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Electron microscopy of primary zoosporogenesis in *Plasmodiophora brassicae*

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Primary zoosporogenesis in resting sporangia of *Plasmodiophora brassicae* that had been incubated for 14 d in culture solution containing turnip seedlings was examined by transmission electron microscopy. A single zoospore differentiated within each sporangium, the differentiation being initiated by the emergence of two flagella in the tight space formed by invagination of the plasma membrane within the sporangium. The differentiating zoospore was similar in intracellular aspects to sporangia within clubroot galls. Then a deep groove formed on the zoospore cell body by further invagination of the plasma membrane. Two flagella appeared to coil around the zoospore cell body in parallel along this groove. Thereafter, the cell body lost the groove and became rounded following the protoplasmic condensation (contraction of cell body) during late development, and assumed an irregular shape at the stage of maturation. Intracellular features in developing and mature zoospores were complicated, being characterized by electron-dense nuclei and mitochondria, microbodies, cored vesicles and various unidentified cytoplasmic vesicles and granules. A nucleolus-like region was observed only in the nucleus of the mature zoospore. A partially opened germ pore was also seen in the sporangium containing the mature zoospore.

Key Words——fine structure; germ-pore; *Plasmodiophora brassicae*; primary zoospore; zoosporogenesis.

Plasmodiophora brassicae Woronin, the causal agent of clubroot disease of crucifers, is a member of the plasmodiophorids (Plasmodiophoromycetes), which are zoosporic obligate parasites and have two major phases in the life cycle. This organism infects host root hairs by primary zoospores, which are released from resting sporangia (primary zoosporangia) in the soil; and infects host cortical cells by secondary zoospores, which are released from secondary zoosporangia formed within host root hairs (Karling, 1968; Ingram and Tommerup, 1972; Dobson and Gabrielson, 1983).

There have been several works on the fine structure of the *P. brassicae* zoospore. Most of these, however, have been conducted on secondary zoospores (Dekhuijzen, 1979; Buczacki and Clay, 1984) while others have been done only on germinating primary zoospores dried and shadowed for preparation (Kole and Gielink, 1962) and on primary zoospores adhering to the surface of host root hairs for infection (Aist and Williams, 1971). There have been no works on the fine structure of primary zoospore *in situ* within the resting sporangium. Little detailed information, therefore, is also available on primary zoosporogenesis, although this knowledge is essential for understanding the biology of the primary zoospore and germination mechanisms of the resting sporangium.

Knowledge of primary zoosporogenesis in other plasmodiophorids is also limited. Two micrographs on the fine structure of the primary zoospore *in situ* within the resting sporangium have been presented only for *Spongospora subterranea* (Lahert and Kavanagh, 1985; Merz, 1997) among the plasmodiophorids. These, however, do not provide enough information to understand primary zoosporogenesis.

In this work we observed a series of ultrastructural changes of zoospores within resting sporangia of *P. brassicae*. This is the first report on primary zoosporogenesis in plasmodiophorids.

Materials and Methods

Resting sporangia Resting sporangia of *Plasmodiophora brassicae* were obtained from clubroot galls of Chinese cabbage (*Brassica rapa* L. subsp. *pekinensis*), which were collected in Hagi, Yamaguchi Pref., Japan and stored at -40°C. Resting sporangia were isolated as described previously (Tanaka et al., 1990).

Induction of zoosporogenesis in resting sporangia Turnip seeds (*Brassica campestris* L. subsp. *rapifera* cv. Taibyo Hikari) were disinfected with sodium hypochloride (active chlorine concentration: 0.25%) for 30 min and washed with tap water for 40 min. About fifty of these seeds were sown on nylon sheet mesh fixed to the upper end in each of two plastic tubes (5 cm in height × 3 cm in diam). These tubes were floated in a deep Petri-dish containing 200 ml of modified Hoagland's solution (1.0 mM $Ca(NO_3)_2$, 1.0 mM KNO_3, 0.4 mM $MgSO_4$ and 0.4 mM KH_2PO_4; pH 6.0) described by Macfarlane (1970). The seeds were covered with filter paper

soaked with the culture solution and germinated at 25°C in the dark. Three days after sowing, the filter paper was removed from the net together with ungerminated seeds. At the same time, the culture solution was replaced with a fresh one, and an adequate volume of the suspension of resting sporangia was added to the culture solution (final concentration of resting sporangia: ca. 1×10^7/ml). Seedlings were continuously grown for 14 d in the laboratory without control of light and temperature.

