A comparison of the death induced by fungal invasion or toxic chemicals in cowpea epidermal cells. II. Responses induced by *Erysiphe cichoracearum*

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Cowpea leaves were inoculated with the plantain powdery mildew fungus, *Erysiphe cichoracearum*, and fresh epidermal cells overlying veins were examined by light microscopy before being cleared or prepared for electron microscopy. Fungal appressoria usually formed a haustorium in the underlying nonhost cell, but only after what appeared to be an unsuccessful penetration attempt that induced a transient cytoplasmic aggregate, a ring of autofluorescence in the plant wall (best seen in cleared tissue), and in two examples observed ultrastructurally, a small penetration peg embedded in a callose-like papilla. The haustorium developed from a different penetration peg and elicited the death of the invaded cell. As reported for the death of cowpea epidermal cells elicited by $CuCl_2$, cytoplasmic changes that occurred rapidly in fresh tissue after cytoplasmic streaming had stopped correlated closely with changes in ultrastructure. Compared with the $CuCl_2$ study, microtubules and Golgi bodies disappeared faster and membranes appeared more disorganized. These data suggest that in cowpea epidermal cells, ultrastructural changes accurately predict the onset of cell death and may also reflect differences in its modes of induction.

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Des feuilles de dolique ont été inoculées avec le *Erisiphe cichoracearum*, champignon causal du oldium du plantain, et des cellules épidermiques fraîches des nervures ont été examinées au microscope photonique avant la décoloration ou les apprêts de la microscopie électronique. Les appressoriums fongiques formaient d'habitude un suçoir dans la cellule sous-jacente non-hôte, mais seulement après ce qui semblait être un essai de pénétration qui avait provoqué un agrégat cytoplasmique transitoire, un anneau d'autofluorescence dans la paroi de la plante (surtout visible dans le tissu décoloré) et dans deux exemples dont on a observé l'ultrastructure, une petite cheville de pénétration qu'enrobe une papille ressemblant à de la callose. Le suçoir provenait d'une chaville de pénétration différente et provoquait la mort de la cellule envahie. Comme rapporté pour la mort des cellules épidermiques du dolique causée par le $CuCl_2$, des changements cytoplasmiques qui surviennent dans les tissus frais après la cessation de la cyclose étaient en étroite corrélation avec les changements ultrastructuraux. Les microtubules et les corps golgiens disparaissaient plus vite et les membranes ont semblé être plus désorganisés qu'au cours des traitements au $CuCl_2$. Ces données suggèrent que dans les cellules épidermiques du dolique, les changements ultrastructuraux prédisent avec précision le commencement de la mort de la cellule et il se peut qu'ils reflètent aussi des différences dans ses modes d'induction.

[Traduit par la revue]

Introduction

The common association of plant cell death with resistance to microbial pathogens makes it of particular interest to phytopathologists. However, cell death induced by either pathological or mechanical damage has not been widely studied in plant tissues (Gahan 1981). Studies that have been performed on plant cell death have indicated that the death process may vary, even within a single tissue (Sigee 1984). Moreover, it is not clear whether differences in ultrastructure of dying cells indicate differences in the death process or its induction (Heath 1972). To add to the information on the changes that occur during plant cell death, the present study was initiated to compare death induced by different agents in a single cell type. A previous paper (Meyer and Heath 1988) reported that death induced by heavy metal salts in cowpea leaf epidermal cells followed an identifiable pattern when observed by light microscopy. Correlated changes in ultrastructure were also observed but only after cytoplasmic streaming had ceased. In this paper, features of cell death, and other responses, elicited by the fungus *Erysiphe cichoracearum* are reported. Cowpea is a nonhost for this fungus, and production of an intracellular haustorium results in death of the invaded plant cell.

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Materials and methods

Methods for maintenance of cowpea (*Vigna sinensis* (Torner) Savi cv. Early Ramshorn) plants, differential interference contrast optics, blue light epifluorescence, and stereological analysis have been described previously (Meyer and Heath 1988). The first leaves of 12- to 15-day-old cowpea plants were used for the study. *Erysiphe cichoracearum* DC. was obtained from naturally infected field-grown plantain (*Plantago major* L.). Some of the infected plantain plants were transferred to pots containing sterilized Pro-mix (Premier Brands Inc., New Rochelle, NY) or soil and kept either in the greenhouse or in a growth chamber maintained at 23°C with a 16-h photoperiod. Plants from all three sources were used to provide conidial inoculum.

For inoculation, infected pieces of plantain leaves were laid onto cowpea leaves and held in place with Vaseline (Chesebrough-Ponds, Inc.) that had been applied to the peripheries of the cut pieces. Some of the leaves used as inoculum sources were laid onto cowpea leaves immediately after being cut from the plantain plants, while others were first placed on wet paper towels in Petri dishes for 1–7 h to encourage conidium formation. The cowpea plants with the plantain leaves on them were covered with clear plastic bags and placed in a 23°C growth chamber overnight. Tissue pieces to be examined were removed from cowpea leaves the next day (17–24 h after inoculation), mounted in distilled water, and examined immediately. All infection sites per leaf piece were examined, and some of those where the underlying epidermal cell appeared alive (i.e., the cytoplasm was still streaming) were selected for continued observation over the next few hours. Over 100 infection sites were examined in detail from at least 30 different cowpea plants over a period of several months. Some tissue pieces were subsequently cleared as described by

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Fernandez and Heath (1986) and about 10% of the infection sites examined in detail in fresh tissue were relocated; the rest could not be identified because of the detachment of the conidium and appressorium during the clearing process. Other sites were examined, however, and a total of 40 infection sites, including those previously observed in fresh tissue, were examined in cleared leaves.

For transmission electron microscopy, tissue pieces were mounted in water and observed by light microscopy. When an observed cell reached the required morphological stage of death, the tissue piece was quickly immersed in fixative; the material was then fixed and embedded following the protocol described by Meyer and Heath (1988). Two cells that were uninfected and healthy and two infected cells each that (*i*) had just stopped their cytoplasmic streaming and (*ii*) were in the period between cessation of streaming and protoplasmic collapse were fixed. Three cells were fixed that had collapsed protoplasts and four cells that were infected but still showed cytoplasmic streaming. As in the previous study (Meyer and Heath 1988) paradermal serial sections were cut from most specimens to examine the peripheral protoplasm under the adaxial cell walls. However, sections of two infection sites were cut perpendicular to the leaf surface to obtain median longitudinal sections of any penetration pegs. Plantain leaves with mature infections were similarly fixed and sectioned at right angles to the leaf surface. Some plantain leaves were also examined by light microscopy 24 h after being inoculated in a manner similar to that described for the cowpea leaves.

