Heat-Induced Ultrastructural Changes in Germinating Spores of *Rhizopus stolonifer* and *Monilinia fructicola*

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ABSTRACT

Germinating sporangiospores of *Rhizopus stolonifer* showed striking ultrastructural changes after exposure to nutrient broth at 52 C for a period of 2.5 min. Nuclei were either disrupted or very granular and disorganized. Mitochondria were distorted or disrupted. The ribosomes, normally distributed rather uniformly throughout the cytoplasm, tended to aggregate. The plasmalemma, while appearing relatively intact, was frequently withdrawn from the spore wall. Effects of heat-treatment on the ultrastructure of germinating conidiospores of *Monilinia fructicola* were qualitatively similar, but not as extreme as the effects described for the *Rhizopus* spore. Globular-shaped bodies were observed in some unheated *Rhizopus* sporangiospores during early stages of germination. These bodies consisted of regularly-spaced granules about 50-60 Å in diam. Phytopathology 60:869-874.

Germinating sporangiospores of *Rhizopus stolonifer* (Ehr. ex Fr.) Lind. and conidiospores of *Monilinia fructicola* (Wint.) Honey are killed when exposed to broth at 52 C for 2 min (14, 15). Dormant spores of these organisms are also relatively labile to heat (15), in contrast to the spores of some microorganisms in which germination may be activated by exposure to high temp, even as high as 100 C (17). The mechanisms underlying heat-resistance and heat inactivation in microorganisms are incompletely understood (11, 13, 16, 17). Heat inactivation of fungus spores is but one facet of the broad subject of heat effects on microorganisms. In the investigation reported here, we studied the effects of heat-treatment on germinating spores of *Rhizopus* and *Monilinia,* at the ultrastructural level.

MATERIALS AND METHODS.—*Source of spores.*—Both *Monilinia* and *Rhizopus* were isolated from decayed peaches, purified, and cultured on potato-dextrose agar at 21 C. Spores were harvested from these cultures after about 1 week, and samples were incubated in nutrient broth (beef-extract 3%, bactopeptone 5%, pH 7.0) at 21 C until about 90% of the spores developed germ tubes whose lengths were 2-3 times the diam of the spore. The spores in the nutrient broth were centrifuged at 35,000 *g* for 15 min and resuspended in nutrient broth to a concentration of about 2×10^5 spores/ml. A 5-ml aliquot of this suspension was pipetted rapidly into 35 ml nutrient broth previously heated to 52 C. After 2.5 min, the flasks were cooled immediately to 0 C in an ice bath. Nonheated spores were likewise cooled and held for the same length of time at 0 C before being prepared for microscopy.

Electron microscopy.—Spores were fixed within 5 min after cooling by adding cold 50% glutaraldehyde to the nutrient broth suspension until a final concentration of 2.5% was reached. After 1 hr, the spores were pelleted by centrifuging at 35,000 *g* for 15 min. The pellets were washed in nutrient broth for 10 min, and a solution of 1% OsO_4 in broth (pH adjusted to 7.0) was added to the pellet and allowed to stand for 2 hr. The OsO_4 solution was removed and the pellets were again washed with nutrient broth for 10 min. The fixed spores were dehydrated by a graded series of isopropyl alcohol (10-100%), and finally by two changes of propylene oxide. All operations up to the 100%-alcohol step were performed at 0-4 C. Some investigators have had difficulty in preserving fungus spore ultrastructure when using OsO_4 as a fixative or postfixative (4). In our hands, glutaraldehyde fixation followed by OsO_4 postfixation gave reasonably consistent results, but membrane contrast was not as great as in Buckley's material fixed in a chrome:osmium mixture (5).

Small pieces of the dehydrated pellets were embedded in Epon 812, generally according to the method of Luft (10). Uniform embedding of the spores was difficult, and a graded series of the complete embedding mixture dissolved in propylene oxide gave better results than the schedule suggested by Luft. Thin sections (600-800 Å) of the embedded material were prepared with an LKB ultramicrotome equipped with a diamond knife. The sections were stained with solutions of uranyl acetate and lead citrate according to a double-staining procedure discussed by Pease (12). A Philips EM-200 electron microscope was used to study the sections. Four separate experiments were performed with both *Rhizopus* and *Monilinia* spores, and the results presented are considered representative of those obtained in all experiments.