Preparation for electron microscopy Resting sporangia were recovered from the culture solution by centrifugation at 1500 rpm for 20 min and fixed with 1% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.0) for 4 h at room temperature. After washing with the same buffer three times, resting sporangia were post-fixed with 2% osmium tetraoxide in the same buffer for 2 h at 5°C, washed with the buffer and embedded in 2% agar. The agar block was cut into about 1-mm^3 pieces and dehydrated in a 50–100% ascending ethanol series. After two 15-min rinses in 100% propylenoxide these specimens were embedded in Spurr's resin (Spurr, 1969). Ultra-thin sections were cut using a Sorvall Porter-Blum MT-2 ultramicrotome, picked up on collodion-coated mesh grids and stained with 2% aqueous uranyl acetate and 0.3% lead citrate. Specimens were observed with a Hitachi H700 or a JEOL JEM 100-B transmission electron microscope operating at 80 kV.

Results

When resting sporangia of *P. brassicae* were incubated for 14 d in the culture solution containing turnip seedlings, the differentiation of primary zoospores was not synchronized among them. Primary zoospores could be observed only in a portion of sporangia, while other sporangia had released zoospores or had not yet differentiated them.

Zoospores showed morphologically different features within incubated sporangia. The process of primary zoosporogensis could be divided into four or five stages on the basis of the shape of the cell body, intracellular aspects and other morphological features in zoospores observed within sporangia. Information on secondary zoosporogenesis in *P. brassicae* and other plasmodiophorids (Dekhuijzen, 1979; Buczacki and Clay, 1984; Talley et al., 1978; Miller and Dylewski, 1983; Miller et al., 1985) was also referred for identification of these stages.

A single zoospore differentiated in each sporangium. Differentiation of the primary zoospore was initiated by emergence of two flagella within the sporangium. Transversely sectioned flagella were seen in the tight space formed between the cell wall and the invaginated plasma membrane in the sporangium (Fig. 1). The zoospore cell was similar in intracellular aspects to mature sporangia within clubroot galls described by Yukawa and Tanaka (1979) but had electron-dense mitochondria and well-developed endoplasmic reticulum.

