Vacuole Dynamics in Fungal Plant Pathogens

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ABSTRACT

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Vacuoles of *Botrytis cinerea* appeared to phagocytize neutral red-containing bodies and lipid bodies. Neutral red appeared to stimulate the formation of endocytic vesicles by the plasmalemma of *B. cinerea* spores. Lipid bodies in spores of *Ceratocystic ulmi* were observed entering vacuoles in living cells, presumably by autophagocytosis. Glycogen granules in *Elsinoë wisconsinensis* spore ground plasm appeared to be engulfed by membranes of the tonoplast and autophagocytized into the vacuole, where they apparently were digested. The widespread occurrence of vacuolar phagocytosis in a variety of fungal pathogens for different purposes suggests that this mechanism may be universal and important in fungal physiology.

Additional key word: lysosomes.

Only recently has it been recognized that plants (like animals) have a lysosomal system (14). The involvement of a lysosomal system (one that compartmentalizes and mobilizes hydrolytic enzymes) in plant diseases was not fully appreciated (19). We now know that lysosomal systems exist in pathogens (7,19) and hosts (14), and that they are involved in host-parasite interactions (11). This paper reports primarily on the activity of autophagic vacuoles in three plant pathogens.

Until recently plant vacuoles have been considered to be metabolically passive organelles serving as a depository for certain nutrients and wastes. Matile and co-workers (13–15) and Coulomb (3,4) introduced the idea that the vacuoles of plants (fungi included) are part of the lysosomal system of the cell; ie, they are single membrane-bounded organelles containing hydrolytic enzymes. As part of the lysosomal system, vacuoles are capable of dynamic interaction with other lysosomal components such as Golgi vesicles, endocytic vesicles, and other vacuoles (19). One way that vacuoles interact with other cellular components is by engulfing them (phagocytosis). Part of this process can be seen with both the light and electron microscope. Fineran (5), Matile (14), and Iten and Matile (8) observed invaginations of the tonoplast and formation of intravacuolar vesicles in meristematic root cells. Griffiths (6) observed such invaginations in the fungus *Fusarium oxysporum*.

