Fine Structure of Phytophthora infestans During Sporangial Differentiation and Germination

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ABSTRACT

Sporangial formation, differentiation, and germination were studied in *Phytophthora infestans*. The cytoplasm of about 90% of the mature sporangia cultured at 20 C contained cleavage vacuoles and well-developed flagella. The sporangia appeared ready for cytokinesis and indirect germination. About 10% of the mature sporangia retained cytoplasm similar to that of vegetative hyphae and had neither basal bodies nor flagella. When sporangia were induced to germinate at 12 C, about 90% released zoospores. The remaining 10% did not germinate. When sporangia from the same lot were induced to germinate at 20 C, however, approximately 10% germinated directly to form germ tubes. Sporangia with germ tubes always had cytoplasm similar to that of immature sporangia. The other 90% did not germinate at 20 C. At the ultrastructural level, about one-third of nongerminating sporangia contained disorganized flagella, indicating resorption. We suggest that flagellar resorption occurs regularly at higher temperatures before sporangia germinate directly, and is a means of increasing spore longevity. We consider mature sporangia containing cleavage vacuoles and flagella to be differentiated from cytoplasm in vegetative hyphae. Flagellar resorption is one evidence of the dedifferentiation which takes place prior to direct germination at warm temperatures. Phytopathology 60:1765-1772.

Additional key words: ultrastructure, potato late blight fungus, spore germination.

Numerous workers since de Bary (2) have confirmed the ability of sporangia of *Phytophthora infestans* (Mont.) d By. to germinate either by zoospores or by germ tubes. This ability to germinate either indirectly or directly is influenced by temp. At 12-15 C, the multinucleate cytoplasm of sporangia is cleaved, and uninucleate zoospores are released; at 20-23 C, multinucleate germ tubes arise from sporangia with undivided cytoplasm. With increasing age, sporangia lose their ability to germinate indirectly. This loss occurs slowly at low temp, and occurs more rapidly at higher temp or following a heat shock of 40 C for 7-10 min (6, 14, 21).

The importance of indirect germination in epidemiological studies emphasizes the need for complete information about sporangial maturation and cytological changes during germination. Although King et al. (14) and Hemmes & Hohl (10) suggest flagellar resorption, this has not been established. Indeed, the lack of flagella in aged sporangia could be interpreted as an associated, and not as a causal, phenomenon. Our study was undertaken to gain a better understanding of sporangial maturation and of changes occurring before and during indirect and direct germination. A preliminary note has been published (8).

MATERIALS AND METHODS.—The original culture of *P. infestans* was provided by J. R. Wallin, Department of Botany and Plant Pathology, Iowa State University. The culture, established from a single zoospore in 1956, has remained virulent. Stock cultures were maintained at 12 C on ground-soybean agar. Working cultures were prepared by placing a cut, fresh-frozen green bean in each test tube with sufficient deionized water to cover half the bean. After autoclaving, the aerial portion was seeded with mycelium, and these cultures were incubated at 20 C.

Attached sporangia and associated hyphae.—Two to 4 days after seeding, small portions from the advancing edge of the mycelium were fixed for 1 to 2 hr at 4 C in 0.1 M phosphate or 0.02 M cacodylate buffered 3% (v/v) glutaraldehyde at pH 7.2-7.4, or were fixed 12 to 45 min at room temp in unbuffered 4% aq potassium permanganate at pH 6. The glutaraldehyde-fixed material was postfixed 1 to 1½ hr at 4 C in 1% (w/v) osmium tetroxide buffered with veronal-acetate at pH 7.2-7.4. Specimens were dehydrated in ethanol and propylene oxide and were embedded in Epon 812 epoxy resin. Blocks containing single sporangiophores were trimmed and mounted for longitudinal sectioning. Glutaraldehyde-osmium fixed specimens were stained in methanol-uranyl acetate (20), and permanganate-fixed specimens were stained with lead citrate (22) or were used unstained. Observations were made by using an RCA EMU-3F electron microscope operating at 50 kv. Sporangia exhibiting cytoplasmic continuity with their sporangiophores were designated "immature". Associated hyphae included in these blocks were also studied.