Results

Light microscopy

Conidia of *Erysiphe cichoracearum* germinated on the surfaces of cowpea leaves and produced appressoria (Figs. 1, 2). A septum separated the appressorium from the very short germ tube (Fig. 2). By 17 h after inoculation, various stages in the penetration of the underlying epidermal cell could be distinguished and in many cases, well-developed haustoria were observed (Figs. 6, 7). However, appressoria were also seen that were only beginning to penetrate the epidermal cell at 24 h after inoculation. In fresh tissue, the first observable response of the plant cell to the fungus was a transient aggregation of cytoplasm, including the nucleus, under the appressorium near the end closest to the septum. In a few cases, a papilla was associated with the plant wall in the center of the aggregate, but it was not clear whether the plant wall had been penetrated. The papilla was sometimes brownish, and faint yellow autofluorescence was seen around its periphery. At sites that were forming or had formed a haustorium, the latter developed at a different position, closer to the tip of the appressorium than did the papilla or cytoplasmic aggregate described above. A small collar of refractive material was commonly seen at the site of penetration of developing haustoria (shown in cleared tissue in Fig. 4) and an extension of the collar completely or partially encased most haustoria that had well-developed bodies (shown in cleared tissue in Fig. 7). Both collars and encasements were often autofluorescent. The plant cytoplasm (Fig. 1) and the nucleus aggregated under the penetration peg area, and this second aggregation persisted after the haustorium formed (Fig. 6). In some haustorium-containing cells observed at 17 h after inoculation, the cytoplasm in the aggregate, the peripheral cytoplasm, and the cytoplasmic strands (Fig. 6) were still streaming, although cells with brown, quiescent, collapsed contents were also seen. Occasionally, an appressorium was observed that had two papillae underneath it and no haustorium; even more rarely, three linearly arranged papillae were seen, the one closest to the appressorium tip being associated with a haustorium.

Anywhere from a few minutes to several hours after living haustorium-containing cells were first observed, cytoplasmic strands decreased in number, and streaming slowed and eventually stopped in the invaded plant cell. The exact timing of this event was difficult to assess, however, since it was affected by the intensity of illumination and streaming often would begin again if the intensity was increased. No change in autofluorescence was observed at this time. Cessation of streaming was often accompanied by a jiggling motion of the cytoplasmic particles. The particles frequently continued jiggling for many minutes after streaming stopped.

Following the cessation of streaming, other changes occurred in the cell. The cytoplasm and nucleoplasm became granular in appearance (Figs. 10, 11). Small particles were seen undergoing Brownian motion in the plant cell vacuole. Sometimes cytoplasmic strands or portions of them remained in position after streaming stopped and continued to jiggle about for some time. Vesicles formed in the cytoplasm of some of the cells.

These changes were followed by collapse of the plant cell protoplast (Figs. 10, 11). This collapse was observed to occur as soon as 6 min after streaming stopped, although in other cells, it took 45 min or longer. Granulation of the haustorial cytoplasm occurred at about the time of plant cell protoplast collapse. The cells with collapsed protoplasts eventually turned brown, sometimes within an hour after collapse. This brown color was observed in collapsed plant cell protoplasts, in plant cell walls, and eventually in haustoria as well (Fig. 11). Sometimes the haustorium browned before the collapsed plant protoplast. Yellow autofluorescence of the protoplast and walls of the plant cell developed after protoplast collapse. Jiggling particles appeared between the plant cell walls and the collapsed cytoplasm and were still seen in the vacuole after the protoplast collapsed. Occasionally, cells were seen with uncollapsed protoplasts, even though the host cell and haustorium had turned brown.

As has been described in other situations (Heath 1984), autofluorescence of epidermal walls underneath appressoria was much brighter in cleared than in fresh tissue, even at the same infection sites. At those sites where there were clear signs of fungal penetration or some response of the plant cell, 50% exhibited a fluorescent ring, up to 8 μm in diameter, in the plant wall at a site corresponding to the position of the first cytoplasmic aggregate seen in fresh tissue near to the septal end of the appressorium (Fig. 5). Less than 20% of these rings contained a detectable papilla when viewed from above, but in the few sites where the curve of the tissue surface allowed the site to be viewed from the side, the plant wall appeared thickened in this area (Figs. 2, 8) and was surrounded by fluorescent material (Figs. 3, 9). Since this fluorescence coated the lower surface of the thickening (Figs. 3, 9), the dark center of the halo of fluorescence seen when the cell wall was viewed from above presumably was caused by the absorption of the underlying fluorescence by the thickened wall. Similar haloes were rarely seen at the site of haustorium formation closer to the appressorium tip. Here the plant wall was more uniformly fluorescent and the fluorescence commonly extended into the collar or encasement associated with the haustorium (Figs. 5, 9). As previously observed for dying cowpea and bean cells (Heath 1984), autofluorescence of collapsed protoplasts was observed in cleared tissue before it could be seen in corresponding sites observed in fresh tissue.

When fresh or cleared samples of host plantain plants inoculated with *E. cichoracearum* were observed by light micros-

copy 24 h after inoculation (Figs. 12–14), no papillae or accompanying fluorescent haloes were observed. There was a slight autofluorescence of anticlinal walls near appressoria. Haustoria had small collars (Fig. 13) and lacked encasements (Fig. 14). Haustoria were about the same size as those formed in the nonhost cowpea cells.

Electron microscopy: fungal development

Sections perpendicular to the cowpea leaf surface were cut through two infection sites. When observed before fixation, the epidermal cell underlying the first appressorium contained streaming cytoplasm, which formed an aggregation near the appressorium tip. However, there was no sign of a haustorium. At the other site, a haustorium was present and the plant cytoplasm was granular and collapsed. At both sites, signs of a papilla could be detected in the region under the appressorium distal from the tip. During fixing and embedding, both appressoria became dislodged from the leaf surface; however, the underlying cells were recognizable by their shape. Serial sections through the first site revealed a single penetration peg (Figs. 15, 16), 0.7 μm wide and 3.5 μm long, that had breached the plant wall and was embedded in a primarily electron translucent papilla that was about 2 μm in diameter. In appearance, this papilla resembled those shown in other situations to contain primarily callose (e.g., Heath and Heath 1971). From its position in the cell, the papilla corresponded to the one detected in fresh tissue. The plant cytoplasm looked normal, with no aggregation around the papilla (Fig. 16). No ultrastructural features were seen in the plant wall around the papilla (Fig. 15) that might correspond to the autofluorescent halo often seen in cleared tissue under this part of the appressorium. No other sign of penetration was observed, although serial sections were cut through the region of the cell corresponding to that under the tip of the appressorium. However, two grids were lost during processing that might have contained a small penetration peg.

At the second site, a small 0.5 μm wide by 2.5 μm long penetration peg (Figs. 17, 18) was observed as well as a small nonseptate haustorium (Fig. 19). The peg had breached the wall and was embedded in a callose-like papilla with a diameter of about 6 μm (Figs. 17, 18). The haustorium had its neck and part of its spherical body encased in a callose-like collar (Fig. 19). The detection of two membranes lining much of the collar indicated that it was a type I collar as described by Littlefield and Heath (1979) and that most of it had formed after the haustorium had breached the plant wall. The cytoplasm of the penetration peg, haustorium, and invaded cell was disorganized (Figs. 17, 19).

As observed for cowpea tissue, conidia and appressoria became detached from the plantain leaf when they were fixed 24 h after inoculation; this problem, together with the low infection rate and lack of relocatable markers, made it impossible to find infection sites (previously observed by light microscopy) in sections cut perpendicular to the leaf surface. Instead, leaves bearing mature infections were fixed and similarly examined. Many haustoria were seen that resembled those described for other powdery mildew fungi (e.g., Gil and Gay 1977). All haustoria observed possessed a septum at the junction of the haustorium neck and body, the body was surrounded by finger-like haustorial lobes, and the neck was encased in a callose-like collar. No callose-embedded penetration pegs resembling those seen in cowpea leaves were observed in spite of the examination of extensive series of serial sections.