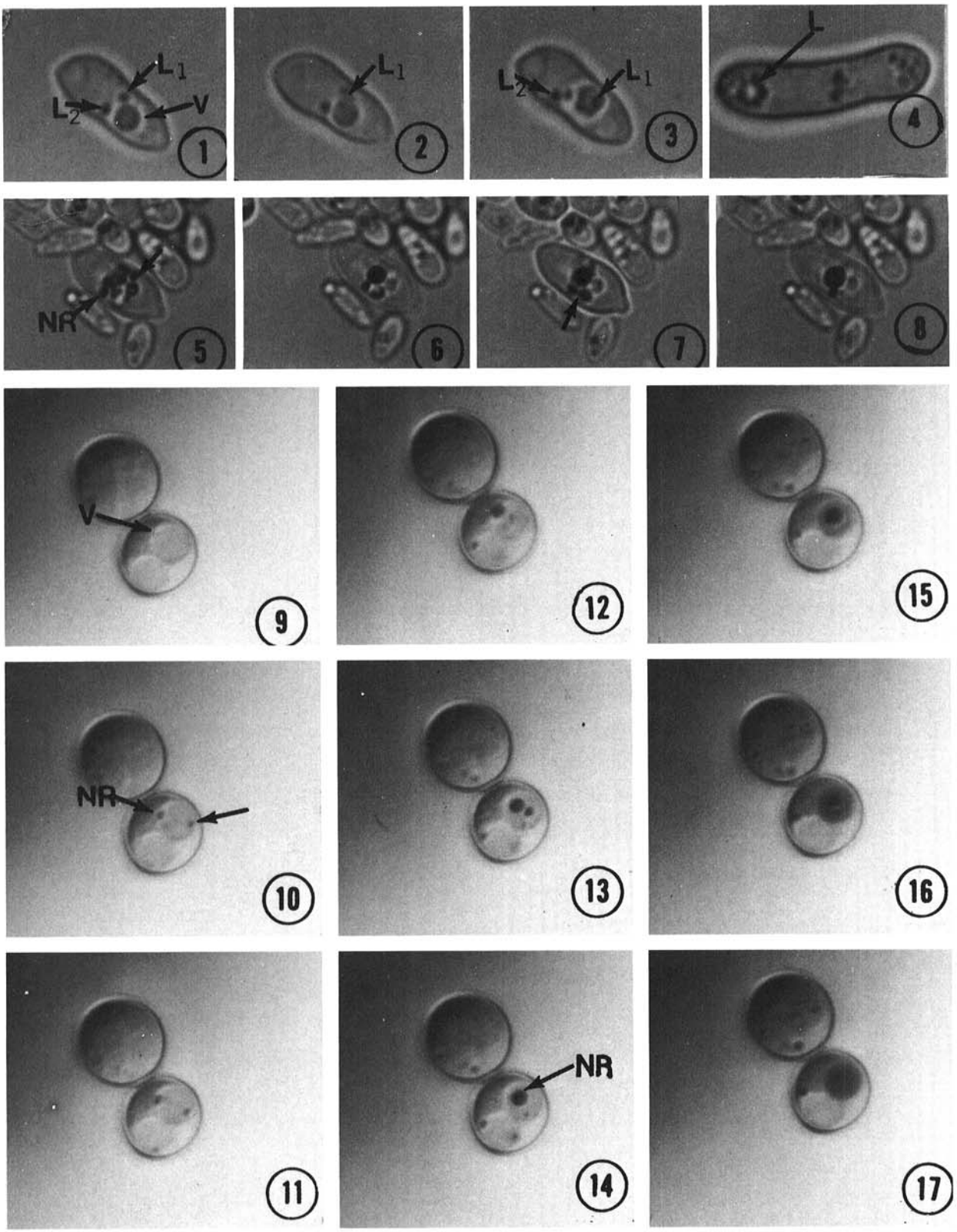
A distinction is made between pinocytosis (to sip) and phagocytosis (to eat). If the material being engulfed is in solution, the process is termed pinocytosis. Phagocytosis connotes the engulfment of solid substances. Sometimes a clear distinction cannot be made between the two processes; endocytosis is used in reference to either process. Although pinocytosis and phagocytosis have long been known in animal cells, their occurrence in higher plants or fungi has only recently been recognized. Two types of phagocytosis have been distinguished: heterophagocytosis, in which exogenous materials are taken into the cell by membrane engulfment; and autophagocytosis, in which normal cellular components are taken into compartments and digested (18). Autophagocytosis in plant cells for the recycling of organelles, membranes, and ground plasm occasionally has been reported (14). Autophagocytosis of lipid bodies (apparently for the digestion and mobilization of lipids) has been observed (16,17).

Pinocytosis by the plasmalemma also has been infrequently reported in plants. Mahlberg (9) demonstrated by the uptake of neutral red that the plasmalemma of plant cells can invaginate and form endocytic structures ("secondary vacuoles") that eventually enter the central vacuole. Mason and Wilson (11) found endocytic vesicles formed by the plasmalemma of *Desmodium* cells adjacent to the parasitic species of the fungus, *Elsinoë*. Wilson (19) was able to follow the apparent endocytic uptake of neutral red into vesicles of *Ceratocystis ulmi* spores that subsequently were incorporated into the vacuole. It also has been demonstrated that vacuoles of *Ceratocystis ulmi, Botrytis cinera,* and *Cryptococcus neoformans* spores take up acridine orange and fluoresce similar to animal lysosomes under ultraviolet radiation (20). This study had as its purpose an investigation of the role of vacuoles in the lysosomal system of fungi and was specifically concerned with the dynamics of vacuoles.