Detached sporangia.—Sporangia became detached from sporangiophores when aerial hyphae from working cultures were dipped directly into fixative. The ability to be easily detached in this manner was assumed characteristic of mature sporangia and was confirmed later by direct observation. These sporangia, detached 4 days after seeding, were fixed by using both fixations and were pelleted at slow speed into depressions in small blocks of 4% agar cast in centrifuge tubes. Dehydration, embedding, microtomy, staining, and microscopy were described previously.

Zoospore release at 12 C.—Vials containing a small amt of deionized water were chilled to 12 C. Sporangia from working cultures were detached directly into the

water, incubated at 12 C, then fixed and pelleted as before at intervals of 15, 30, 60, and 90 min. After 2 hr in vials at 12 C, the germination percentage for several hundred sporangia was determined by light microscopy.

Germination at 20 C.—Vials containing a small amount of deionized water were maintained at 20 C. Sporangia from working cultures were detached into the water, immediately exposed to a 40-C heat shock for 7-10 min (21), then rapidly cooled to 20 C. Sporangia were fixed and pelleted as before, immediately after cooling to 20 C and after 4, 8, 13, and 20 hr of incubation in water at 20 C. Germination percentage was determined after 22 hr at 20 C in the same manner as described for germination at 12 C.

RESULTS.—*Vegetative hyphae and immature sporangia.*—The walls of both hyphae and immature sporangia were approximately 0.1 μ thick and appeared electron-transparent, except for a thin, relatively electron-dense outer surface (Fig. 1-A). The plasma membrane was appressed to the inner surface of the wall (Fig. 1-A), and the appearance of nuclei, dictyosomes, lomasomes, mitochondria, endoplasmic reticulum, and ribosomes was comparable to structures previously described in hyphae of Oomycetes (3, 7, 9, 17). A structure resembling a longitudinally-sectioned centriole or procentriole was seen near a nucleus (Fig. 1-A), but neither basal bodies nor flagella were observed in these immature sporangia and hyphae.

The cytoplasm contained many large vacuoles (Fig. 1-A). When fixed in glutaraldehyde and osmium, vacuolar contents appeared electron-transparent except for occasional profiles of electron-dense lipoidal inclusions ≤ 0.5 μ in diam (Fig. 1-A). Scattered throughout the cytoplasm were other lipid bodies ≤ 2 μ in diam similar to those previously described in hyphae of Oomycetes (3, 7, 17).

Mature sporangia.—The distinction between mature and immature sporangia was based not only on the ease of detaching sporangia in water, but also on the presence of a basal plug. During sporangial development, hyphal-type cytoplasm was present until shortly before basal plug formation: then, within a very short time, the basal plug was formed, and the cytoplasm assumed characteristics of mature sporangia. These sporangia were easily detached when placed in water. The wall (approximately 0.3 μ thick) often appeared faintly layered. The vesicular layer described for *P. erythroseptica* (6) was not observed. In about 10% of the 36 mature sporangia examined, the cytoplasm appeared similar to that found in immature sporangia. In the remaining 90%, however, striking differences were observed (Fig. 1-B). Nuclear profiles were pyriform in shape, with the narrowed end oriented to the periphery of the sporangium and associated with typical paired basal bodies (Fig. 3-A). Flagella in vacuoles near the sporangial wall axially adjoined the basal bodies (Fig. 3-A). Nuclei, basal bodies, and flagella of *Phytophthora* have been described previously (11, 12, 13, 18, 23). Profiles of filaments resembling mastigonemes (approx 25 nm) in diam were observed and occasionally were associated laterally with the flagellar membrane (Fig. 2-A).

After glutaraldehyde and osmium fixation, cleavage vacuoles are electron-transparent and similar to the flagellar vacuoles, except for the absence of flagella. These vacuoles appeared homologous with the vesicular components of dictyosomes (Fig. 2-B). Some of the lipoidal inclusions within vacuoles showed striations of 30 to 100 A periodicity (Fig. 2-C). After permanganate fixation, the striations were not evident, and these vacuoles were more electron-dense than the cleavage vacuoles (Fig. 2-B). Microtubules, approx 15 nm in diam, in membrane-bound packets similar to those previously reported in *Phytophthora* (14, 18, 23), were observed randomly in the cytoplasm (Fig. 2-D). Lomasomes were not observed.