In cowpea tissue sectioned paradermally to examine the ultrastructure of the plant cytoplasm, the fungus was not always seen because of the angle of the section. When observed, haustoria had unlobed, spherical bodies and were completely or partially encased in callose-like material continuous with the collar. Fungal cytoplasm appeared disorganized only in those cells where the plant cytoplasm was also disorganized.

Electron microscopy: plant ultrastructure

Healthy, uninfected cells

Healthy control cells (Figs. 20, 29) contained ribosomes (many in the form of coiled polyribsomes), microtubules, mitochondria, microbodies, vesicles, endoplasmic reticulum (ER), Golgi bodies, and uniform ground cytoplasm. The tonoplast and plasmalemma often appeared discontinuous when fixed and were not used as indicators of cell death.

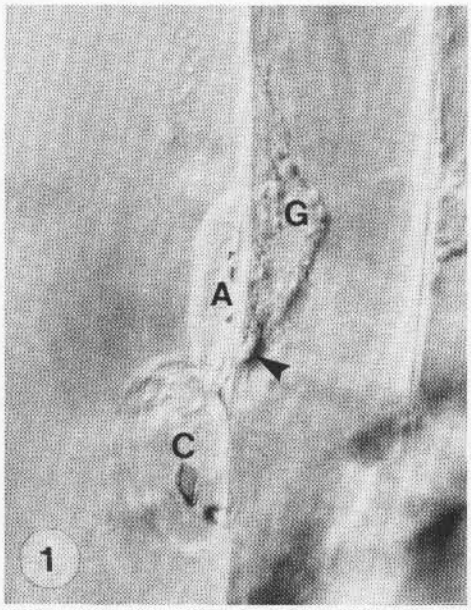
Infected cells with streaming cytoplasm

Cells containing haustoria with the plant cell cytoplasm still streaming at the time of fixation resembled healthy, uninfected cells in their ultrastructure (compare Figs. 21 and 22 with 20).

Infected cells just after streaming stopped

Some coiled polyribosomes were seen in one cell (Figs. 23), and the average number per unit area (2.2) was not significantly different from that of healthy, uninfected cells (2.1). However, coiled polyribosomes were completely lacking in the other cell (Fig. 24), which appeared to be at a later stage of degeneration. Microtubules were not observed in either cell. Mitochondria were present, but the cristae varied from reasonably healthy looking (Fig. 23) to disorganized or absent (Fig. 24). In the less healthy appearing cell, the membranes forming the envelopes of some mitochondria were dark and (or) had electron-opaque spots on them (Fig. 24). Sometimes