The zoospore at the stage of early development

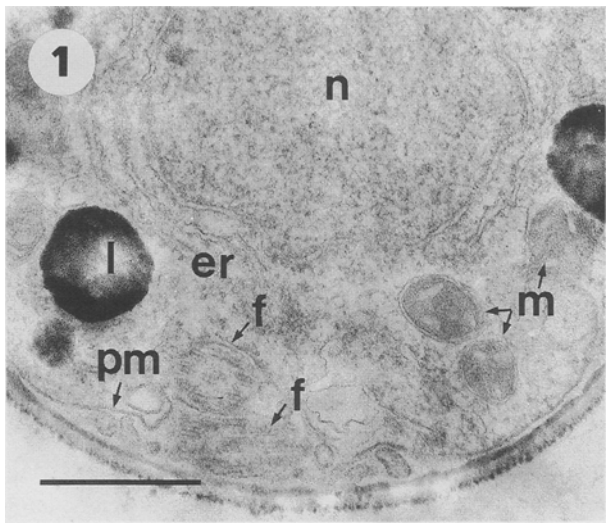
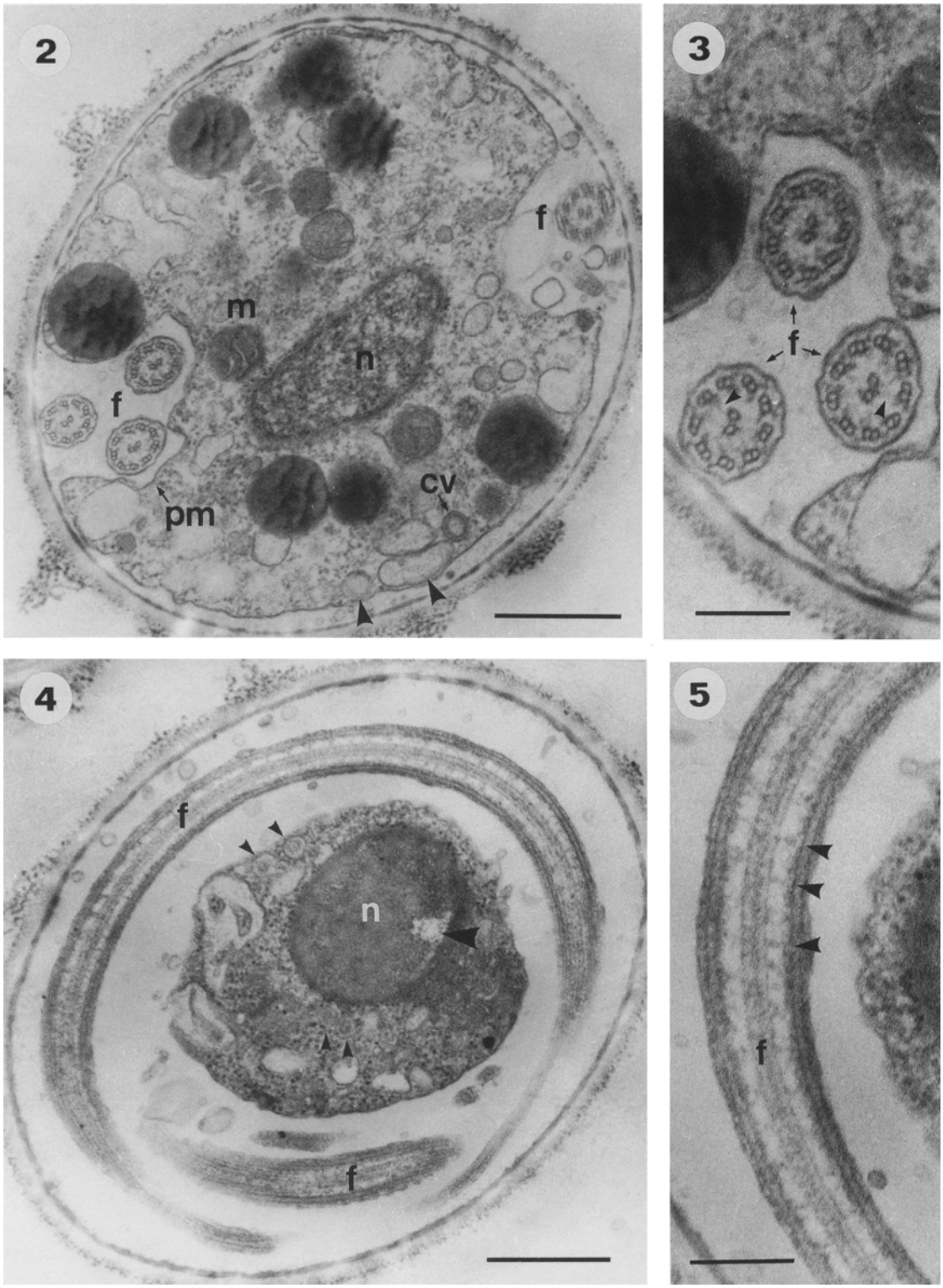