Zoospore release at 12 C.—At 12 C, about 30% of the mature sporangia released zoospores within 90 min. During formation of zoospores, the cleavage vacuoles became arranged radially, approximating the future planes of cytokinesis. The margins of flagellar vacuoles fused with the coalescing cleavage vacuoles (Fig. 3-B). In numerous instances, profiles of cytoplasmic elements were observed between the membranes of this system and the intact plasma membranes (Fig. 3-B). At later stages, similar profiles of cytoplasm were not seen, and the flagella lay between the plasma membrane and sporangial wall. Membrane-limited inclusions resembling the spherosomelike bodies reported by Ho et al. (11), and the microbodies reported by Hemmes & Hohl (10), were apparent in permanganate-fixed material of immature, mature, and germinating sporangia (Fig. 3-B).

Germination at 20 C.—At 20 C, about 10% of the sporangia germinated by germ tubes. The remainder did not germinate. Sporangia with germ tubes always had cytoplasm similar to that of immature sporangia and vegetative hyphae (Fig. 4-A). A newly formed inner wall was observed between the sporangial wall and the plasma membrane. The wall was most apparent in the apical region (Fig. 4-A), and tapered away to nothing in the body of the sporangium. This differs from *P. parasitica,* in which the newly formed inner wall was reported to completely surround the sporangial protoplast (10). About one-third of the nongerminating sporangia contained disorganized flagella or flagellar vacuoles with vesicular material (Fig. 4-C). We assumed that this condition indicated a stage of flagellar resorption. In these sporangia, the striated lipoidal inclusions appeared to change to uniformly dense lipoidal inclusions. Perinuclear centrioles and intranuclear microtubules (approx 17 nm in diam) were observed in some sporangia with germ tubes (Fig. 4-B), suggesting that some of the nuclei were fixed during intranuclear mitosis.

DISCUSSION.—The vacuolate cytoplasm present in immature sporangia is similar to that found in hyphae and germination tubes. The presence of flagella, striated inclusions, and cleavage vacuoles distinguishes the cytoplasm of most mature sporangia from that of hyphae and immature sporangia. The occurrence of these

