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Ultrastructural Changes of Chinese Cabbage Root Tissues Associated with Pathogenesis of *Plasmodiophora brassicae*

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Roots of Chinese cabbage (*Brassica campestris* var. *chinensis*) seedlings infected with *Plasmodiophora brassicae* were examined by light and electron microscopy to reveal histopathological changes related to pathogenesis in the susceptible host. The pathogen colonized the cortex and partly the stele as well, invading up to the xylem. Gall tissues could be differentiated from the initially infected tissues, involving less compact organization and new vascular development. The infected cells were much hypertrophied, and contained one to several plasmodia. Except cellular hypertrophy, no pathological ultrastructural modification was noted in the infected cells. Infected cytoplasm became dense with ground cytoplasm, inconspicuous central vacuole, and increased cellular organelles such as mitochondria and dictyosomes. There were two types of nuclear states of plasmodium, uninucleate and multinucleate. Both plasmodia were structurally similar, filled with lipid droplets, bounded with envelope, and containing mitochondria, endoplasmic reticulum, and sometimes small vacuoles. Plasmodial fragmentation, which may be regarded as a way to discharge plasmodial materials into host cytoplasm, commonly occurred, forming plasmodial fragments by outgrowth of plasmodial cytoplasm and regional compartmentalization. Plasmodial fragments were degenerated sometimes followed by forming chains of spherical vesicles especially in the uninucleate plasmodial state. These ultrastructural features indicate the biotrophic nature of the pathogen associated with its pathogenesis in the susceptible host.

Keywords **:** Chinese cabbage, pathogenesis, *Plasmodiophora brassicae*, plasmodium, ultrastructure.

The club root of crucifers such as Chinese cabbage, cabbage, and radish is caused by *Plasmodiophora brassicae*, and distributed worldwide. Recently the disease has spread to major Chinese cabbage-growing areas in Korea such as Yeoncheon, Pyeongtaik, Pyeongchang, and Taibaik, causing severe losses of the vegetable production.

The most pronounced symptoms of susceptible plants infected with *P. brassicae* are the formation of root galls with the reduced fine root system. Kim et al. (1999) reported that microscopic galls were formed on Chinese cabbage seedling roots 13 days after inoculation, and that the amount of root hairs was reduced in proportion to the index of galling. These clubs inhibit nutrient and water transport, cause foliar chlorosis and poor growth of the plant, and increase the susceptibility to wilting. In some weeks the clubbed roots become decayed and eventually dead.

The spores of *P. brassicae* can remain infectious for at least 15 years (Mattusch, 1977). Control methods for the clubroot disease like chemical application (Datnoff et al., 1987) and use of resistant cultivars (Wit and Van de Weg, 1964; Williams, 1966b; Toxopeus and Janssen, 1975) are hardly efficient due to their persistence in soil and genetic variation (Hansen, 1989). Cultural practices, especially the application of calcium and boron, and liming to decrease soil acidity, may reduce disease pressure (Myers and Campbell, 1985; Oh et al., 1997), but are often not sufficient to keep the crop healthy.

In the disease cycle of *P. brassicae* in a susceptible host, two types of infection occur; the primary infection on root hairs and the secondary on the main root (Dylewski, 1990). The development of *P. brassicae* in crucifer root hairs has been described by Cook and Schwartz (1930), Ayers (1944), MacFarlane (1952), and Williams et al. (1971). Ultrastructures of the primary infection process have been well documented, illustrating penetration of the encysted zoospore into host cell with the aid of adhesorium, stachel and schlauch (Aist and Williams, 1971). Nuclear division of plasmodia occurs in infected root hairs, accompanying their swellings.

It is the secondary infection stage that plant reactions can be differentiated between susceptibility and resistance since Voorrips (1992) observed no correlation between resistance to root hair infection and resistance to clubroot development. However, there have been only a few structural and ultrastructural studies related to pathogenesis of *P. brassicae*. In the secondary infection, infected cells of main roots contained multinucleate plasmodia surrounded with a dis-

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crete plasmodial envelope (Williams and Yukawa, 1967). The plasmodium contained abundant lipid droplets, accompanying fragmentation of vacuoles in infected cells that were replaced by host cytoplasm and plasmodia, but ultrastructural events during the process of galling were not considered. They only examined ultrastructural features of full developed club galls. Therefore, this study was focused on the ultrastructural modifications associated with the secondary pathogenesis before and after the full development of the club gall in Chinese cabbage infected with *P. brassicae*. This study would provide a basic information on the plasmodial infection process and life cycle which can be used for the development of control methods such as cultural practices and breeding of resistant cultivars.

