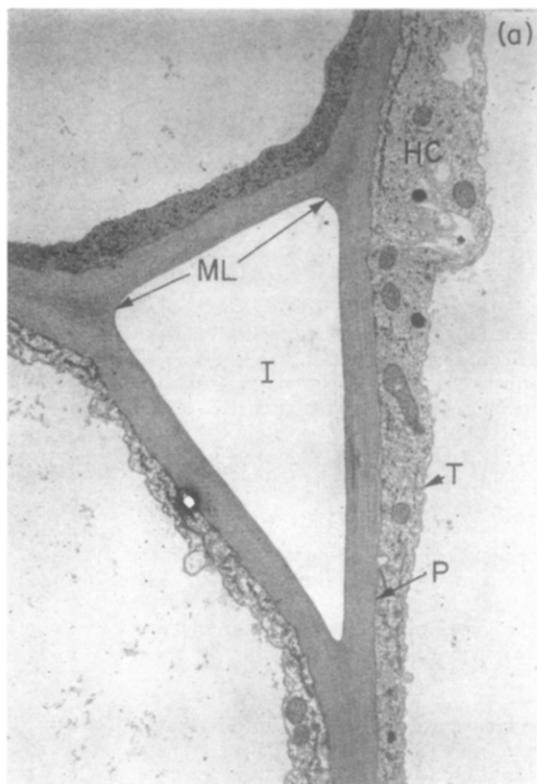
The most frequently observed site of root penetration by the charcoal rot fungus is between the epidermal cells [Plate 1(d)]. Intercellular colonization is accompanied by a conspicuous change in the middle lamella. In some cases clean fractures are found with no evidence of cellular debris in the space between the two primary cell walls [Plate 2(a)]. More commonly, however, the middle lamella appears eroded with irregular granules of moderately electron dense material embedded in an electron-lucent matrix [Plates 2(b), 3(a) and 3(b)].

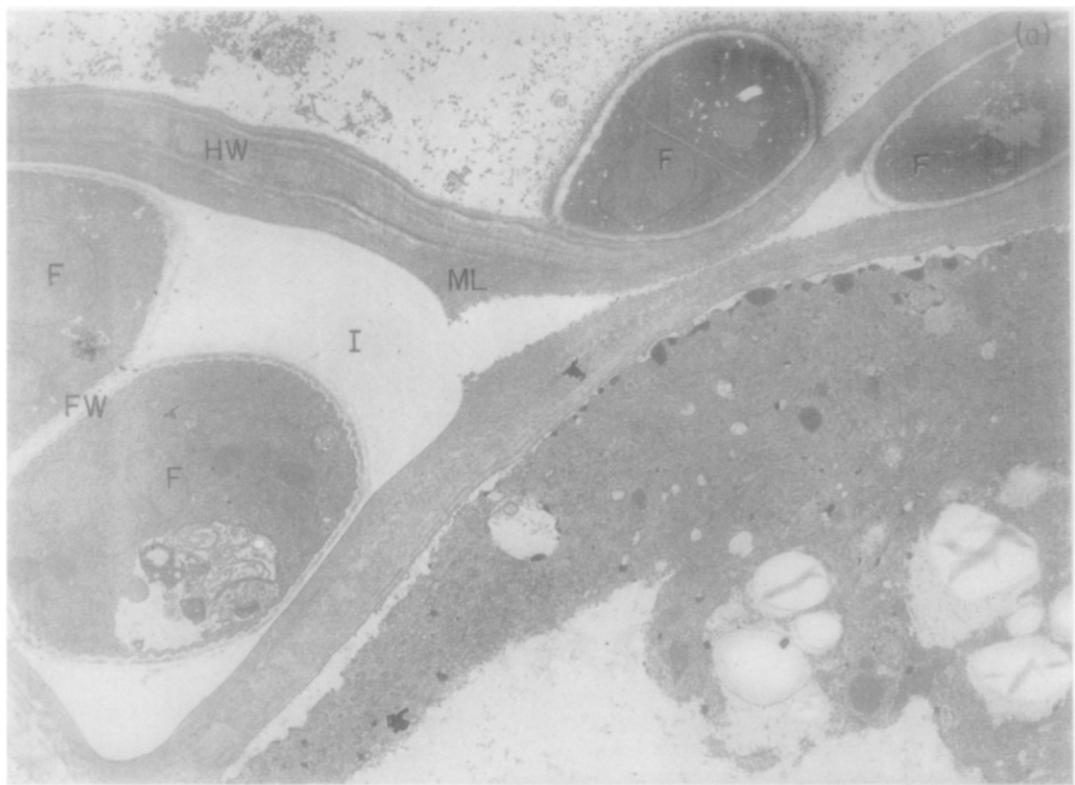
The laminated construction of host cell walls is evident in infected cortical tissue. Three to five electron dense zones oriented parallel to the plasmalemma alternate with less dense layers. These striations extend for considerable distances in cell walls surrounding intercellular spaces which contain hyphae [Plates 2(b) and 3(a)]. Cell walls undergoing more advanced stages of disintegration reveal lighter stained patches within the wall [Plate 3(b)]. At this stage, the crisp parallel laminations in the cell walls are lost.