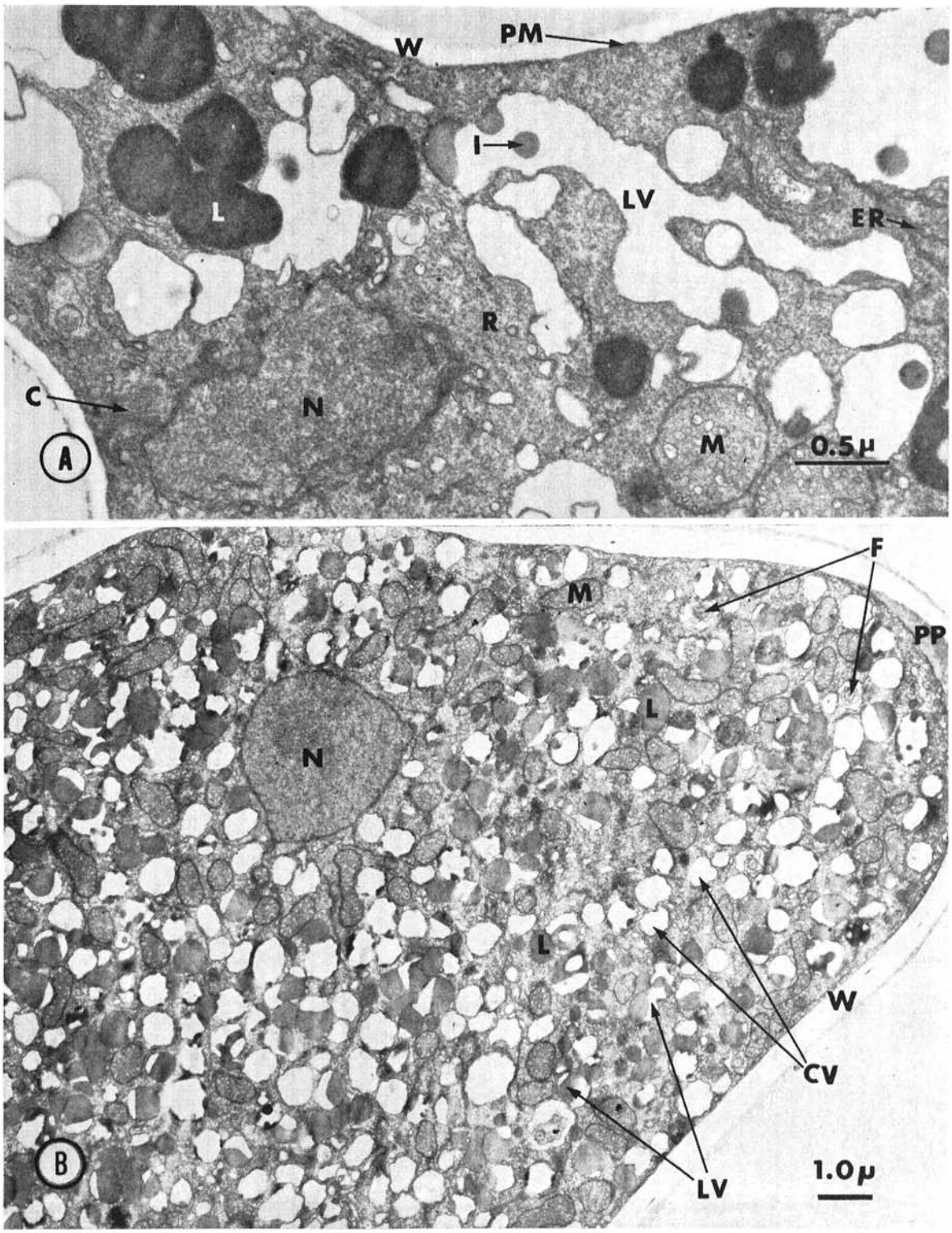

Fig. 1. A) Immature sporangium of *Phytophthora infestans*. Sporangium wall (W); plasma membrane (PM); vacuole (LV) with lipoidal inclusions (I); lipid body (L); mitochondrion (M); endoplasmic reticulum (ER); ribosomes (R); nucleus (N); centriole (C). Glutaraldehyde-osmium fixation and uranyl acetate staining (×30,000). **B)** Mature sporangium with flagella. Sporangium wall (W); papillar plug (PP); nucleus (N); flagella (F); cleavage vacuoles (CV); vacuoles (LV) with lipoidal inclusions; lipid body (L); mitochondrion (M). Glutaraldehyde-osmium fixation and uranyl acetate staining (×9,000).