FIGS. 1–9. Infection sites of *E. cichoracearum* in cowpea observed by interference contrast light microscopy, or by blue light to detect autofluorescence. Figs. 1–3. The same infection site observed in fresh tissue (Fig. 1) or cleared tissue (Figs. 2, 3). The conidium (C) has formed an appressorium (A). ×820. Fig. 1. Note the papilla (arrow) in the plant cell near the conidium end of the appressorium and the aggregation of cytoplasm (G) closer to the appressorium tip. The cytoplasm was still streaming when the tissue was fixed and cleared, and the fungus probably would have formed a haustorium near the appressorium tip. Fig. 2. Note the apparent thickening of the plant wall (large arrowheads) adjacent to two slight projections of the appressorium, and the septum (small arrowhead) separating the appressorium from the germ tube. Fig. 3. Note the strong autofluorescence around the rim of the wall thickening closest to the germ tube (cf. Fig. 2). Figs. 4 and 5. Surface focus of a haustorium penetration site in cleared tissue. ×960. Fig. 4. A refractive collar (arrowhead) can be seen around the haustorial neck at the site of penetration. Fig. 5. Autofluorescence at the same focal plane as Fig. 4. The collar and associated plant wall are autofluorescent and there is a conspicuous nonfluorescent area with a fluorescent periphery to the left. Fig. 6. A living, invaded cell observed in fresh tissue that was showing streaming in the cytoplasm surrounding the haustorium (large arrowhead) as well as in cytoplasmic strands (small arrowhead) traversing the vacuole. ×630. Fig. 7. A haustorium (H) in cleared tissue. Note the partial encasement (arrowhead). ×780. Fig. 8. Higher focal plane than that illustrated in Fig. 7 to show appressorium (A) and a thickening of the plant wall (arrowhead) not at the site of haustorium penetration. ×780. Fig. 9. Autofluorescence of the cell shown in Fig. 8. Note the autofluorescence of the encasement and the plant cell wall, except for the thicker region detectable in Fig. 8. ×780.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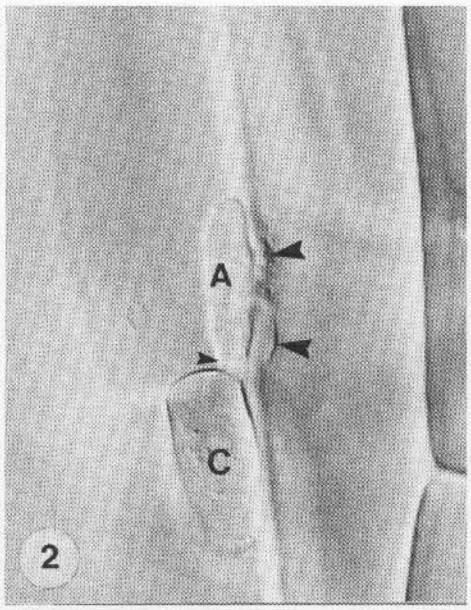
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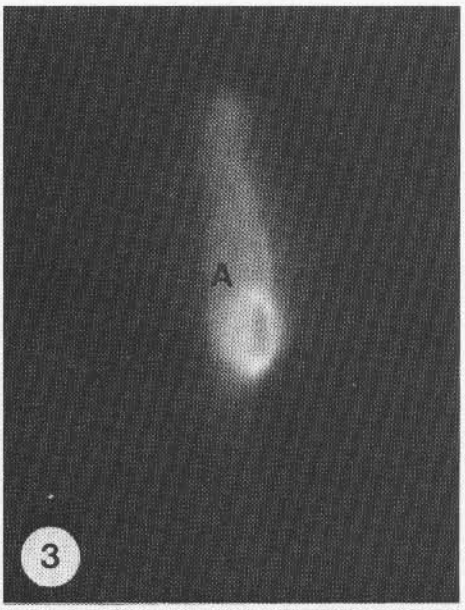
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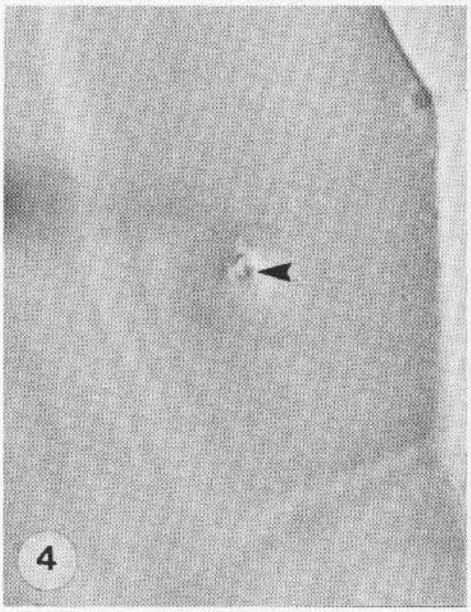
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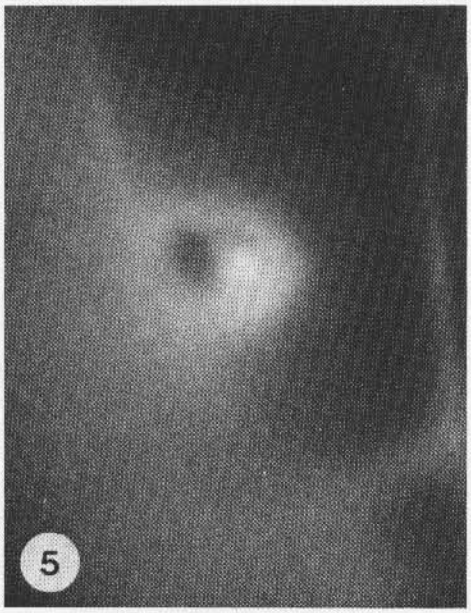
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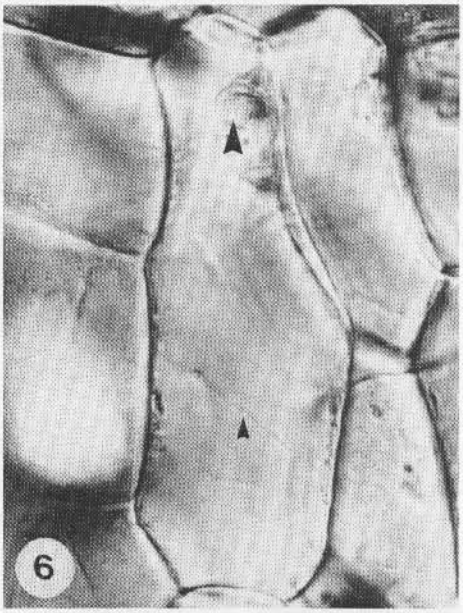
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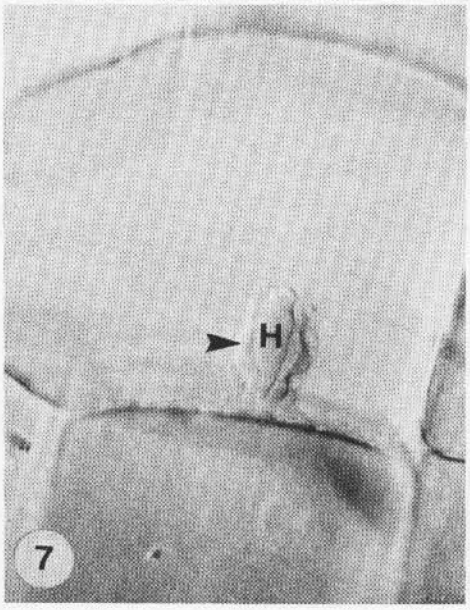
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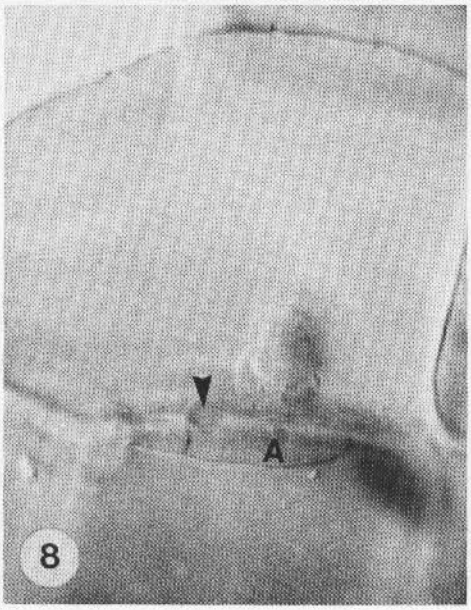
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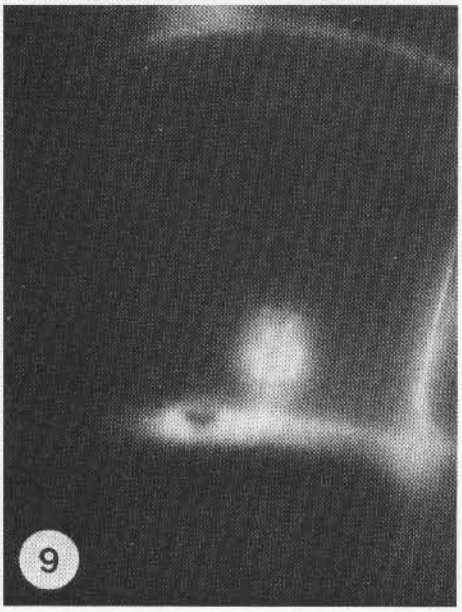
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the double membrane structure of the envelopes was no longer discernible. The mitochondria appeared somewhat rounded in section (Fig. 24). Microbodies were present (Fig. 23), although some had electron-opaque spots in the membranes. Vesicles were observed, along with membranous structures that did not resemble the vesicles seen in healthy cells. Endoplasmic reticulum (ER) was present in one cell but was not seen in the other cell. Golgi bodies were not seen in either cell. The ground cytoplasm was neither uniform nor dense (Fig. 24).

Cells after streaming stopped but before protoplasmic collapse

During fixation, the cytoplasm or portions of it pulled away from the cell wall. If cytoplasm was not present just under the adaxial walls of these cells, sections were examined starting from the area where the cytoplasm first appeared.

Coiled polyribosomes and microtubules were absent (Figs. 25, 26). Mitochondria were present (Fig. 26), but some had cristae, while others lacked them. Electron-opaque spots were present in mitochondrial envelopes, and the double membranes often appeared as a single darkened membrane (Fig. 26). Microbodies were not recognizable in one cell; in the other cell, they were present but had electron-opaque spots in the outer membranes. ER could not be positively identified, although a number of large membrane-bound vesicles were present (Fig. 25). Electron-opaque spots were present on some of the membranes and in the cytoplasm (Figs. 25, 30). Golgi bodies were not seen. The ground cytoplasm was somewhat uniform in one cell (Fig. 25) but not in the other. Small membranous structures were observed between the cell wall and the protoplasm that had pulled away from it, and one cell also had electron-opaque granules just under the adaxial cell wall (Fig. 30).

Cells with collapsed protoplasts

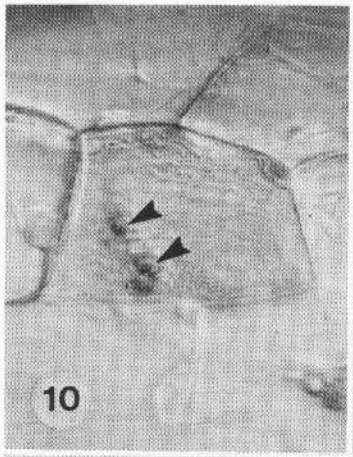
These cells did not have cytoplasm just under their adaxial walls. Ultrastructurally, the contents of these cells appeared similar to the contents of cells observed during the period prior to protoplasmic collapse. Coiled polyribosomes and microtubules were absent (Figs. 27, 28). Most of the mitochondrial cristae were not present, and the envelopes appeared as a single darkened structure, often with electron-opaque spots (Figs. 27, 28). Structures that may have been microbodies were present, but ER and Golgi bodies could not be identified. Electron-opaque spots were associated with some membranes, and some membranes were darkened. The ground cytoplasm was not uniformly dense. Small membranous structures, bits of cytoplasm, and in one cell, electron-opaque particles were present between the cell wall and the collapsed protoplasts (Fig. 31). The plasmalemma and tonoplast were more obvious here than in previously described samples, possibly because of the membrane darkening and the collapse of the protoplast.