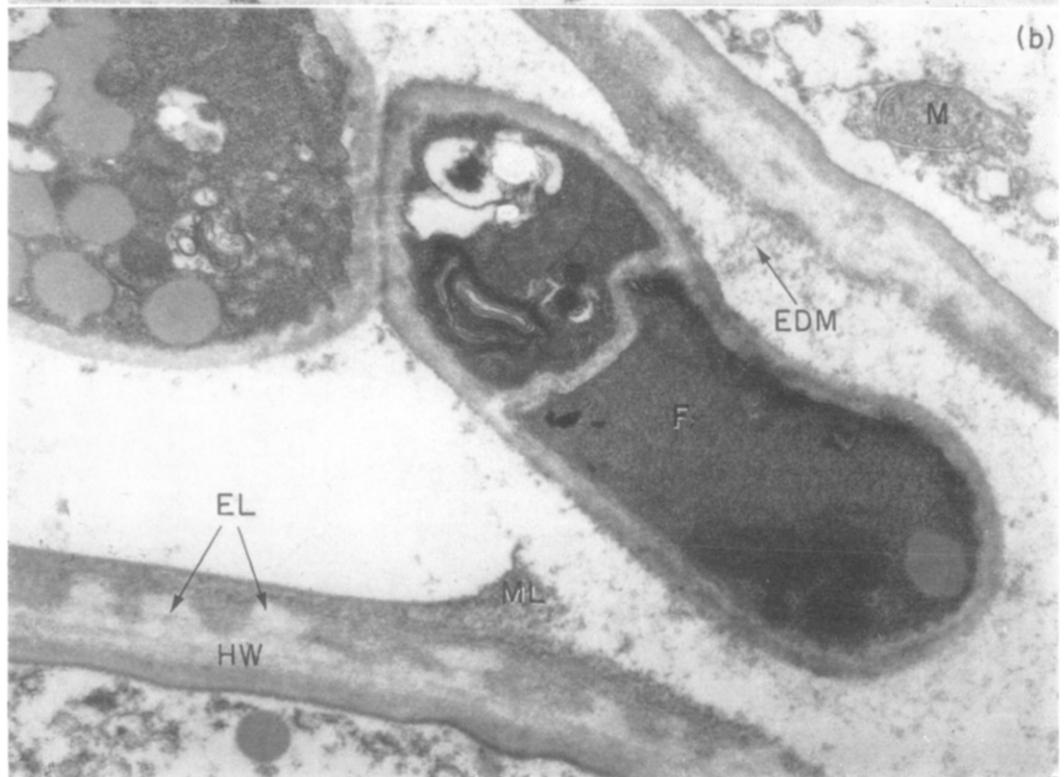
Plate 4(a) shows an intracellular hyphal strand of *M. phaseolina* penetrating an interior cell wall. There is an accumulation of electron dense material in the wall matrix at a point midway along the length of the cell wall making contact with the fungus. The host wall is distorted in the direction of penetration and an electron dense layer of flocculent material covers the outer surface of the fungus except in the area where the hypha contacts the host wall.