RESULTS.—*Unheated rhizopus sporangiospore.*—The germinating sporangiospore has a thick wall, consisting of an extremely electron-dense outer layer, a thick, moderately dense middle layer, and a thin inner layer (Fig. 1-A). The outer and middle layers are thickened along certain lines to form ridgelike structures. The results presented here generally corroborate those of Hawker & Abbott (8) concerning the *Rhizopus* sporangiospore wall.

Numerous mitochondria, some nuclei, structures re-

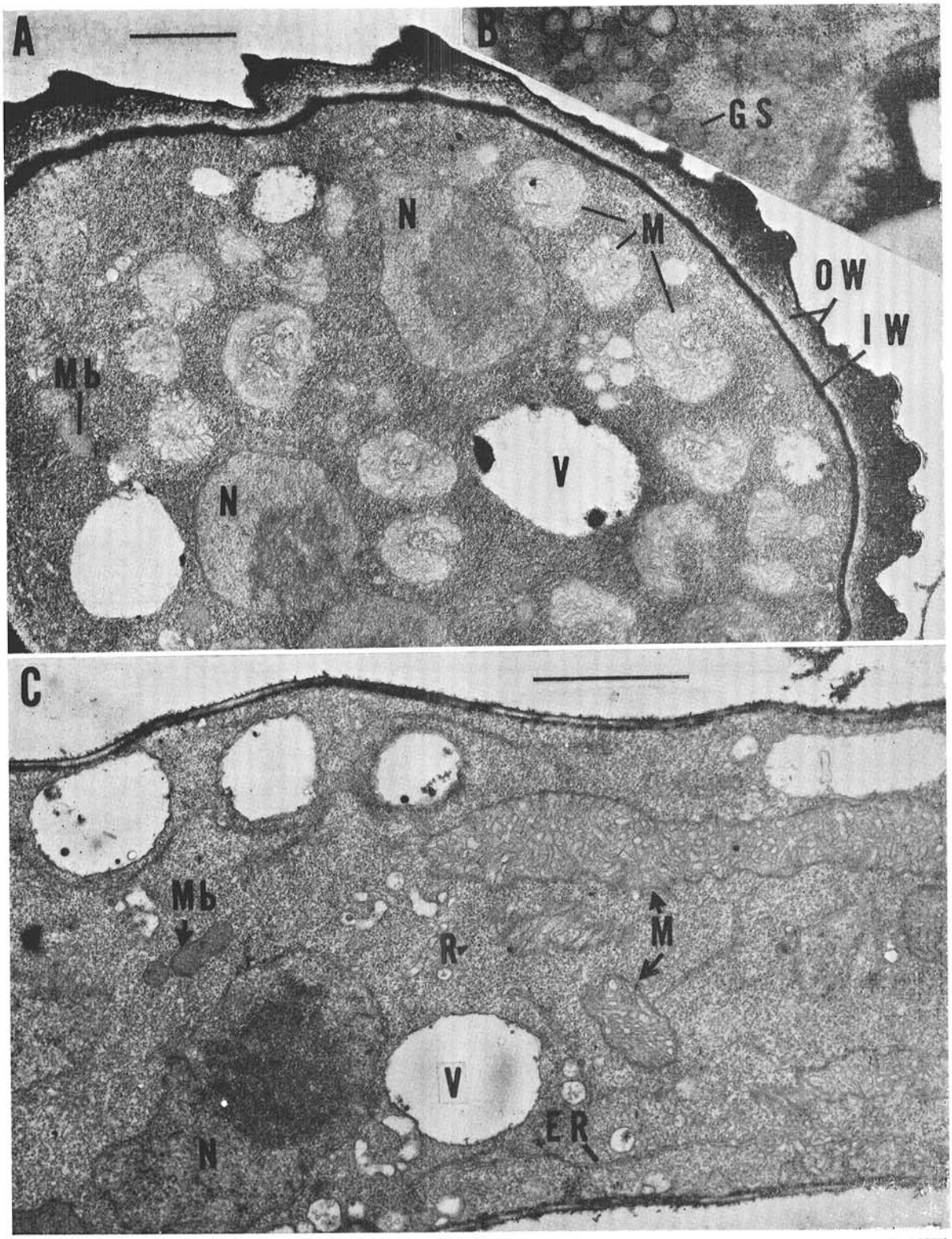

Fig. 1. A) Cross section through a germinated sporangiospore of *Rhizopus stolonifer*. Note the outer wall (OW) consisting of two distinct layers, the thin inner wall (IW), the nuclei (N), the vacuoles (V), the microbodies (Mb), and the mitochondria (M). The marker in this and subsequent figures indicates a length of 1 μ. **B)** Portion of a swollen sporangiospore in an early stage of germination, showing globular-shaped bodies (GS) with ordered substructure. The magnification in (B) is 2.6 times that in (A). **C)** Portion of *Rhizopus stolonifer* germ tube, showing microbodies (Mb), ribosomes (R), endoplasmic reticulum (ER), and other organelles as in the spore shown in (A).

sembling microbodies (1, 7), and vacuoles are evident in both the spore and the germ tube shown in Fig. 1-A, C. Ribosomes are numerous and are distributed rather uniformly throughout the cytoplasm. Endoplasmic reticulum is easily detected in the germ tube of Fig. 1-C but is difficult to resolve in the spore of Fig. 1-A, due to the density of ribosomes and low membrane contrast. Globular-shaped bodies containing regularly spaced granules about 50-60 Å in diam are present in early stages of germination before the inner spore wall is fully developed (Fig. 1-B). These bodies do not appear to be membrane-bounded, and do not resemble any structure previously reported in fungi.