Materials and Methods

Observation of infected root samples. Chinese cabbage (*Brassica campestris*) roots naturally infected with *P. brassicae* were collected and observed with naked eye or under a stereomicroscope. Close examination could differentiate the infected roots into 3 arbitrary groups depending on the degree of galling; gall index 1: slight swelling of root surface, gall index 2: gall tissue formation (different from original root) and cracking of root surface, and gall index 3: full development of gall tissue (larger than root width). The infected roots sampled were hand-sectioned about 10-30 μm thick with a razor blade. The sections were stained with 1% toluidine blue O (in 50% ethanol) briefly (for few seconds) and washed in distilled water. The stained sections were observed under a light microscope (Axiophot, Zeiss, Germany).

Light (LM) and transmission electron microscopy (TEM) of infected cells. The inoculated root samples were fixed with Karnovsky's fixative in 2% paraformaldehyde and 2% glutaraldehyde in 0.05 M cacodylate buffer at pH 7.2, washed in the same buffer, and post-fixed in 1% osmium tetroxide for 2 hours. The fixed root samples were washed briefly in distilled water and stained *en bloc* in 0.5% uranyl acetate overnight. They were dehydrated in a graded ethanol series (30%, 50%, 70%, 80%, 95%, and 100%), and embedded in a Spur's epoxy resin (Spurr, 1969). Sections 1-2 μm thick were cut with a glass knife on an ultramicrotome (MTX, RCM, Inc., Tucson, AZ, USA) and mounted on glass slides. The sections were stained by Quickie staining method (Harris, 1978) before observation under the light microscope (Axiophot, Zeiss, Germany).

Also ultrathin sections of 80-90 nm in thickness were made with a diamond knife mounted on the ultramicrotome. Sections were mounted on copper grids, and stained with 2% (w/v) uranyl acetate and with Reynolds' lead citrate for 7 min, respectively. Sections were observed under a transmission electron microscope (JEM-1010, JEOL, Ltd., Tokyo, Japan) at 80 kV.

Results

Gall morphology and light microscopy. Various degrees of galling were observed in Chinese cabbage roots infected with *P. brassicae*, of which the aboveground symptoms were poor growth and wilting. Galls were microscopic to huge in size. The tiny galls were swelling of the original root surface; however, in large galls the root surface was cracked and gall tissues, which were composed of looser tissues, appeared to be bulged out of the root surface.

The root epidermis beneath root hairs was colonized initially by plasmodia (data not shown). In gall index 1 (Fig. 1A, B), plasmodia invaded into and colonized in the cortex (Fig. 1A), and in more advanced case, plasmodia reached the stele near xylem vessels (Fig. 1B). In the infected cortex, periclinal and oblique cell plates crossing parenchyma cells were frequently found. In the stele, cells were considerably enlarged, compared to unaffected normal cells (Fig. 1B), indicating that the plasmodia invaded into the center of the root. The plasmodia contained one nucleus as revealed in larger magnification (Fig. 1B insert) at this stage of infection.

In gall index 2 (Fig. 1C-E), unusual tissues, probably gall tissues, that can be differentiated from surrounding tissues were formed in the cortex and partly in the stele, and grow outward like a lateral root (Fig. 1C). In the gall tissues, differentiation of new vascular bundle including xylem vessels was frequently found (Fig. 1D). Semithin sections of the gall tissue area showed an array of xylem vessels surrounded by parenchyma cells containing multinucleate plasmodia (Fig. 1E). Eventually large gall tissues produced and differentiated from the initially infected tissues, which was designated as gall index 3 in this study (Fig. 1F). Galls were composed of looser tissues than normal tissues (data not shown).

Electron microscopy of club root. Plasmodia of *P. brassicae* were completely immersed in host cytoplasm, and composed of plasmodial envelope and numerous cell organelles such as endoplasmic reticulum and mitochondria, and especially prominent lipid droplets (Fig. 2 and 3). The plasmodial cytoplasm could be easily differentiated from that of host by the prominence of the lipid droplets. Two types of plasmodia were noted in infected root cells; one was uninucleate (Fig. 2) and the other multinucleate (Fig. 3), which were at the states of gall indices 1 and 2, respectively.