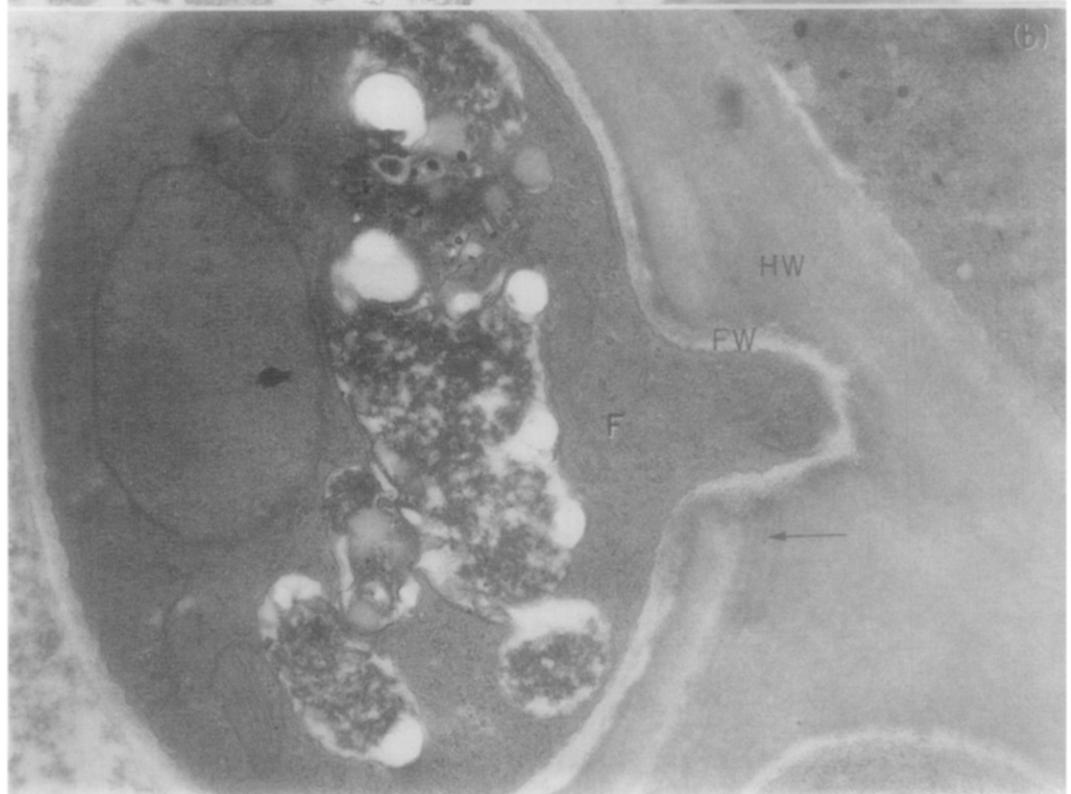
Penetration through the thickened portion of a primary wall can occur by the production of a narrow infection peg [Plate 4(b)]. Electron dense material is present within and in advance of the electron lucent wall of the penetration peg. The laminated areas in the host cell wall which has been penetrated do not show any inward bending in the direction of penetration.