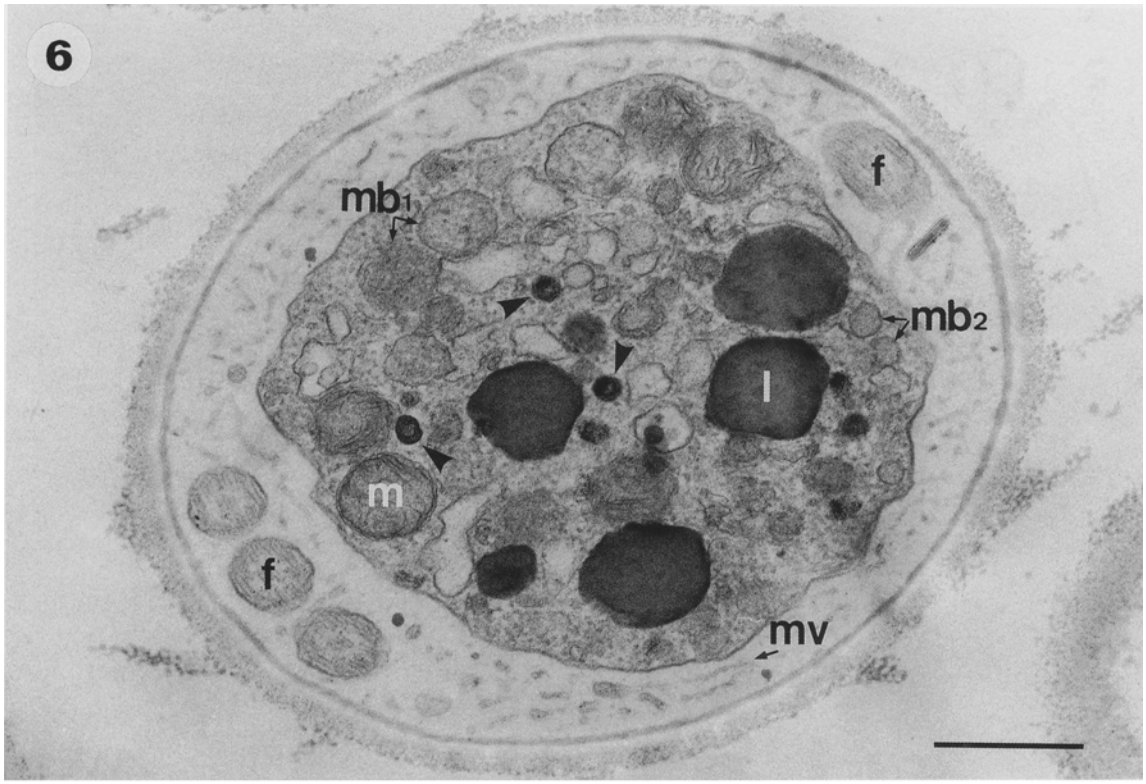
Fig. 1. A differentiating primary zoospore within the resting sporangium. Transversely sectioned flagella (f) are seen in the tight space between cell wall and invaginated plasma membrane (pm). Bar=0.5 μm.