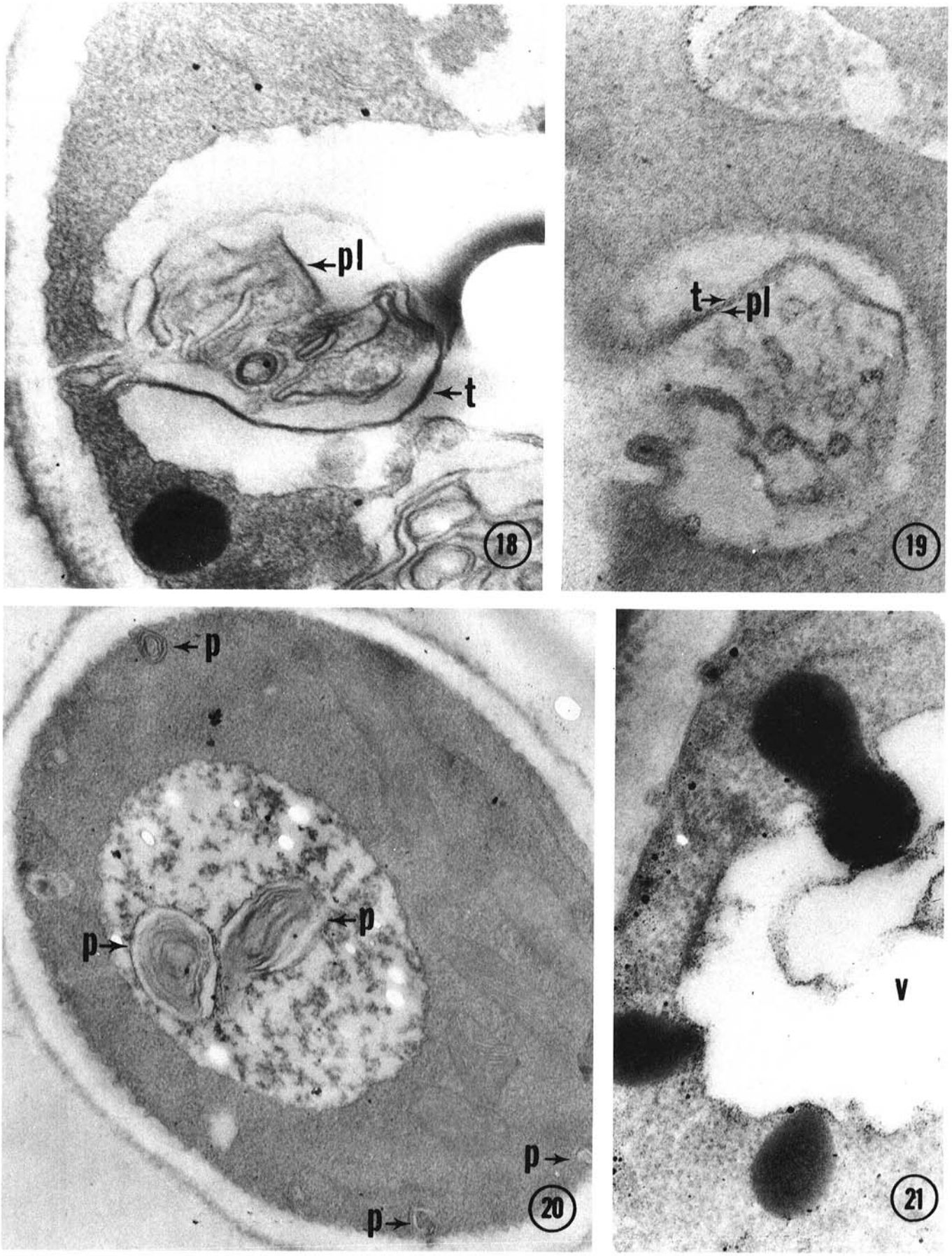
MATERIALS AND METHODS

For light microscopic observations of the uptake of neutral red spores of *B. cinerea* Pers. ex. Fr. and *C. ulmi* (Buism.) C. Moreau from 4- to 6-day-old cultures on PDA were placed on a microscope slide. A drop of water was added, and a coverslip was placed over the spores and sealed to the slide with paraffin along its two longer sides. A drop of neutral red in aqueous solution (1:200,000, w/v) was placed at the edge of the coverslip and allowed to diffuse under it. For observations of lipid body movement in *C. ulmi* spores, the fungus was grown on slides dipped in potato dextrose agar. After 4 days, a coverslip was placed over the growth and sealed to the slide with paraffin (Figs. 1–4). Photographs were taken on Kodak Tri-X film through Nomarski interference contrast or bright-field optics in a Zeiss Photomicroscope equipped with an electronic-strobe flash.