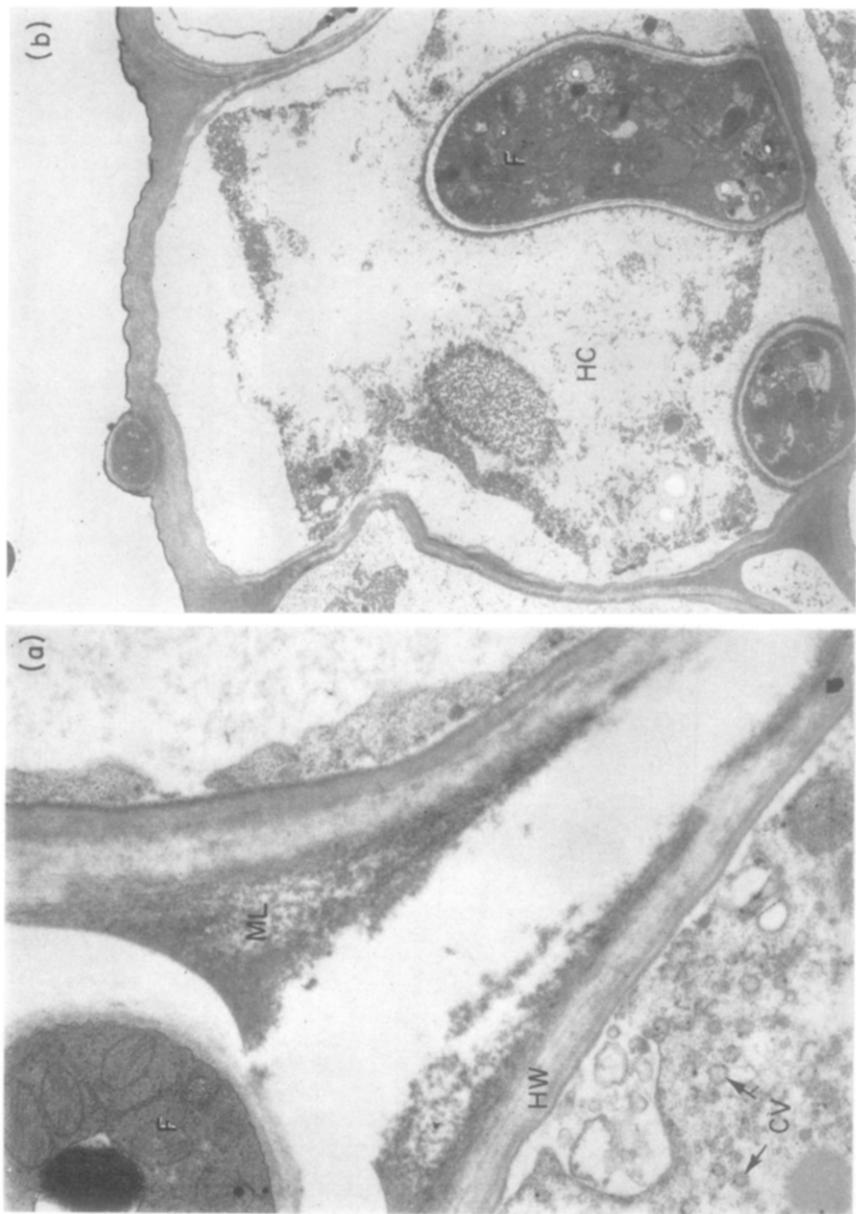
Infection of seedling soybean root tissue results in marked cytoplasmic alterations. The plasmalemma separates from the cell wall and the thin band of cytoplasm appears disorganized and is no longer closely associated with the cell wall [Plates 2(b) and 3(a)]. Numerous vesicles appear within the cytoplasm [Plate 5(a)]. The











cytoplasm is completely disrupted without recognizable organelles in cells containing intracellular hyphal strands [Plates 2(a) and 5(b)].

DISCUSSION

The *in vitro* and *in vivo* production of cellulolytic and pectolytic enzymes by the charcoal rot fungus has been reported [1, 2, 5, 6, 9]. High levels of pectinase activity and significant reductions in the pectin content of plants infected with *M. phaseolina* [6] are highly suggestive of their involvement in tissue disintegration. The results reported here provide visual evidence of the significance of pectolytic activity in the development of charcoal rot disease of soybean.

Growth of *M. phaseolina* in soybean roots during the early stages of disease development is predominantly intercellular through intercellular spaces and the middle lamella. These would be the growth patterns expected of a pectolytic organism which gains entry into a root predominantly between epidermal cells. The rapid longitudinal growth of this fungus is probably attributable to the abundance of large, continuous intercellular spaces along this axis, the availability of large quantities of suitable substrates, favorable moisture conditions and the lack of resistance to its growth. In areas of tissue breakdown [Plates 2(b), 3(a) and 3(b)] fungal growth seemed to occur unrestricted as indicated by the absence of distorted fungal cells. Where evidence of enzymic activity was lacking [Plate 2(a), upper right], fungal growth between walls resulted in a flattening of the hyphal cell.

Nourishment for continued fungal growth through soybean roots is probably derived from breakdown products of the pectin components contained in the middle lamella and primary walls. The disintegration of this important constituent results in a weakening of the wall and subsequent intracellular colonization. The electron lucent patches occurring in walls [Plate 3(b)] are interpreted as areas of enzymic breakdown of the protopectin matrix surrounding cellulose microfibrils.