Heated sporangiospore.—The wall of the heated spore appears similar to that of the unheated spore (Fig. 2), but rather drastic changes occur in the cellular contents during or after 2.5 min at 52 C. The cytoplasm shows aggregates of ribosomes, sometimes containing membranes (Fig. 2), in contrast to the normal spore that shows a rather uniform distribution of ribosomes (Fig. 1). No intact mitochondria are evident in the figures presented, although occasionally a mitochondrion was not completely disrupted. Nuclei are severely damaged, appearing disrupted in many cases and in other cases extremely granular (Fig. 2-A, B). The germinated spore shown in Fig. 2-C shows no recognizable mitochondria or nuclei.

Unheated Monilinia fructicola conidiospore.—The spore wall of the unheated conidiospore has a thin, electron-dense outer layer and a relatively thick electron-transparent layer (Fig. 3-A). It is similar in appearance to the wall of the *Botrytis cinerea* conidiospore in electron micrographs published by Hawker & Hendy (9) and Buckley et al. (4). The ER was easily demonstrated in the *Monilinia* conidiospore. Mitochondria were numerous in the germinating conidiospores, and were pleomorphic. Small peripheral vacuoles were observed near the plasmalemma, a finding also reported for the conidiospore of *Botrytis cinerea* (9). Dense granular bodies resembling microbodies of animal and plant cells (1, 7) and glyoxysomes from plants (3) were also demonstrated in the unheated *Monilinia* conidiospore (Fig. 3-A). They are similar in appearance to the microbodies observed in the *Gilbertella persicaria* zygospore (2). Frequently, micrographs of *Monilinia* conidiospores showed numerous vacuolelike bodies which in many cases contained electron-dense material (Fig. 3-A, inset). They are similar in appearance to the lipid bodies of *Gilbertella* (2).

The heated conidiospore.—The cell wall of the conidiospore shows no signs of structural alteration due to heat treatment (Fig. 3-B). Some alterations are evident in the internal ultrastructure of the spore, although they are by no means as severe as those observed in heated *Rhizopus* sporangiospores. Aggregates containing ribosomes are present in the heated conidiospore (Fig. 3-B). The nuclei and mitochondria both appear to be damaged, although they are not completely disrupted.