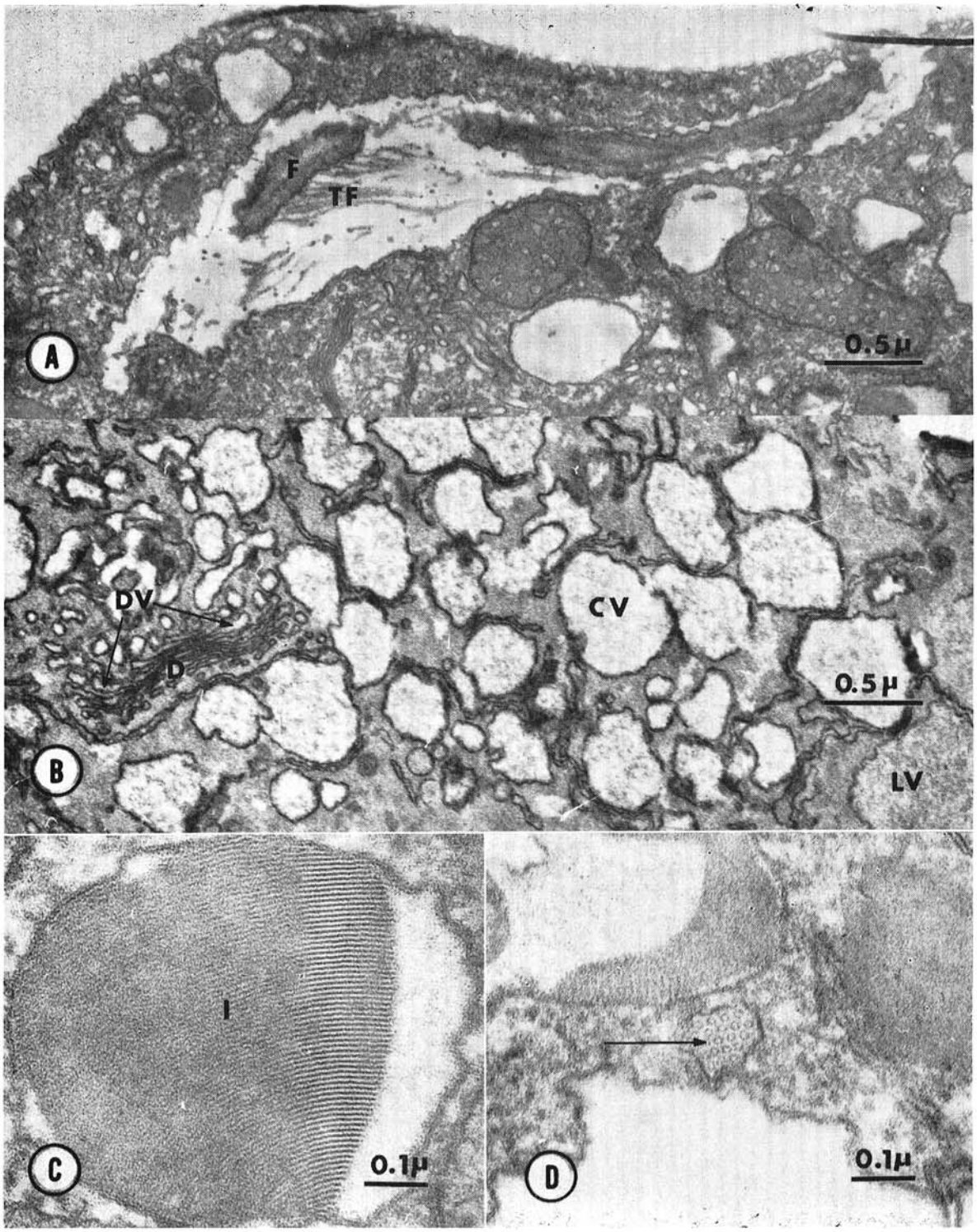

Fig. 2. Fine structure of a mature sporangium of *Phytophthora infestans* before exposure to germination conditions. **A)** Filaments (TF) attached laterally to a flagellum (F). Glutaraldehyde-osmium fixation and uranyl acetate staining (×30,000). **B)** Cleavage vacuoles (CV) surrounding dictyosomes (D). Vacuole (LV); dictyosome vesicles (DV). Potassium permanganate fixation and lead staining (×30,000). **C)** Vacuole with lipoidal inclusions. Myelinlike lipoidal inclusion (I). Glutaraldehyde-osmium fixation and uranyl acetate staining (×100,000). **D)** Membrane-bound packet of microtubules (arrow). Glutaraldehyde-osmium fixation and uranyl acetate staining (×100,000).