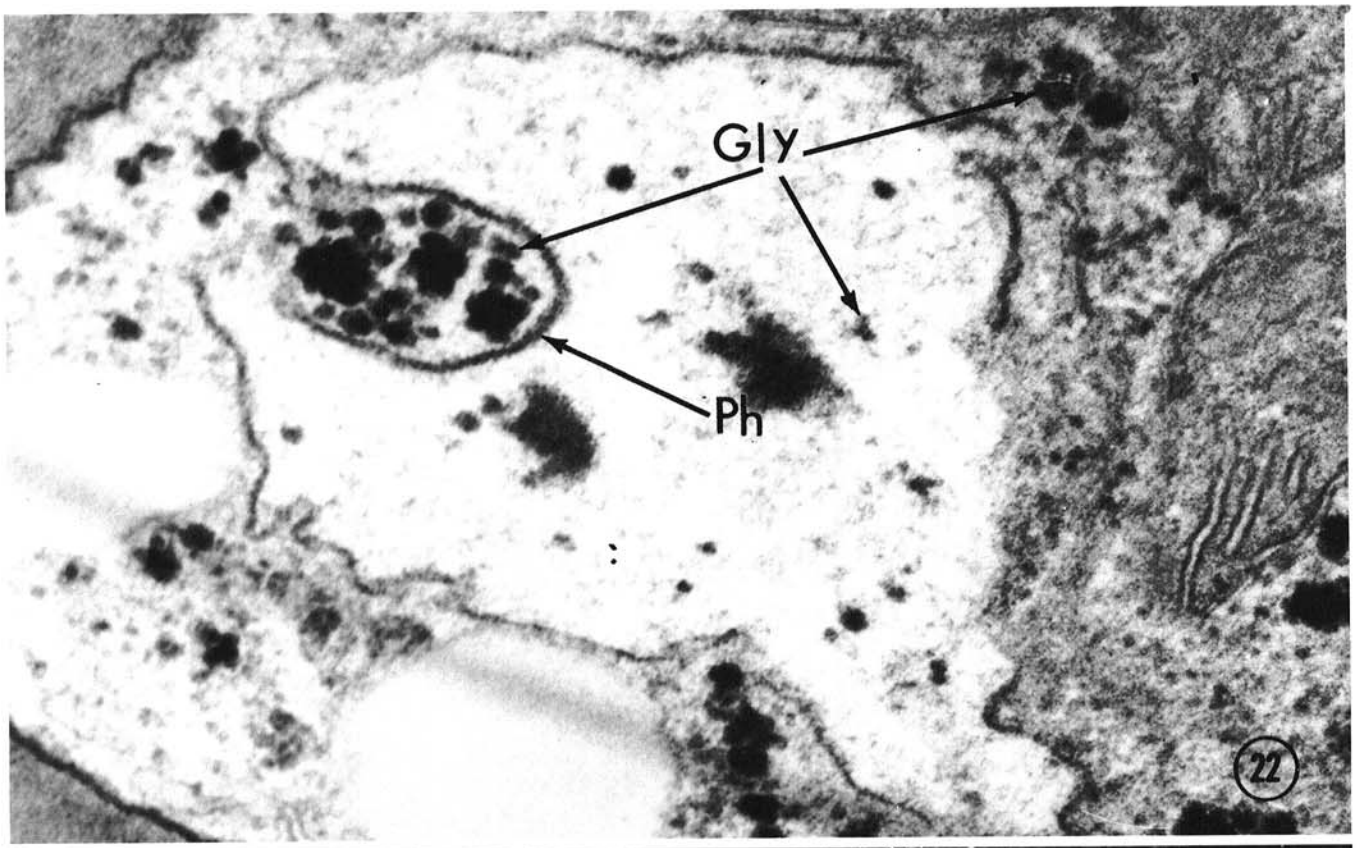
For electron microscopic study of vital-dye uptake, *B. cinerea* spores were placed in a solution of neutral red (1:40,000, w/v) for 3–15 min. They were then examined with a light microscope to confirm that neutral red had localized within vacuoles. If it had, the spores were prepared for electron microscopy according to the