Caution must always be exercised in the interpretation of electron micrographs, particularly those of fixed and embedded materials. We considered the possibility that heat treatment altered the properties of spores in such a way that fixation or embedding was incomplete, and resulted in the derangements attributed to heat. However, the ultrastructural features described for heated spores were not observed in material that was poorly fixed or incompletely embedded. While it is still possible that we are dealing with indirect effects of heating on ultrastructure, all present evidence indicates that the following conclusions and discussion are justified.

DISCUSSION.—The observed derangements in heated spores, either damage to nuclei and mitochondria, aggregation of cytoplasmic components, or a combination of these two derangements, might produce a lethal effect. Therefore, on the basis of these results, thermal death of *Rhizopus* and *Monilinia* spores cannot be linked to a single first event. Perhaps a simple relationship is not to be expected, since death by moist heat is generally assumed to be a denaturation process. Rahn (13) discussed the basis of this assumption, which is that the high Q_{10}-value of death rate is characteristic of protein denaturation, a process likely to be rather nonspecific. Nevertheless, it is remarkable that such drastic effects occurred in the heated *Rhizopus* sporangiospore after a period of only 2.5 min at 52 C. This is a relatively short exposure to heat, compared to a 3-day exposure at 55 C required to kill conidia of *Penicillium chrysogenum* (17). According to some theories, heat-resistant cells have proteins that resist denaturation, or have some factor such as high fat content or low electrolyte content which acts as a mitigating influence against heat denaturation (11, 13, 17). While the spores of both *Rhizopus* and *Monilinia* were killed by exposure to the same heat treatment, the ultrastructural changes in *Monilinia* were much less severe than in *Rhizopus,* indicating basic differences in the chemistry of the two organisms.

The aggregation of ribosomes appears to be a different phenomenon than the "mottled cytoplasm" effect induced in *Rhizopus* sporangiospores by anaerobiosis (6). In the latter case there were empty spaces in the cytoplasm, but ribosomes were not in higher concentration, where present, than in the normal spore cytoplasm. The aggregation of ribosomes after heat treatment probably reflects a denaturation process.

While the primary purpose of this investigation was to study the effect of heat on the ultrastructure of *Rhizopus* and *Monilinia* spores, certain noteworthy cytological observations were made. Globular-shaped structures consisting of subunits 50-60 Å in diam were observed in *Rhizopus* sporangiospores in early stages of germination when the inner spore wall was not fully developed. We speculate that these structures are involved in the synthesis of the inner wall. It is doubtful that these globular structures are related to the "S inclusions" reported by Buckley et al. (5), since S inclusions apparently were membrane-bounded, and were observed at later stages of germination than were the globular-shaped bodies reported here. The electron micrographs presented here do not show structures resembling S inclusions, but we did observe such structures in other micrographs.