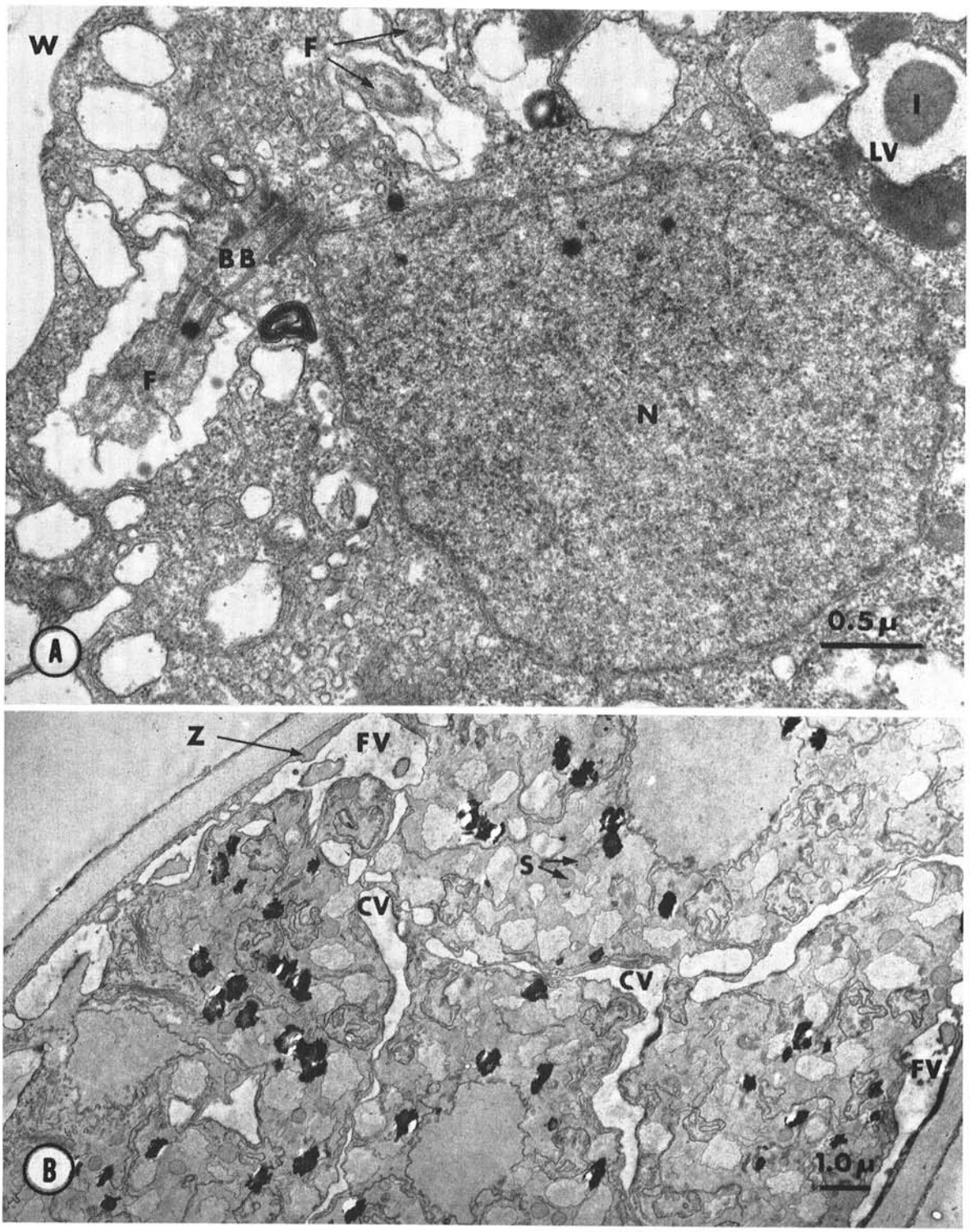

Fig. 3. **A)** Nucleus of a mature sporangium of *Phytophthora infestans* with flagella. Sporangium wall (W); nucleus (N); basal body (BB); flagella (F); vacuole (LV) with lipoidal inclusions (I). Glutaraldehyde-osmium fixation and uranyl acetate staining (×30,000). **B)** Fine structure of a mature sporangium during exposure to germination conditions at 12 C. Cleavage vacuoles (CV); flagellar vacuoles (FV); zone of cytoplasm (Z) between flagellar vacuoles and plasma membrane; membrane limited inclusions (S) resembling spherosomes. Potassium permanganate fixation (×9,000).