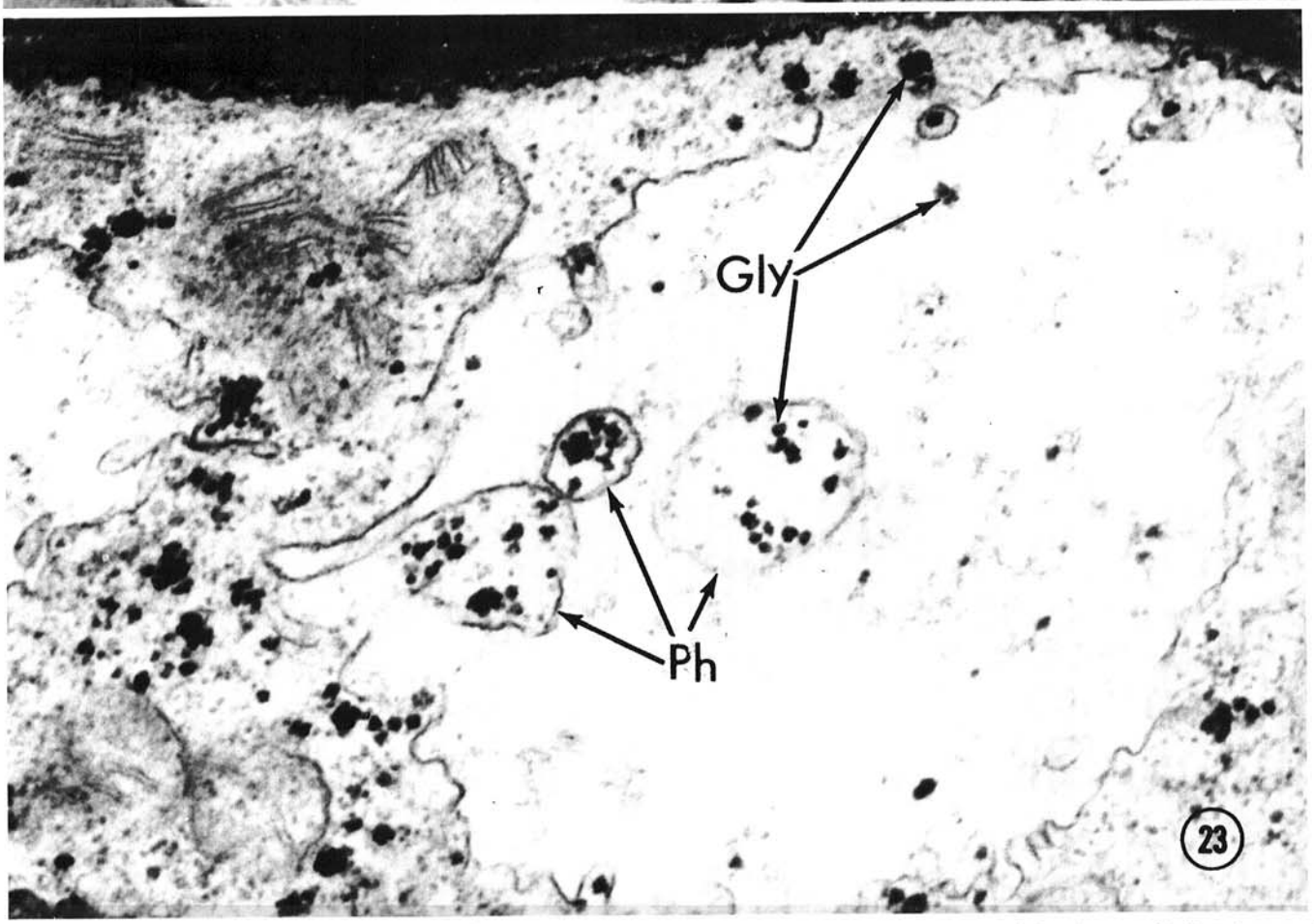


Figs. 1–17. Interaction of spore vacuoles in *Ceratocystis ulmi* and *Botrytis cinerea* with lipid bodies and neutral red dye. **1–3,** Entry of two lipid bodies into the vacuole of a *C. ulmi* spore (elapsed time, 10 min; bright-field microscopy ~ ×4,375). **1,** Two lipid bodies (L_1 and L_2) outside vacuole and a large lipid body within the vacuole. **2,** Dumbbell-shaped lipid body (L_1) half in and half out of the vacuole. Compare with Fig. 21. **3,** One lipid body L_1 has entered the vacuole and fused with large lipid body within. Another lipid body L_2 is half in and half out of the vacuole. L_2 subsequently entered the vacuole and also fused with the large lipid body. **4,** Spore of *C. ulmi* with a large number of lipid bodies (L), some congregated around a vacuole (bright-field microscopy, ~ ×4,375). **5–8,** Ten-minute sequence showing fusion of numerous small neutral red bodies (NR) in the vacuole of *C. ulmi* spore to form one large body. Arrow in Fig. 7 shows point of fusion of two bodies (bright-field light microscopy ~ × 4,375). **9–17,** Nomarski interference contrast microscopy showing a twenty-minute sequence following addition of ~ 1/20,000 (w/v) neutral red solution to *Botrytis cinerea* spores (~ X3,500). **9,** Very small vesicles (V) are seen along the outside of the tonoplast 15–30 sec after exposure of the spores to neutral red dye. **10,** Vesicles fuse, forming small spherical neutral red bodies which enlarge, 2–5 min. **13–15,** Bodies formed by fusion also enlarge and fuse, 10–12 min. **17,** Neutral red body continues to increase in size until it can no longer be observed, 20 min.



Figs. 18–21. Electron micrographs showing invaginations of plasmalemma and lipid bodies into the vacuole of *Botrytis cinerea* spores. **18–19,** Spores treated with neutral red. Plasmalemma (pl) and tonoplast (t) are closely appressed. **18,** (~ × 42,500). **19,** (~ × 85,000). **20,** Spore treated with neutral red that has similar multilaminated bodies (p) that are protruding into the ground plasm and vacuole (~ × 21,250). **21,** Lipid bodies (dark staining) around vacuole of *B. cinerea* spore. One appears to be entering the vacuole by phagocytosis (~ × 105,000).