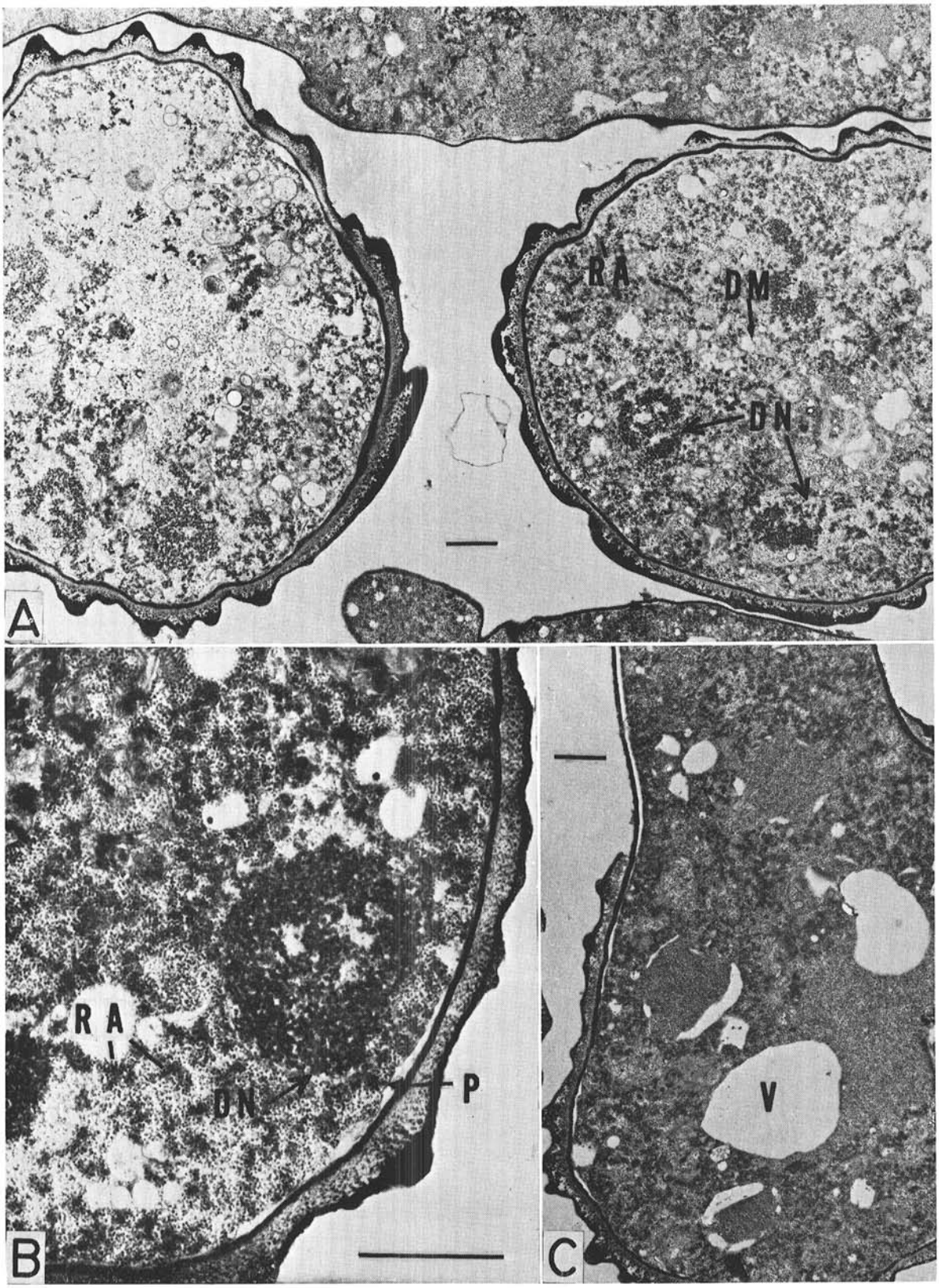

Fig. 2. Sections through germinating *Rhizopus stolonifer* sporangiospores heated at 52 C for 2.5 min. **A)** Note very granular, damaged nuclei (DN), membranes, and vesicles scattered throughout the cytoplasm, ribosomal aggregates (RA), and damaged mitochondria (DM). **B)** Enlarged portion of a heated sporangiospore showing granular nucleus, plasmalemma (P) withdrawn from the spore wall, and ribosomal aggregates. **C)** Section of sporangiospore and germ tube showing no recognizable nuclei or mitochondria.