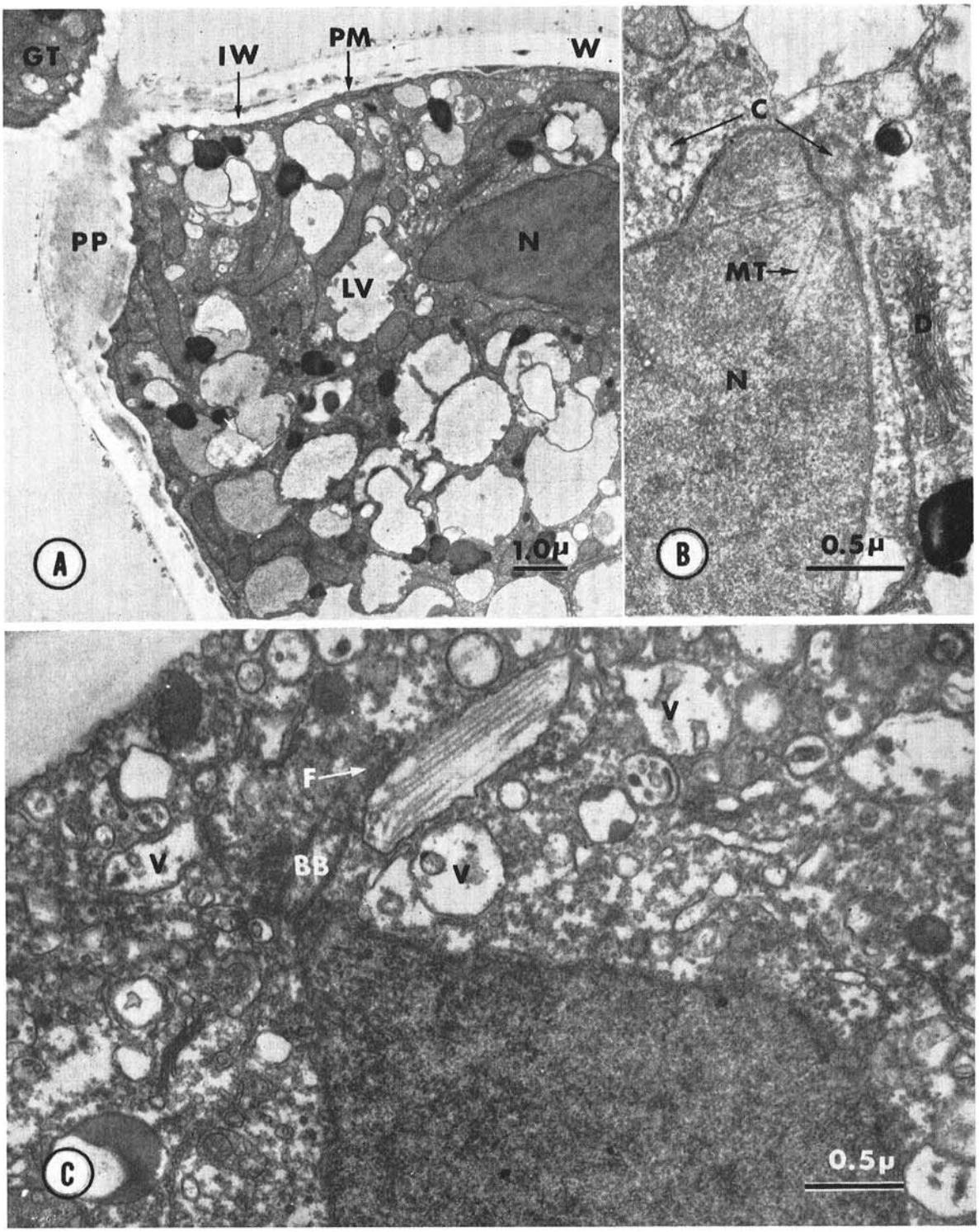

Fig. 4. Fine structure of mature sporangia of *Phytophthora infestans* during exposure to germination conditions at 20 C. Glutaraldehyde-osmium fixation and uranyl acetate staining. **A)** Germ tube (GT); papillar plug (PP); sporangium wall (W); inner wall (IW); plasma membrane (PM); vacuole (LV); nucleus (N) (×9,000). **B)** Nucleus (N); centrioles (C); intranuclear microtubule (MT); dictyosome (D) (×30,000). **C)** Basal body (BB); disorganized flagellum (F); vesicular material (V) within vacuoles. Glutaraldehyde-osmium fixation and uranyl acetate staining (×30,000).

changes at or near the time of basal plug formation agrees with our definition of mature sporangia as those possessing basal plugs. Aging of sporangia as related to an inability to germinate indirectly must then be considered as being initiated at time of basal plug formation.