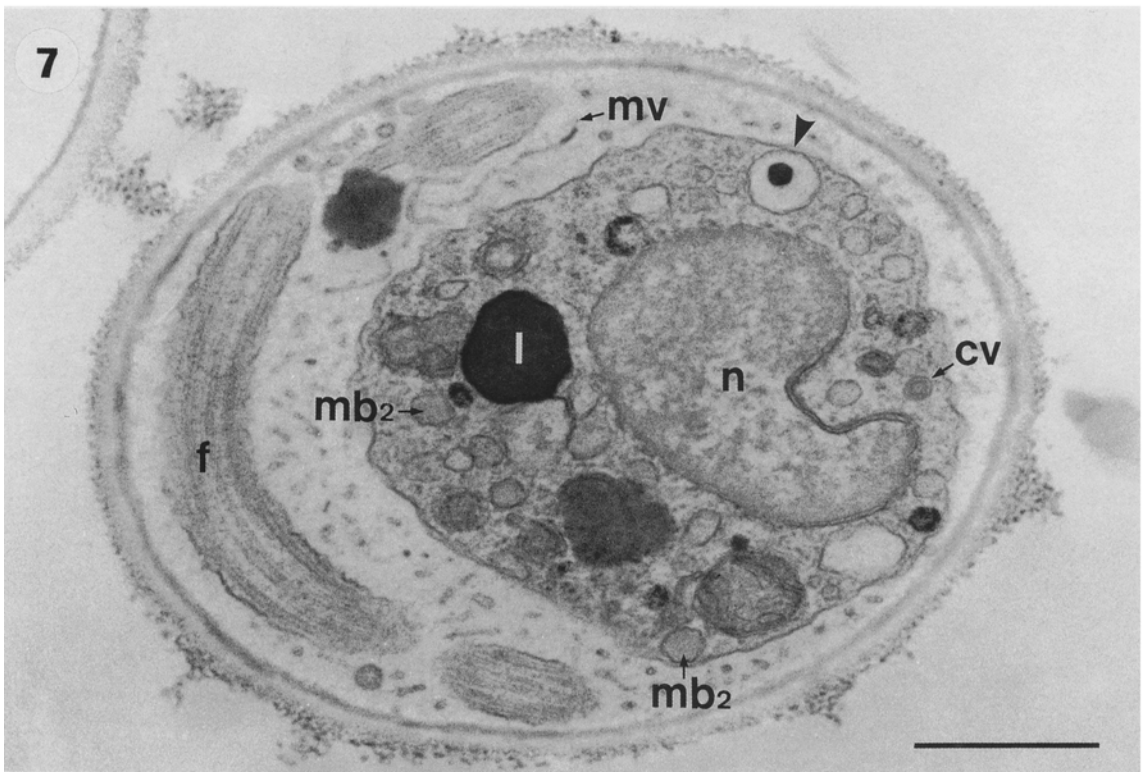
exhibited complicated intracellular aspects with the further invagination of the plasma membrane within the sporangium (Fig. 2). Transversely sectioned flagella were clearly observed in the invaginated portions of the plasma membrane (Figs. 2, 3). The zoospore cell body appeared to be constricted in the middle by the invagination of the plasma membrane, and flagella were arranged in parallel along the inside of a deep groove formed on the cell body. This feature corresponded well in three-dimensional configuration with observations of the zoospore shown in Fig. 4. Longitudinally sectioned flagella coiled around the zoospore cell body, which appeared to be small, indicating that the cell body was sectioned at its constricted middle portion (Fig. 4). Intracellularly these zoospores were characterized by an electron-dense nucleus without nucleolus, electron-dense mitochondria, lipid droplets and cored vesicles (Figs. 2, 4). Unidentified vesicles containing electron-opaque and -dense materials were also observed in these zoospores (Figs. 2, 4). An electron-translucent area was sometimes seen in the nucleoplasm (Fig. 4), as described for free swimming primary zoospores of *Polymyxa graminis* (Barr and Allan, 1982) and secondary zoospores of *Ligniera verrucosa* (Miller et al., 1985). Flagella were the whiplash type and showed the typical 9+2 configuration of microtubules (Figs. 3, 5). Radial spokes were seen unclearly between central pairs and outer doublets of microtubules in these flagella (Figs. 3, 5).

Zoospores at the stage of late development are shown in Figs. 6 and 7. The process of protoplasmic condensation (contraction of cell body) had begun in these zoospores, and a wide periplasmic space had developed in the sporangia containing them. Numerous microvillus-like structures were observed within this space (Figs. 6, 7). Although flagella coiled in parallel around the cell body, the groove was not seen on the cell body (Fig. 6). Intracellularly these zoospores were