We suggest that penetration of interior soybean root cell walls by *M. phaseolina* occurs as a result of mechanical pressure and/or chemical softening. Chemical softening of cell walls as the primary means of penetration by *Pyrenopeziza terrestris*, another soil borne fungal plant pathogen, was implicated by Hess [3]. His interpretation was based on the accumulation of electron dense products at the fungus cell, host cell interface and the irregularity in shape of fungal strands penetrating host walls. The pectic enzymes produced by *M. phaseolina* may account for the absence of laminations within walls [Plate 3(b)] and the accumulation of cellular debris at the point of apparent wall disruption [Plate 4(a)]. Mechanical pressure as a mechanism in wall penetration is also indicated in Plate 4(a) by the flattened walls at the host-fungus interface. In Plate 4(b), penetration seems to be primarily chemical. This interpretation is based on the appearance of the laminar wall striations around the narrow infection tip. A folding or inward bending of these striations in the direction of penetration would be expected if mechanical pressure were a significant factor.

Colonization of cortical tissue results in a number of host ultrastructural alterations which vary in degree depending on the duration of the host-parasite association. During the initial stages of pathogenesis [Plate 2(b)], the erosion of the middle lamella is accompanied by what appears to be a loosening of the cell wall matrix. At the same time the dense inner layer of the wall, thought to be the middle lamella

becomes more evident. Cytoplasmic organelles in cells adjacent to fungal strands are not visibly altered. The plasmalemma, however, has begun to pull away from the cell wall. By the time tissue breakdown has reached the stage shown in Plates 3(a) and 3(b), mitochondrial structure has become partially disrupted. Intracellular colonization [Plate 5(b)] is characterized by a complete disorganization of cytoplasmic structures in host cells. Disruption of membrane function is indicated by the disorganized state of the cytoplasm in Plates 2(a) and 5(b). Investigations by Lai *et al.* [4] on permeability changes in *Phaseolus aureus* Roxb. associated with infection by *Rhizoctonia solani* pointed out that membrane alteration, as detected by electrolyte leakage, occurred 14 to 18 h after inoculation. Their efforts to isolate the permeability altering material resulted in the identification of two endopolygalacturonase-type enzymes, but commercial pectinases containing polygalacturonase did not, however, alter permeability. They concluded that alteration of permeability is not a general characteristic of pectolytic enzymes. The principal responsible for membrane alteration in the charcoal rot-soybean complex is unknown but a role for pectolytic enzymes appears to be indicated and this question needs further investigation.

REFERENCES

1. CHAN, Y. H. & SACKSTON, W. E. (1969). Mechanisms of pathogenesis in *Sclerotium bataticola* on sunflowers. I. Production and translocation of a necrosis-inducing toxin. *Canadian Journal of Botany* **47**, 1147-1151.
2. CHAN, Y. H. & SACKSTON, W. E. (1970). Mechanisms of pathogenesis in *Sclerotium bataticola* on sunflowers. II. Pectolytic and cellulolytic enzyme production *in vitro* and *in vivo*. *Canadian Journal of Botany* **48**, 1973-1077.
3. HESS, W. M. (1969). Ultrastructure of onion roots infected with *Pyrenopeziza terrestris*, a fungus parasite. *American Journal of Botany* **56**, 832-846.
4. LAI, M. T., WEINHOLD, A. R. & HANCOCK, J. G. (1968). Permeability changes in *Phaseolus aureus* associated with infection by *Rhizoctonia solani*. *Phytopathology* **58**, 240-245.
5. MATHUR, S. B. (1968). Production of toxins and pectolytic enzymes by two isolates of *Sclerotium bataticola* Taub. and their role in pathogenesis. *Phytopathologische Zeitschrift* **62**, 327-333.
6. RADHA, K. (1953). The enzymic activity of *Macrophomina phaseoli* (Maubl.) Ashby. *Proceedings of the Indian Academy of Sciences* **37**, 231-234.
7. REYNOLDS, E. S. (1963). The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *Journal of Cell Biology* **17**, 208.
8. SPURR, A. R. (1969). A low-viscosity epoxy resin embedding medium for electron microscopy. *Journal of Ultrastructure Research* **26**, 31.
9. THORNBERRY, H. H. (1938). Pectinase activity of certain microorganisms. *Phytopathology* **28**, 202-205.
10. YOUNG, P. A. (1949). Charcoal rot of plants in east Texas. *Texas Agricultural Experiment Station Bulletin* **712**. 33p.