Discussion

Light microscopy of living epidermal cells indicated that the powdery mildew fungus, *Erysiphe cichoracearum*, caused a response of the nonhost cowpea before the initiation of the haustorium. This response occurred at a different position under the appressorium from that at which the haustorium eventually developed, and was represented by a transient aggregation of cytoplasm under the end of the appressorium close to the germ tube. Usually this was followed by the formation of a refractive papilla or, more often, an autofluorescent ring in the plant wall that was best seen after the tissue was cleared. Whether the latter corresponded to the edges of a flattened papilla that was not visible when the leaves were viewed from above is not clear, although in the few cells observed from the side, the ring appeared to be caused by the presence of a nonfluorescent, thicker cell wall that absorbed the autofluorescence emitted from its lower surface. These autofluorescent rings closely resemble those reported at haustorium-lacking sites in susceptible barley infected with *Erysiphe graminis* f.sp. *hordei* (Koga et al. 1980). However, in the two sites examined ultrastructurally, no structure corresponding to this ring could be observed in the plant wall, although narrower, callose-like papillae were adpressed to the inner wall surface.

The fact that both of these papillae contained penetration pegs suggests that the responses described above occur in response to unsuccessful penetration attempts by the fungus. Nevertheless, most appressoria did form a haustorium but usually from a penetration peg formed closer to the appressorium tip. Although the plant wall and the collar surrounding the haustorium wall were often autofluorescent, a ring-like halo was not observed. Such haloes, and any detectable signs of penetration other than at the site of haustorium formation, were also absent from susceptible plantain leaves infected with the fungus; this suggests that haustorium formation was not preceded by any unsuccessful attempts at penetration in the host plant. These observations raise interesting questions concerning the function of the first penetration attempt in nonhost cowpea cells. Since it seemed consistently unsuccessful whereas the second attempt commonly succeeded, it is possible that the first penetration peg influenced the cell such that resistance to the second invasion was lowered. To our knowledge, no similar phenomenon has been described before for powdery mildew fungi. Both *E. graminis* f.sp. *hordei* and *E. cichoracearum* have been reported to try and penetrate hosts and nonhosts via a second appressorial lobe if the first attempt