At the uninucleate state of plasmodium, no aberrant cytoplasmic features were observed in infected cells by the pathogen infection. The infected cells contain healthy-looking nucleus and cell organelles such as mitochondria and protoplasts. Cell walls between neighboring infected cells were thin compared to those between infected and uninfected cells (Fig. 2A). Increase of cytoplasm was noted, accompanying breakdown of central vacuole (Fig. 2C), increased mitochondria (Fig. 2A-2D) and sometimes

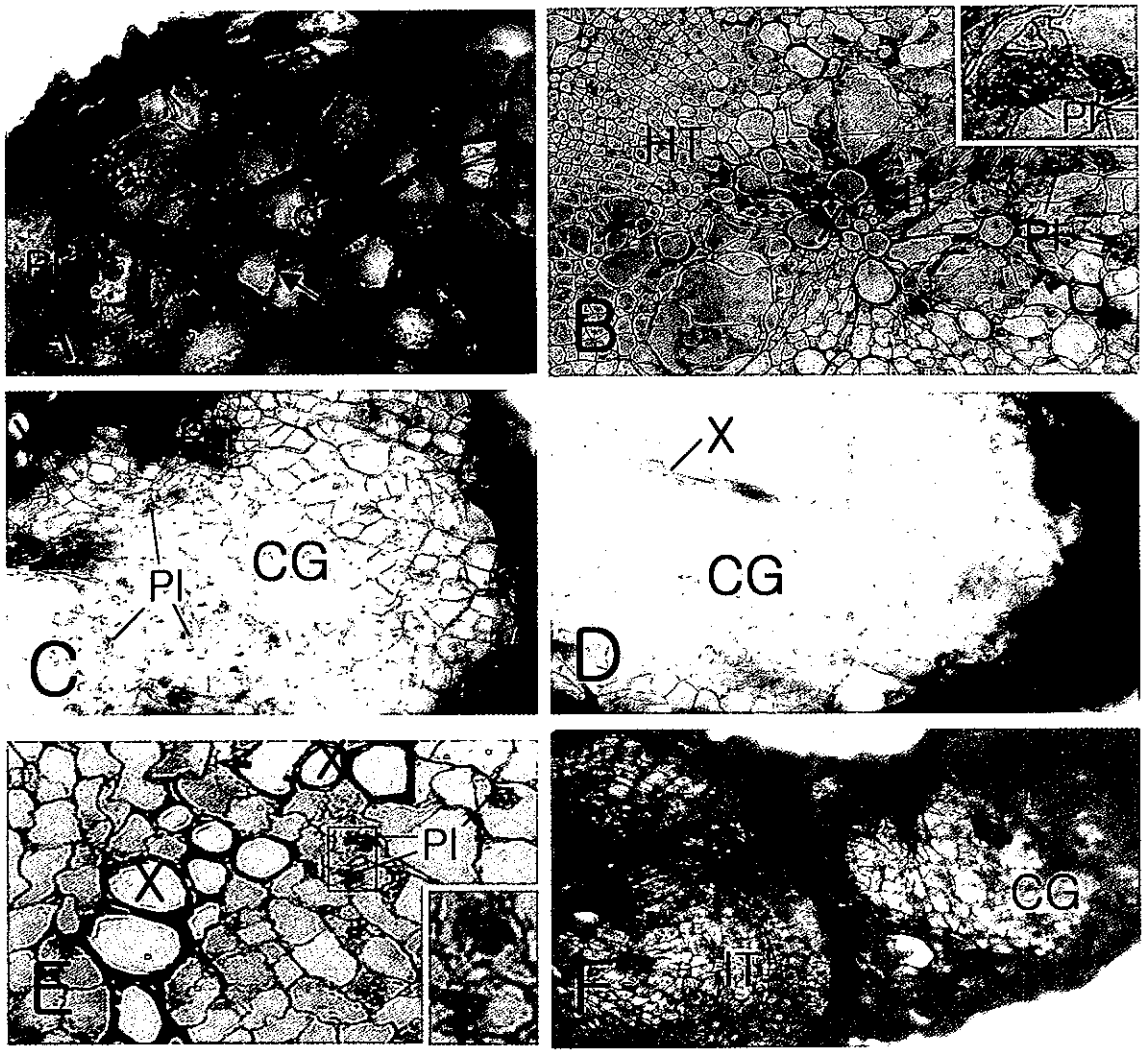

Fig. 1. Hand-sectioned (C, D, F) and microtome-sectioned (A, B, E) specimens of Chinese cabbage roots infected with *Plasmodiophora brassicae* with gall indices 1 (A, B), 2 (C, D, E), and 3 (F). **(A)** The outer cortical cells of the infected tissue containing uninucleate plasmodia (Pl). Note cell wall (arrow) crossing cortical cells, suggesting cell division has been stimulated. **(B)** Infection of *P. brassicae* in the stele. Note hypertrophied cells in the infected tissue (IT) near xylem vessels (X), compared to healthy normal tissue (HT). Plasmodia (Pl) are uninucleate (see **insert** <x1,000>). **(C, D)** Formation of club gall tissue (CG). Note plasmodia (Pl) in the gall tissue of **C**, distributing in the cortex and stele, and xylem differentiation in **D**. X: xylem, Ph: phloem. **(E)** Differentiated xylem vessels (X) in gall tissue containing multinucleate plasmodia (Pl) in parenchyma cells (see **insert** <x1,000>). **(F)** Formation of club gall tissue (CG) which can be differentiated from the original infected root tissue (IT). The club gall is composed of loose tissue. Magnification = 400 x (A, B, C, D, E); 200 x (F).

together with dictyosomes and vesicles (Fig. 2B). Fragmentation of plasmodium was common. Small parts of plasmodium were protruded outward and detached from the plasmodium, forming vesicles. Often a chain of tiny spherical vesicles was formed on the membrane of the plasmodial vesicle, and discharged to ground cytoplasm or near cell wall when the vesicles were degenerating with membranes ruptured. Division of plasmodium occurred, separating into two plasmodial segments containing each nucleus (Fig. 2C). The infected cells adjacent to xylem vessels usually contained more than one plasmodium (Fig. 2D), and their structural features were similar to those of infected cortical cells. Cell wall ingrowths, which are usually formed in giant and syncytial cells formed by nematodes adjacent to xylem vessels, were not observed throughout the study.

The structural features of multinucleate plasmodium were identical to those of uninucleate one except for electron density of lipid droplets. Lipid droplets in the multinucleate plasmodium was less electron-dense than those in the uninucleate (Fig. 3A-C). Infected cells were much enlarged compared to uninfected cells, while the size of nucleus seemed to be little enlarged (Fig. 3A). Cytoplasm became