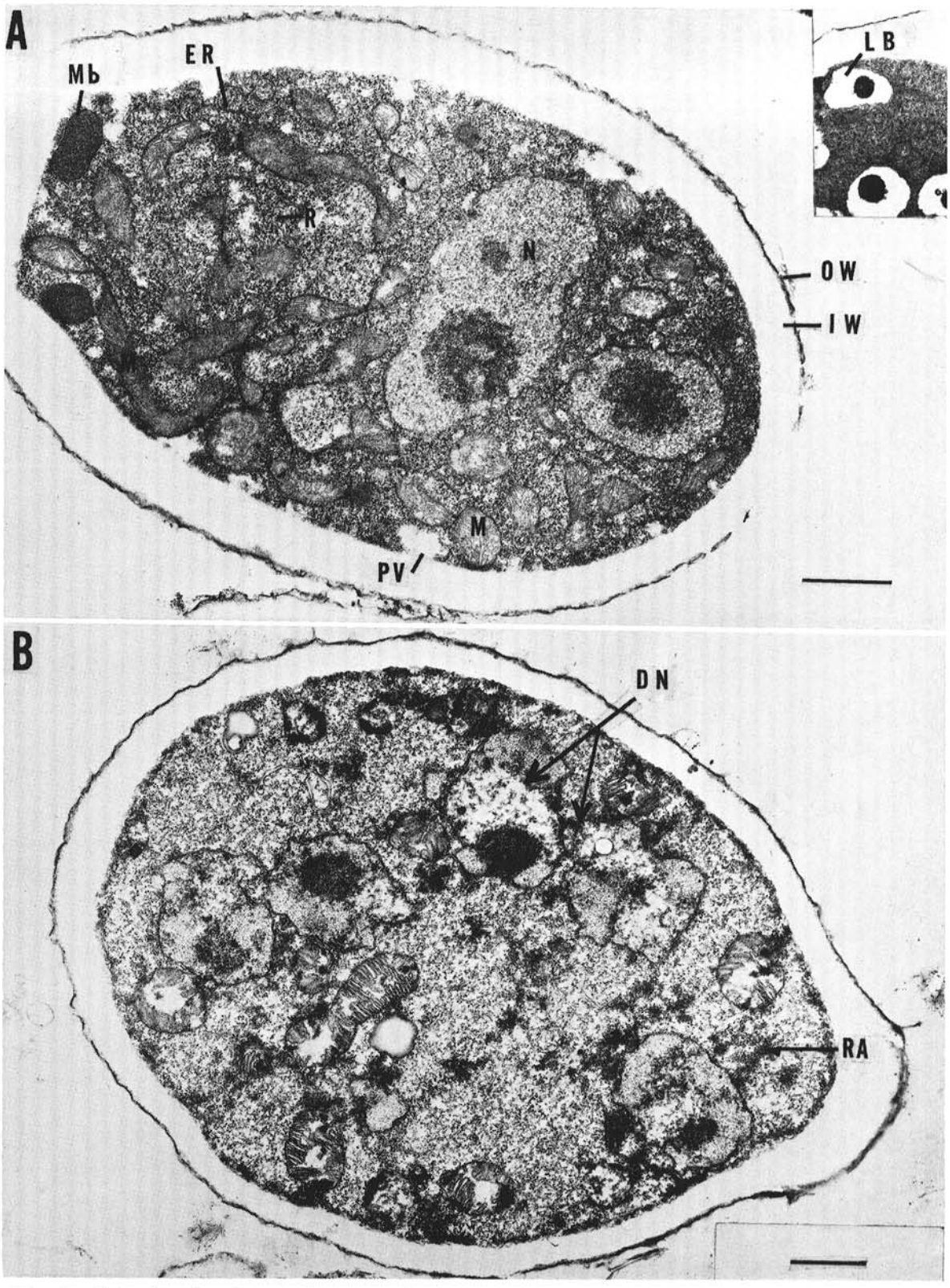

Fig. 3. **A)** Section through a germinating conidiospore (unheated) of *Monilinia fructicola* showing a thin outer wall (OW), a relatively thick, electron-transparent inner wall (IW), nuclei (N), ribosomes (R), endoplasmic reticulum (ER), mitochondria (M), peripheral vacuoles (PV), and microbodies (Mb). Inset shows a portion of another conidiospore containing several lipid bodies (LB). **B)** Conidiospore heated at 52 C for 2.5 min showing damaged nuclei (DN) and ribosomal aggregates (RA).

The ultrastructure of the *Monilinia* conidiospore wall resembled that of the *Botrytis cinerea* conidiospore. Features of the cytoplasm and organelles were similar to those of other fungus spores in general. Organelles resembling microbodies, and inclusions resembling lipid bodies noted in other organisms, were more numerous in the *Monilinia* conidiospore than in the *Rhizopus* sporangiospore. We believe these to be the first such observations reported for the *Monilinia* conidiospore.

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