Figs. 22 and 23. Electron micrographs of vacuoles of *Elsinoë wisconsinesis* with apparent autophagocytized vesicles containing glycogen. **22,** Protuberance of ground plasm into the vacuole, forming an apparent phagosome (Ph). Glycogen granules (Gly) are apparent in the phagosome, ground plasm, and vacuole (~ ×72,000). **23,** Autophagic vesicles (phagosomes, Ph) containing glycogen. The membranes of the phagosomes appear to be in different stages of digestion and some glycogen granules (Gly) can be seen free in the vacuolar sap (~ × 58,800).

modified procedure of Aist and Williams (1). The ultrastructural preparations of *Elsinoë wisconsinensis* were made as described by Mason and Wilson (11).

RESULTS

Uptake of neutral red by *B. cinerea* and *C. ulmi*. *Light microscopic observations.* One way to observe endocytosis directly by vacuoles is to monitor the uptake of vital dyes with the light microscope (Figs. 5–17). When hundreds of *B. cinerea* spores were studied in this manner, localization of neutral red was first apparent in small vesicles along the inner surface of the tonoplast (Fig. 9). These small vesicles fused, forming one or two large neutral red bodies within the vacuole (Fig. 10). Uptake of neutral red through the spore wall, plasmalemma, and spore ground plasm was not observed with the light microscope. Addition of NaF to the dye inhibited its localization, indicating that energy is required for the uptake process. Germination tests indicated that *B. cinerea* spores were viable after uptake and localization of neutral red. Some of the neutral-red bodies within the vacuole eventually shrank and disappeared, simultaneously staining the vacuolar sap (Figs. 16 and 17). Apparently the neutral-red bodies rupture, releasing dye into the vacuolar sap.

Wilson (19) previously described the uptake of neutral red into *C. ulmi* spores through endocytic vesicles that apparently were phagocytized by the vacuole. Figs. 5–8 illustrate the fusion of several such neutral red-containing phagosomes into two bodies.