Well-developed basal bodies and flagella were present in the cytoplasm of 90% of mature sporangia cultured at 20 C, even though the opt temp for indirect germination is 12-15 C. King & Butler (13) noted differentiation of flagella in *P. infestans* before chilling for indirect germination. They reported no structures that could be interpreted as the "hairs" (mastigonemes) on the tinsel flagellum, and suggested that these were formed at a later stage of zoospore formation, perhaps after cleavage and immediately before release. Our observations of flagella, some with mastigonemes, indicate an early and quite complete development in mature sporangia before exposure to conditions initiating zoospore release. We conclude that mature sporangia containing flagella are, except for completion of cytokinesis, immediately ready for indirect germination, provided that the requirements of temp and moisture are met.

The presence of striated inclusions has concerned numerous investigators (6, 11, 12, 14, 18, 23). Evidence from several sources suggests that such configurations represent myelinlike ordered arrays of hydrated lipid molecules (5, 15, 19). Williams & Webster (23) hypothesized that these lipids are used as an energy source or as a synthetic pool. In *P. infestans*, the ordered arrays were observed only in sporangia containing flagella and in zoospores. They were not present in hyphae and immature sporangia. In sporangia that appeared to be undergoing flagellar resorption, the striated inclusions also appeared to be changing, possibly into uniformly dense inclusions. The reason for these changes is not clear. If these inclusions represented an immediately available energy source, then the altered structures are even more puzzling. Since the dedifferentiated sporangium is a more entropic structure, perhaps this represents a change to a long-term source of reserves.

The sequence of zoospore cleavage was similar to that previously described in *Phytophthora* (8, 12, 14, 23). Cleavage vacuoles and flagellar vacuoles coalesce, and the membranes of this system appear to fuse with the plasma membrane, delimiting the zoospores and freeing the flagella. There is general agreement among investigators concerning the sequence of zoospore cleavage; however, the reports of the origin of cleavage vacuoles differ. Williams & Webster (23) noted that the great bulk of membrane used for cleavage vacuoles in *P. capsici* appeared to come from transformation of a central vacuole. Although Hohl & Hamamoto (12) found a central vacuole in young sporangia in *P. parasitica*, they hypothesized that cleavage "vesicles" originate from the Golgi apparatus. We observed a central vacuole in young sporangia of *P. infestans*, which agrees with the observation of King et al. (14). We did find evidence, however, that cleavage vacuoles originate from dictyosomes (Fig. 2-B).

There is considerable evidence suggesting resorption of flagella before germination by germ tube. Taylor et al. (21) and Aragaki et al. (1) reported over 50% germination when sporangia were stimulated to germinate directly, and over 80% zoospore release when sporangia were stimulated to germinate indirectly. Some workers have suggested that age of sporangia affects mode of germination; as age increases, a smaller percentage germinates indirectly (4, 6, 14, 16). King et al. (14) reported that, after aging, sporangia do not contain flagella, and they suggested that the absence of flagella in old sporangia indicates flagellar degeneration. Hemmes & Hohl (10) claimed that, after exposure of sporangia of *P. parasitica* to conditions favorable for direct germination, flagella form and then degenerate before germ tube formation. We suggest that the usual sequence of differentiation in 80-90% of the sporangia of *P. infestans* consists of a change from the highly vacuolate cytoplasm in immature sporangia to a cytoplasm containing flagella, cleavage vacuoles, and striated inclusions. These sporangia germinate indirectly at 12-15 C. Of the sporangia germinating directly at 20 C, some may represent the 10-20% that have never formed flagella. On the other hand, evidence of flagellar resorption and the large percentages of directly germinating sporangia (1, 21) indicate that some sporangia undergo dedifferentiation. We suggest that this dedifferentiation occurs regularly before direct germ tube formation. Perhaps zoospore release would be the more primitive mode of germination, and direct germination would be an evolutionary adaptation. Obviously, the potato late-blight organism is well adapted to conditions present in the plant canopy. During the warmer portions of the day, sporangia containing flagella, striated lipoidal inclusions, and cleavage vacuoles are formed and detached. On a substrate with a film of moisture, the mature sporangia germinate in cool evening temp by means of zoospores and provide the max chances for successful infection. When evening temp are warm, or moisture is lacking, the sporangium undergoes changes that, while not offering opportunities for multiple infection, do permit greatest spore longevity until favorable conditions return. Further study could involve the effect of heat shocks such as might occur during the warmest part of the day, and the consequences of this phenomenon on flagellar resorption.

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