FIGS. 10 and 11. Two infection sites in fresh tissue of cowpea observed by interference contrast optics. ×540. Fig. 10. The protoplast has collapsed and the cell has begun to turn brown. Two brown papillae (arrowheads) have formed under the appressorium, one probably associated with a haustorium. Fig. 11. Similar stage of cell death as shown in Fig. 10 except that the haustorium (arrowhead) is brown. FIGS. 12–14. Infection site in plantain observed in cleared tissue, using interference contrast optics. ×525. Fig. 12. Surface view of the conidium (C) and appressorium (A). Fig. 13. Lower focus to show penetration site and collar (arrowhead) around the base of the haustorial neck. Fig. 14. Lower focus still to show the haustorial body (arrowhead). FIGS. 15–19. Electron micrographs of two infection sites in cowpea. Fig. 15. The first penetration attempt in a cell that was still showing cytoplasmic streaming at the time of fixation. Note the short penetration peg and papilla (arrowhead). ×3720. Fig. 16. Detail of Fig. 15 to show the penetration peg (P) and callose-like papilla (C). Note the different staining pattern of the wall (W) around the penetration peg. × 25 240. Fig. 17. The first penetration attempt into a cell that was brown with granular cytoplasm at the time of fixation. Note the papilla (C) deposited on the plant wall (W) at the tip of the penetration peg. ×6470. Fig. 18. Detail of the penetration peg (P) and papilla (C) shown in Fig. 17. × 25 800. Fig. 19. The haustorium (H) surrounded by a collar (C) formed by the same appressorium that formed the penetration peg shown in Figs. 17 and 18. ×7000.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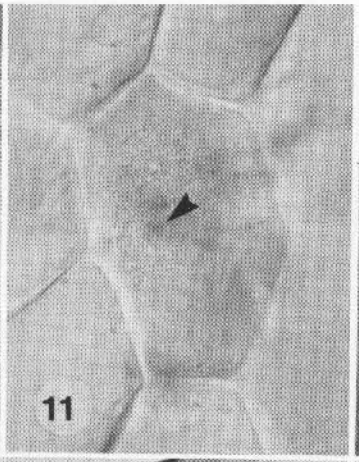
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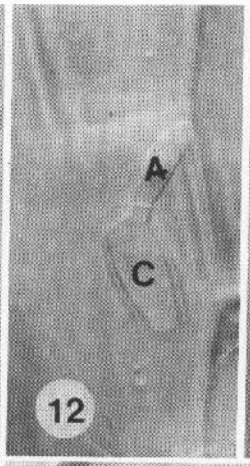
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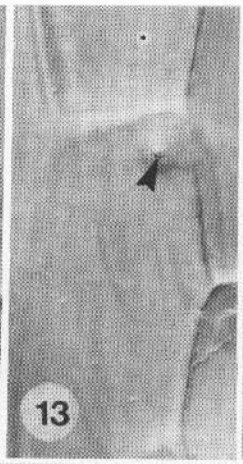
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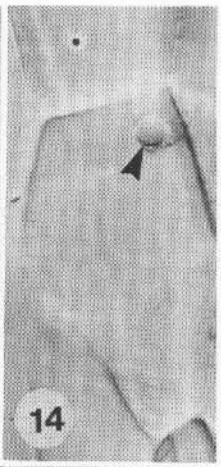
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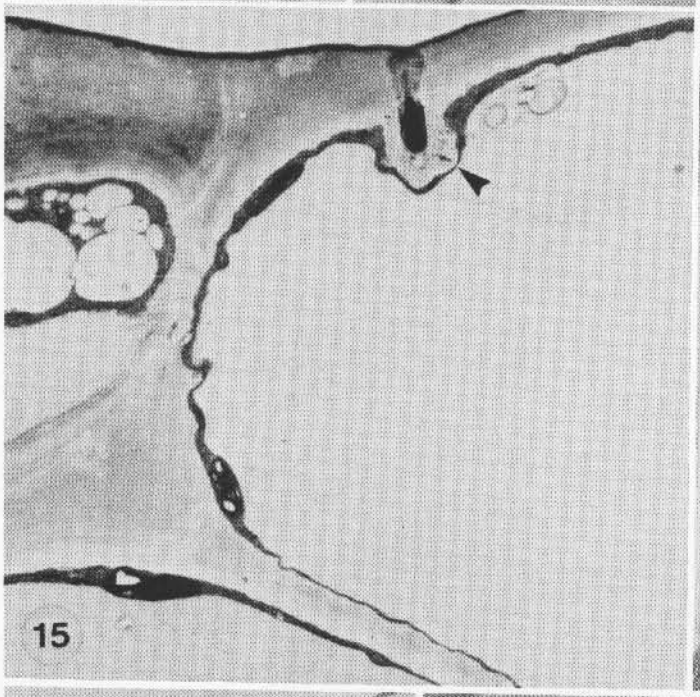
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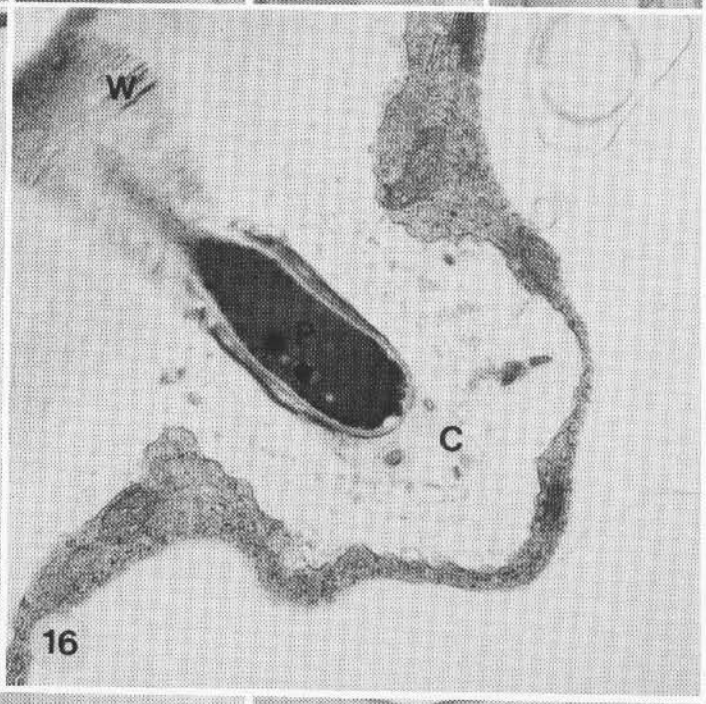
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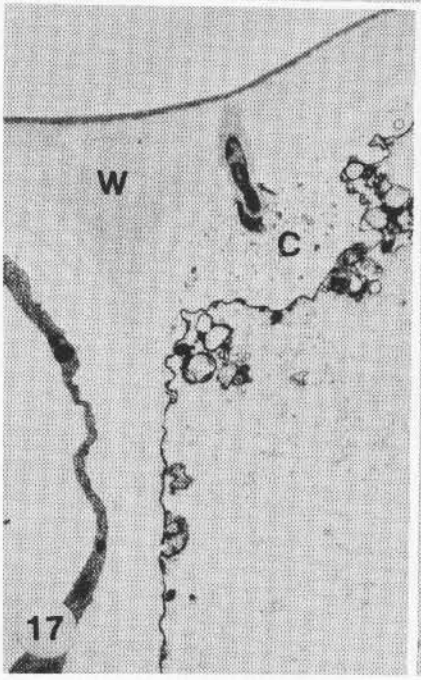
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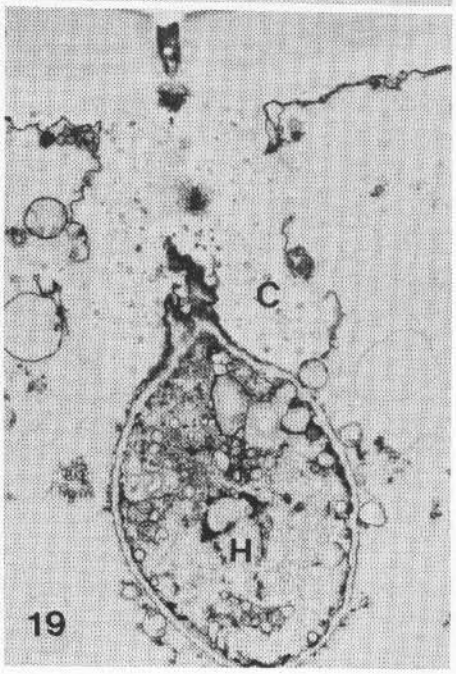
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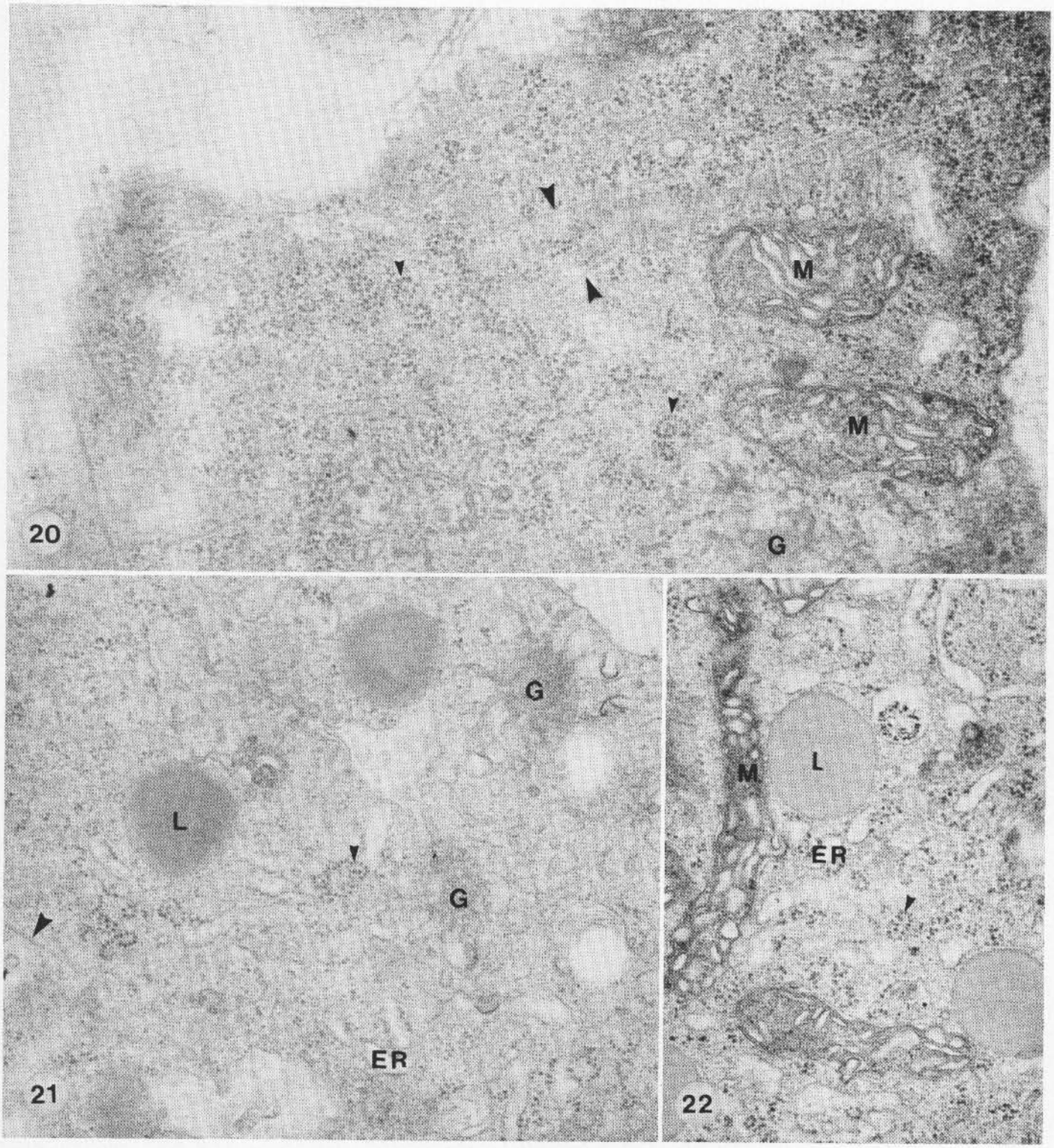
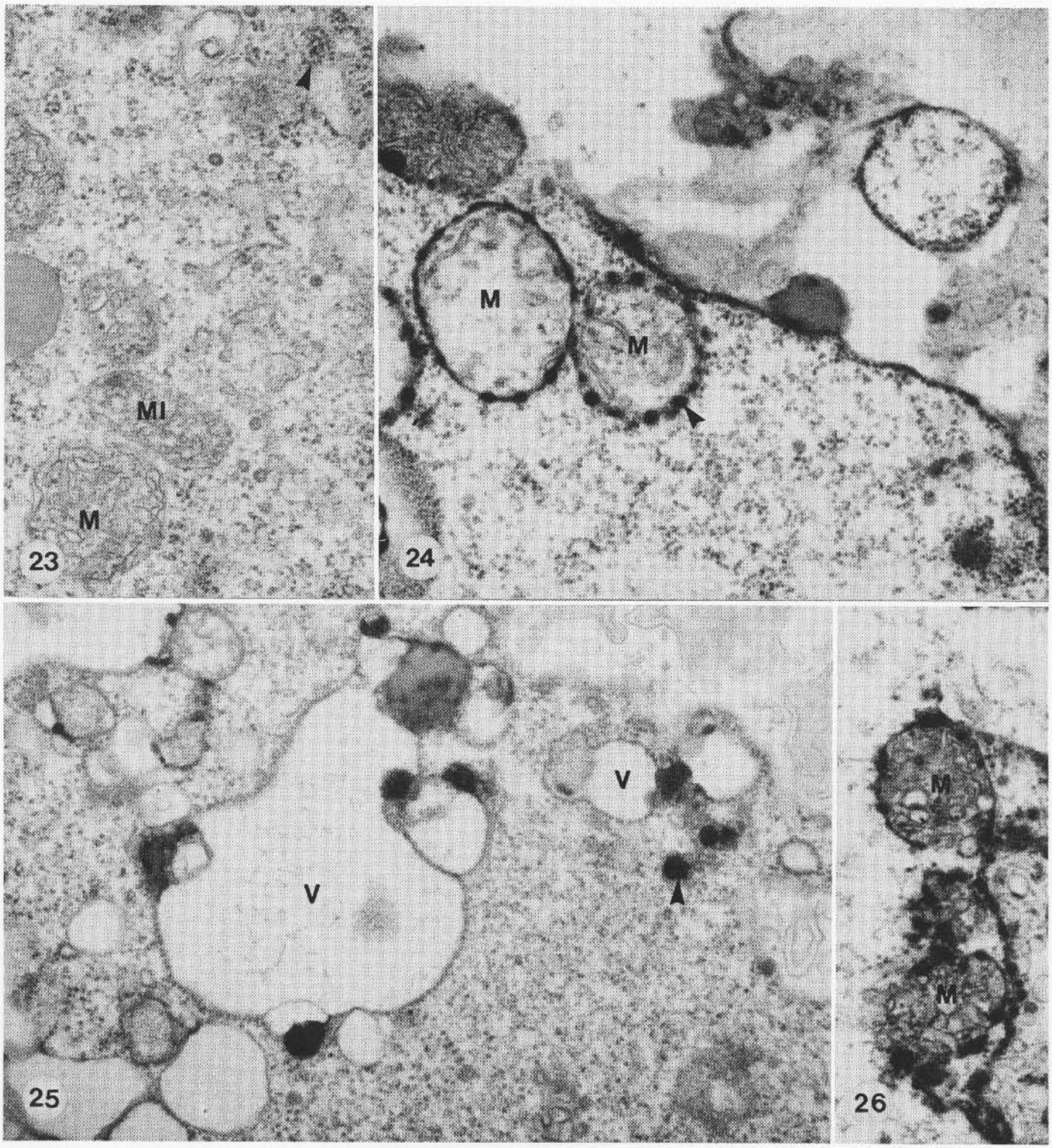
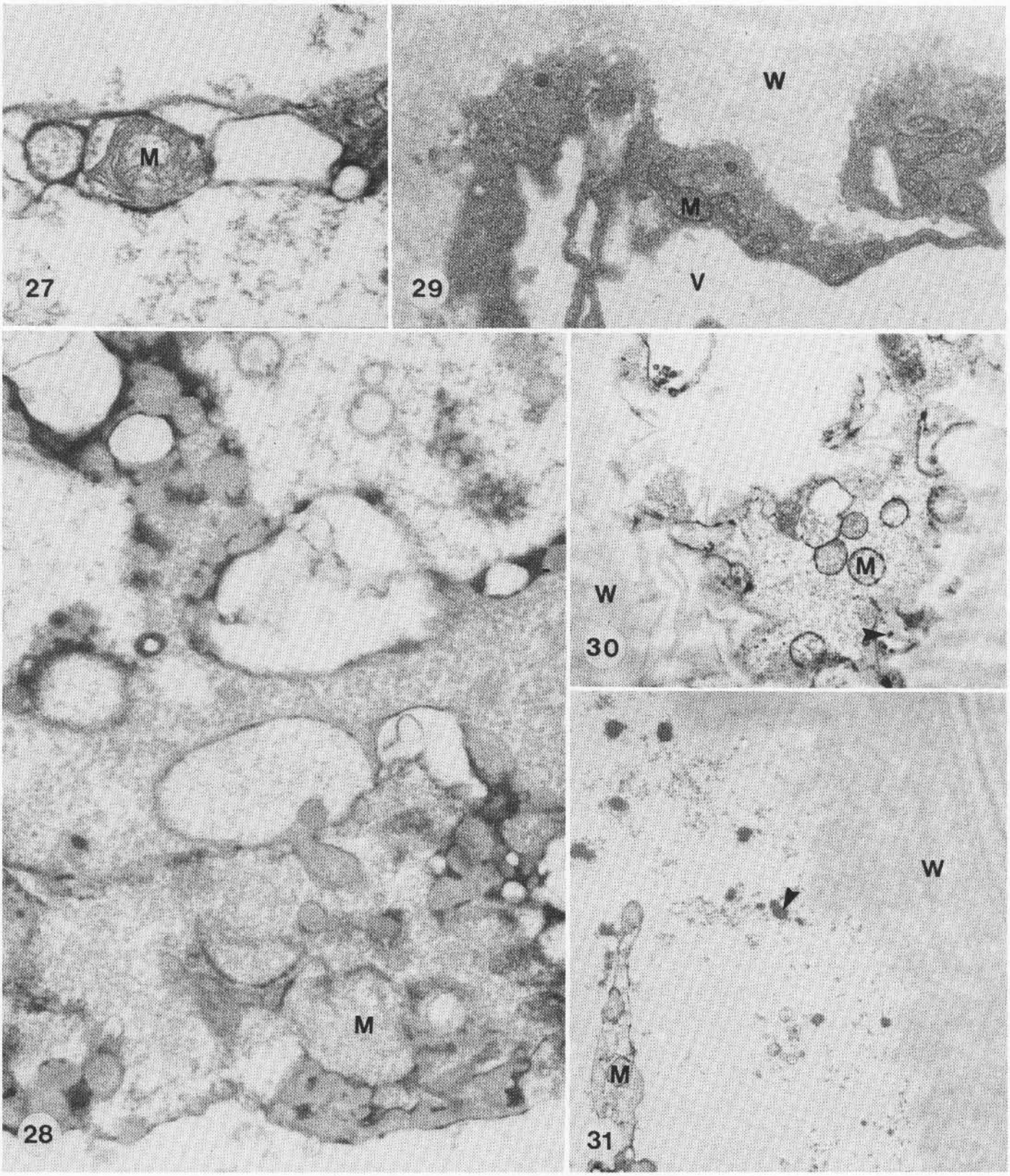