Electron microscopic observations. Spores of *Botrytis cinerea* treated with neutral red had numerous invaginations of the plasmalemma (Figs. 18–20). Such invaginations were infrequently seen in untreated spores. Some invaginations had endocytized to the extent that small vesicles with a multilaminar consistency were formed in vacuoles (Figs. 18 and 20). Other vesicles of a similar appearance were found within the ground plasm of spores treated with neutral red and may have been formed from endocytosis of the plasmalemma. Such structures also could represent damage from the vital dye. Matile (12) found that acridine orange induced the formation of aberrant invaginations of the plasmalemma which resulted in the separation and elimination of cytoplasm.

The most direct evidence of phagocytosis of endocytic vesicles by vacuoles of *B. cinerea* was the observation of plasmalemma invaginations protruding into vacuoles (Figs. 18 and 19). Similar observations were reported for *Tradescantia virginiana* stamen-hair cells, in which peripheral vesicles have been found protruding into the central vacuole of hair cells (10) and in *Desmodium* cells parasitized by *Elsinoë wisconsinensis* (11).

Autophagocytosis of lipid bodies by *C. ulmi* and *B. cinerea* vacuoles. Lipid bodies in the spores of *C. ulmi* are distinguishable because they are birefringent with dark-field optics and fluoresce bright white when stained with Nile blue (21). They frequently are clustered around vacuoles (Fig. 4) and some are in vacuoles (Figs. 1–3). On several occasions and in different spores, lipid bodies of *C. ulmi* spores were observed entering the vacuole of living cells, presumbably by autophagocytosis. Figs. 1–3 is a photographic sequence during a period when two lipid bodies entered a vacuole. Upon entering the vacuole lipid bodies may fuse with one another or remain distinct.

Lipid bodies in *B. cinerea* appear as bright white spheres when viewed with dark-field optics and as colorless spheres when viewed with Nomarski interference contrast optics. They commonly were distributed along the periphery of the spore protoplast and sometimes distributed around vacuoles. In electron micrographs, lipid bodies appeared to be moving into vacuoles by autophagocytosis (Fig. 21). Similarly Bauer and Sigarlakie (2) observed apparent autophagocytosis of lipid bodies (spherosomes) by yeast vacuoles.

Autophagocytosis of glycogen by *Elsinoë wiconsinensis*. Mason and Wilson (11) described the balanced parasitic relation of the fungus, *E. wisconsinensis*, on its host *Desmodium*. Autophagocytosis observed in *Elsinoë* involved the movement of glycogen from the ground plasm into the vacuole. In the present study, various stages of autophagocytosis of glycogen by the vacuole were visible in electron micrographs (Figs. 22 and 23). The tonoplast appeared to form small involuted protuberances containing glycogen granules (Fig. 22). The involuted protuberances appeared to break away from the tonoplast, forming autophagosomes within the vacuole (Fig. 22). Various stages of degradation of the membrane surrounding the glycogen could be seen in the vacuole along with free glycogen granules (Fig. 23).

DISCUSSION

We were not able to delineate the entire pathway whereby exogenous substances such as neutral red are taken up by fungal cells and eventually deposited in the vacuole. However, our light- and electron microscope observations both indicated that such substances may be taken up into endocytic vesicles that subsequently enter the vacuole. The pathways indicated in these studies may play an important role in normal uptake and transport of certain substances by fungal cells. If stored materials in fungal cells such as glycogen and lipid were digested in situ without having been compartmentalized, the hydrolyses involved might be destructive to the ground plasm. The autophagocytosis and subsequent digestion of lipid bodies and glycogen by fungal vacuoles probably provides a means of protection against such injury.

The ability of fungal cells to compartmentalize foreign material and transport it to a lytic compartment (the vacuole) also may be important as a defense against toxins, including fungicides. Conceivably toxic materials could be compartmentalized in vesicles that are subsequently phagocytized by vacuoles, in which the toxins could be hydrolyzed and detoxified. Wilson (19) has proposed such a mechanism for the activity and detoxification of fungicides.

Heterophagy has not been generally recognized as occurring in plant cells. However, the uptake of neutral red in root hairs of *Tradescantia* (9) and spores of *Ceratocystis ulmi* (19) can be interpreted as heterophagy. Also, the digestion of bacteroids in pea roots is considered by Matile (14) to be heterophagy. More attention needs to be given heterophagy and its role in normal and abnormal physiology of plant cells.

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