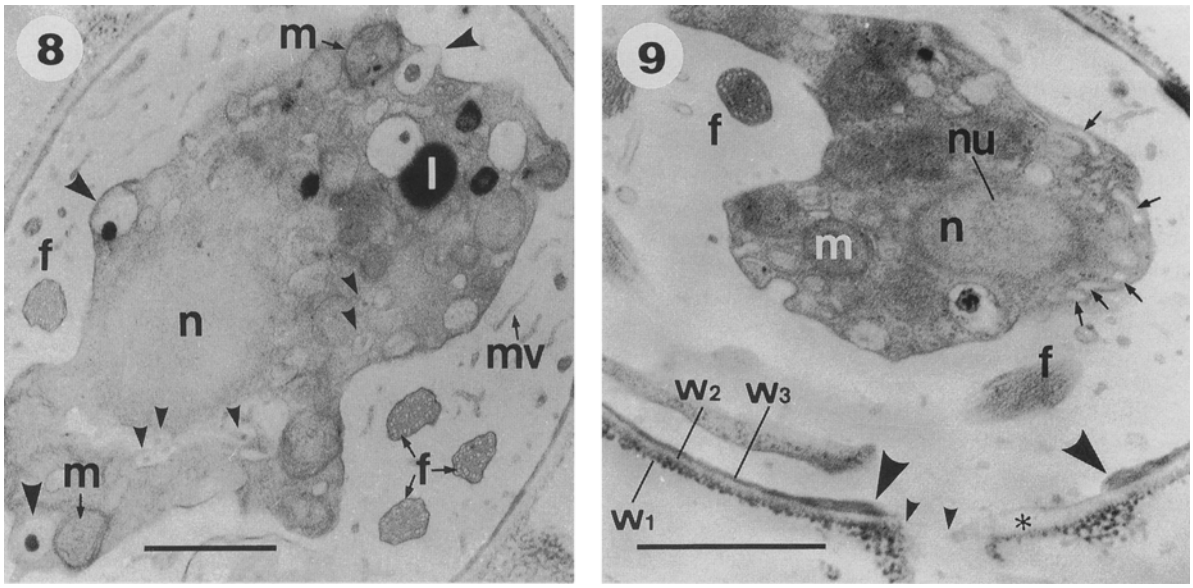


Figs. 2–5. Primary zoospores at the early stage of development and their flagella. 2. A zoospore showing transversely sectioned flagella (f) in a deep groove formed on the cell body by invagination of plasma membrane (pm). Electron-dense nucleus (n) and mitochondria (m), cored vesicles (cv) and other kinds of unidentified vesicles (arrowheads) are seen in the cell. Bar=0.5 μm. 3. Transversely sectioned flagella (f) showing radial spokes (arrowheads) between central pairs and outer doublets of microtubules. Bar=0.2 μm. 4. A zoospore sectioned at the constricted middle portion of the cell body. Longitudinally sectioned flagella (f) coil around the cell body at its constricted portion. Bar=0.5 μm. 5. A longitudinally sectioned flagellum showing the arrangement of radial spokes (arrowheads) between central pairs and outer doublets of microtubules. Bar=0.2 μm.





Figs. 6–7. *Primary zoospores* at the stage of late development within resting sporangia. 6. A zoospore showing a spherical cell body without the groove. Flagella (f) coil around the body in parallel. Numerous microvillus-like vesicles (mv) are present in the periplasmic space. Large and small microbodies (mb_1 and mb_2, respectively) and small electron-dense bodies (arrowheads) are rich in the cell. Bar = 0.5 μm. 7. A small microbody (mb_2)-rich zoospore cell. A vesicle containing an electron-dense granule (arrowhead), several cored vesicles (cv) and a few lipid droplets (l) are seen in the cell. Bar = 0.5 μm.



Figs. 8–9. Mature primary zoospores within resting sporangia. 8. An irregular-shaped, condensed cell body of the zoospore. Many vesicles contain electron-dense granules (large arrowheads) and small particles (small arrowheads). Bar = 0.5 μm. 9. A zoospore and a partially opened germ pore in the resting sporangium. The zoospore cell has a nucleus (n) with a nucleolus-like region (nu) and a cluster of flattened vesicles (arrows) in the cell. A partially opened germ pore (small arrowheads) can be seen in a thickening (asterisk) of the middle (w_2) wall layer in the area surrounded by the thickened ring (large arrowheads) of the innermost (w_3) wall layer. Bar = 0.5 μm.

characterized by an electron-dense nucleus without nucleolus, spherical mitochondria, relatively few lipid droplets, abundant microbodies (large and small), small electron-dense bodies, cored vesicles and unidentified vesicles containing electron-dense granules (Figs. 6, 7).