FIGS. 20–22. Electron micrographs of cowpea cells. M, mitochondrion; G, Golgi body; L, lipid droplet; ER, endoplasmic reticulum; small arrowhead, coiled polyribosomes; large arrowhead, microtubule. × 38 900. Fig. 20. Uninfected, healthy cell. Figs. 21 and 22. Haustorium-containing cells that showed cytoplasmic streaming at the time of fixation.

at penetration failed (Johnson et al. 1982); however, in none of these interactions was the second penetration attempt particularly common or successful. Appressoria of *E. pisi* apparently sequentially elicit two cytoplasmic aggregates in barley coleoptile cells, but both of these appear to develop near to the appressorium tip and only one penetration peg is formed (Kunoh et al. 1985). More similar to the cowpea situation perhaps are the two penetration pegs initiated by the conidia of *E. graminis* f.sp. *avenae* on oats (Kidger and Carver 1981) and by *E. graminis* f.sp. *hordei* on barley (Kunoh 1982). One peg is formed by the primary germ tube and the other by the appressorium. As in the first penetration attempt described for



FIGS. 23–26. Electron micrographs of cowpea cells containing haustoria. × 38 900. Fig. 23. Cell fixed shortly after cytoplasmic streaming had stopped. Microtubules have disappeared but some coiled polyribosomes (arrowhead) are visible. The mitochondria (M) have a less dense matrix compared with Figs. 20 and 22 and somewhat dilated cristae. Microbodies (MI) are still detectable. Fig. 24. Cell fixed shortly after cytoplasmic streaming had stopped. Coiled polyribosomes have disorganized; many mitochondria (M) have few or no cristae and electron-opaque envelopes associated with lipid-like spots (arrowhead). Figs. 25 and 26. Cells fixed some time after streaming had stopped but before protoplast collapse. Note the absence of polyribosomes, microtubules, and ER. There are numerous unidentified vesicles (V) and electron-opaque spots on many of the membranes and in the cytoplasm (arrowhead). Cristae of the mitochondria (M) are dilated or absent.



FIGS. 27 and 28. Electron micrographs of haustorium-containing cowpea cells fixed after protoplast collapse. Mitochondria (M) are the only recognizable organelles. × 38 900. FIGS. 29–31. Grazing sections of cowpea cells taken just under the outer epidermal wall (W). M, mitochondria; V, vacuole. ×9340. Fig. 29. Uninfected cell showing a typically dense cytoplasm. Fig. 30. Haustorium-containing cell fixed shortly after cytoplasmic streaming stopped. Note the more disorganized mitochondria and the less dense cytoplasm compared with Fig. 29, and also the electron-opaque granules (arrowhead) just under the wall. Fig. 31. Haustorium-containing cell fixed after protoplast collapse. Note the clumps of electron-opaque material (arrowhead) between the collapsed protoplast and the cell wall.

E. cichoracearum on cowpea, a haustorium never develops from the primary germ tube and the peg may be embedded in a papilla (Kunoh 1982). However, in *E. graminis* f.sp. *hordei*, the penetration peg does not appear to be mandatory for successful invasion from the appressorium (Kunoh 1982) and it may induce a transient state of resistance in the cell (Kunoh 1982; Woolacott and Archer 1984). In addition, there is some evidence that it has a role in water uptake in arid environments (Carver and Bushnell 1983). In contrast, the presence of autofluorescence beneath the primary germ tubes of *E. graminis* f.sp. *avenae* did appear to increase the success of subsequent haustorium formation by the appressorium (Kidger and Carver 1981).

The first sign of cell death observed by light microscopy in haustorium-containing cowpea cells was the slowing and cessation of cytoplasmic streaming. This process followed a series of steps somewhat similar to those described by Bushnell (1981) in his index of streaming used to define the responses of incompatible barley coleoptile cells invaded by *Erysiphe graminis* f.sp. *hordei*. Unlike barley, in cowpea cells there were few cytoplasmic strands left in the cells prior to the time streaming stopped; most of the cytoplasm was located peripherally in the cell or aggregated around the haustorium. In those areas, however, the organelles underwent sluggish movement, vibration, and finally no movement, much as Bushnell described.

This was followed by a period, sometimes quite short, when observable changes occurred in the plant cell cytoplasm. These changes included granulation of the plant cell cytoplasm, formation of vesicles in some cells, and appearance of particles undergoing Brownian motion in the vacuole. Following this period, the protoplast collapsed away from the plant cell walls. This series of events is similar to that observed by Hazen and Bushnell (1983) in an incompatible barley host infected with the powdery mildew fungus *E. graminis* f.sp. *hordei* and resembled that induced in cowpea cells by heavy metal salts (Meyer and Heath 1988). However, the time period from the cessation of streaming to protoplast collapse tended to be more variable than for a given concentration of metal salt.

As in $CuCl_2$-treated cells (Meyer and Heath 1988), the ultrastructure of fungus-invaded cells closely reflected their appearance in fresh tissue. Infected cells fixed before cytoplasmic streaming stopped looked ultrastructurally similar to healthy uninfected cells. However, detectable changes were seen shortly after streaming stopped. Compared with $CuCl_2$-treated cells, the main difference in the ultrastructural degeneration of the fungus-invaded cells was the faster disappearance of microtubules and Golgi bodies. It is possible that such differences were due to the fact that it was more difficult to determine when cytoplasmic streaming had finally ceased in fungus-infected cells, therefore possibly causing a greater period to elapse before the tissue was fixed. However, since microtubule-lacking invaded cells otherwise appeared to be at the same stage of degeneration as $CuCl_2$-treated cells in which microtubules were seen (compare Fig. 23 with Figs. 9, 11, and 12 in Meyer and Heath, 1988), it is possible that the difference was real. In addition, fungal-induced death seemed to affect membranes in a manner dissimilar to $CuCl_2$-induced death. Differences included the presence of electron-opaque, lipid-like spots in many of the membranes, the appearance of mitochondral envelopes as thick darkened structures, and the absence of cristae in a number of mitochondria, all suggestive of membrane disorganization.

In summary, this and the previous paper (Meyer and Heath 1988) have demonstrated that the first sign of cell death in fresh tissue, the cessation of cytoplasmic streaming, cannot clearly be detected by electron microscopy after conventional fixation. However, the subsequent morphological alterations that are observed in the plant cytoplasm appear to be closely mirrored in changes in ultrastructure seen after the tissue is fixed. Whether these changes completely reflect those in the unfixed cell is unknown, since it cannot be proved from the present study that the fixation procedure did not cause some changes in ultrastructural detail. Nevertheless, our observations provide a measure of confidence that ultrastructural studies may be used in, for example, examining the timing of cell death relative to fungus invasion (e.g., Heath 1982). In addition, the differences observed in cell death elicited by the fungus and copper chloride suggest that there is a relationship between the cell's mode of death and its ultrastructure after conventional fixation. This raises the possibility that future studies will eventually allow such ultrastructural observations to be translated in terms of biochemical events.

Acknowledgements

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