The mature zoospore had an irregular-shaped and fully condensed cell body (Figs. 8, 9), as described for mature secondary zoospores of *L. verrucosa* (Miller et al., 1985). Intracellular features were complicated, characterized by an electron-dense nucleus with a nucleolus-like region (Fig. 9), unidentified vesicles containing electron-dense granules and small particles (Fig. 8) and a cluster of flattened vesicles (Fig. 9). Zoospores shown in Figs. 8 and 9 differed slightly in the degree of maturation. The zoospore shown in Fig. 8 had abundant microvillus-like structures as well as developing zoospores (Figs. 6, 7) in the periplasmic space, while the zoospore shown in Fig. 9 had few. In addition, flagella coiled around the cell body in parallel in Fig. 8, but no such arrangement of flagella was seen in Fig. 9. These two zoospores appeared to be at early and late stages of maturation, respectively.

A partially opened germ-pore was also observed in the sporangium containing the mature zoospore (Fig. 9). This pore was located in the area (thickening of the middle (w_2) wall layer) surrounded by the thickened ring of the inner (w_3) wall layer, which has been reported for mature sporangia within clubroot galls by Yukawa and Tanaka (1979).

Discussion

The process of primary zoosporogenesis in *P. brassicae* was initiated by the emergence of flagella within resting sporangia, and thereafter the cell body shape and intracellular features of zoospores changed frequently following the protoplasmic condensation during development and maturation. This process is fundamentally similar to that of secondary zoosporogenesis in *P. brassicae* and other plasmodiophorids (Dekhuijzen, 1979; Buczacki and Clay, 1984; Talley et al., 1978; Miller and Dylewski, 1983; Miller et al., 1985). However, formation of the groove on cell bodies and emergence of flagella in this groove, which were observed for primary zoospores in the present work, have not previously been described for secondary zoospores of any plasmodiophorid. This mode of development may be unique to the primary zoospore. Two flagella appeared to coil in parallel around the cell body in developing zoospores and a part of mature ones, suggesting that flagella are not yet functional and may become so only in completely mature zoospores.

The intracellular features were relatively simple in the differentiating zoospore but very complicated in developing and mature ones. The developing zoospores had electron-dense nuclei without nucleoli. Such nuclear features are also commonly seen in the developing secondary zoospores of *P. brassicae* and other plasmodiophorids (Dekhuijzen, 1979; Buczacki and Clay, 1984; Talley et al., 1978; Miller and Dylewski, 1983; Miller et al., 1985) and most of the electron-dense nuclear content has been presumed to be chromatin (Barr and Allan, 1982). On the other hand, a nucleolus-like region was observed only in the nucleus of mature zoospore in the present work. Lehert and Kavanagh (1985) have

reported the nucleus with a nucleolus in the mature primary zoospore of *Spongospora subterranea*. The nucleolus may appear only at a limited stage during primary zoosporogenesis.

Various kinds of vesicles and granules were seen in developing and mature zoospores. Although their functions can not be determined at the present time, the appearance of these organelles may reflect the active metabolism in these zoospore cells. Microbodies were also abundant in the developing primary zoospores in which lipid droplets were relatively poor (Figs. 6, 7). Microbodies (glyoxysomes) convert fatty acids stored as lipids to glucose in germinating plant seeds (Beevers, 1969; Tolbert and Essner, 1981) and are often abundant among the lipid droplets that are decreasing in size or number during the spore germination in many plant pathogenic fungi (Maxwell et al., 1977). The metabolic pathway of fatty acids associated with microbodies may be also present in the primary zoospore of *P. brassicae* as well as in other many organisms.

Each mature resting sporangium within clubroot galls has a single closed germ pore, which is plugged by the thickening of the middle (w_2) wall layer in the area surrounded by a thickened ring of the inner (w_3) wall layer (Yukawa and Tanaka, 1979). In the present work, the partially opened germ pore was seen in this area of the incubated sporangium (Fig. 9). This suggests that the completely opened germ pore first develops at the stage of maturation of zoospores in the sporangium. The cell wall of resting sporangia contains a chitin component (Buczacki and Moxham, 1983; Tanaka et al., 1993). The thickening of the middle (w_2) wall layer is also known to be susceptible to chitinolytic enzymes (Yano et al. 1994). The chitinolytic processes may be involved in the formation of the opened germ pore in the resting sporangium of *P. brassicae*.

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