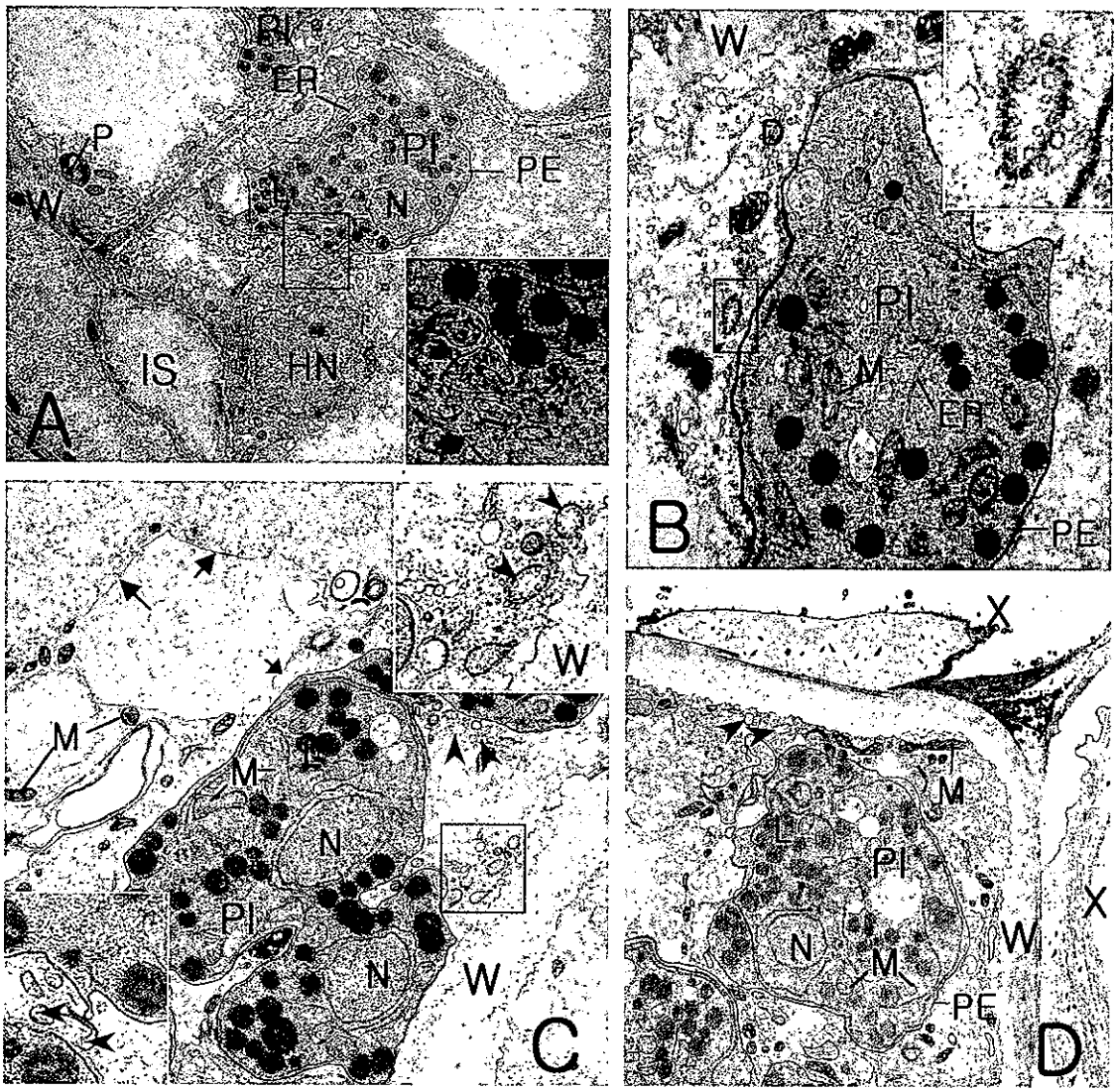

Fig. 2. Electron microscopy of the uninucleate state of plasmodia (Pl) and Chinese cabbage root infected with *Plasmodiophora brassicae* at the state of gall index 1. **(A)** Infected cortical cell containing a uninucleate plasmodium involving numerous lipid droplets (L). The plasmodium was bounded with plasmodial envelope (PE). The infected cortical cell has a normal-looking nucleus (HN), and its cytoplasm appeared to be increased. The thin cell walls (W) between similar cells indicate the cellular division has happened just beforehand. P: plastid, W: cell wall. IS: intercellular space. x 3,000. **Insert**: higher (x 9,000) magnification of the rectangle region of **A**, showing plasmodial fragments detached from the main plasmodium. **(B)** An outer cortical cell in root tissue with slight swelling of root surface, showing increased cytoplasm of the infected cell and a young plasmodium with less numerous lipid droplets. In the infected cortical cell, numerous vesicles, probably derived from dictyosomes (D) appeared to be associated with the cell wall (W). M: mitochondria. ER: endoplasmic reticulum, x 12,000. **Insert**: higher magnification (x 45,000) of the rectangle region of **B**, showing the formation of a chain of spherical vesicles along double membrane of a plasmodial fragment. **(C)** Plasmodial (Pl) division, showing nuclear division and cytoplasmic division. Arrows indicate vacuolar fragmentation probably by cytoplasmic increase of the infected cell. Increased mitochondria (M) indicate the infected cell is highly metabolic. N: plasmodial nucleus. L: lipid droplets. x 7,500. **Upper insert**: higher magnification (x 17,000) of the rectangle region of **C**, showing chains of spherical vesicles formed around vacuolar membranes (arrowheads) which appeared to be degenerated and associated with cell wall (W). **Lower insert**: another portion of the infected cell showing the outgrowth of plasmodial cytoplasm and its separation from the plasmodium (arrowheads). x 15,000. **(D)** Infection of the pathogen at the cell near xylem vessels (X), suggesting that the plasmodium invaded deep into the stele. Note no cell wall ingrowths were formed on the cell wall adjacent to xylem vessels. N: plasmodial nucleus, PE: plasmodial envelop, M: mitochondria, Arrowheads indicate plasmodial fragments near cell wall (W). x 5,000.

denser with increase of ground cytoplasm and organelles such as mitochondria, and sometimes full of vesicles mostly driven from dictyosomes (Fig. 3C). Fragmentation of plasmodium was also common; however, plasmodial

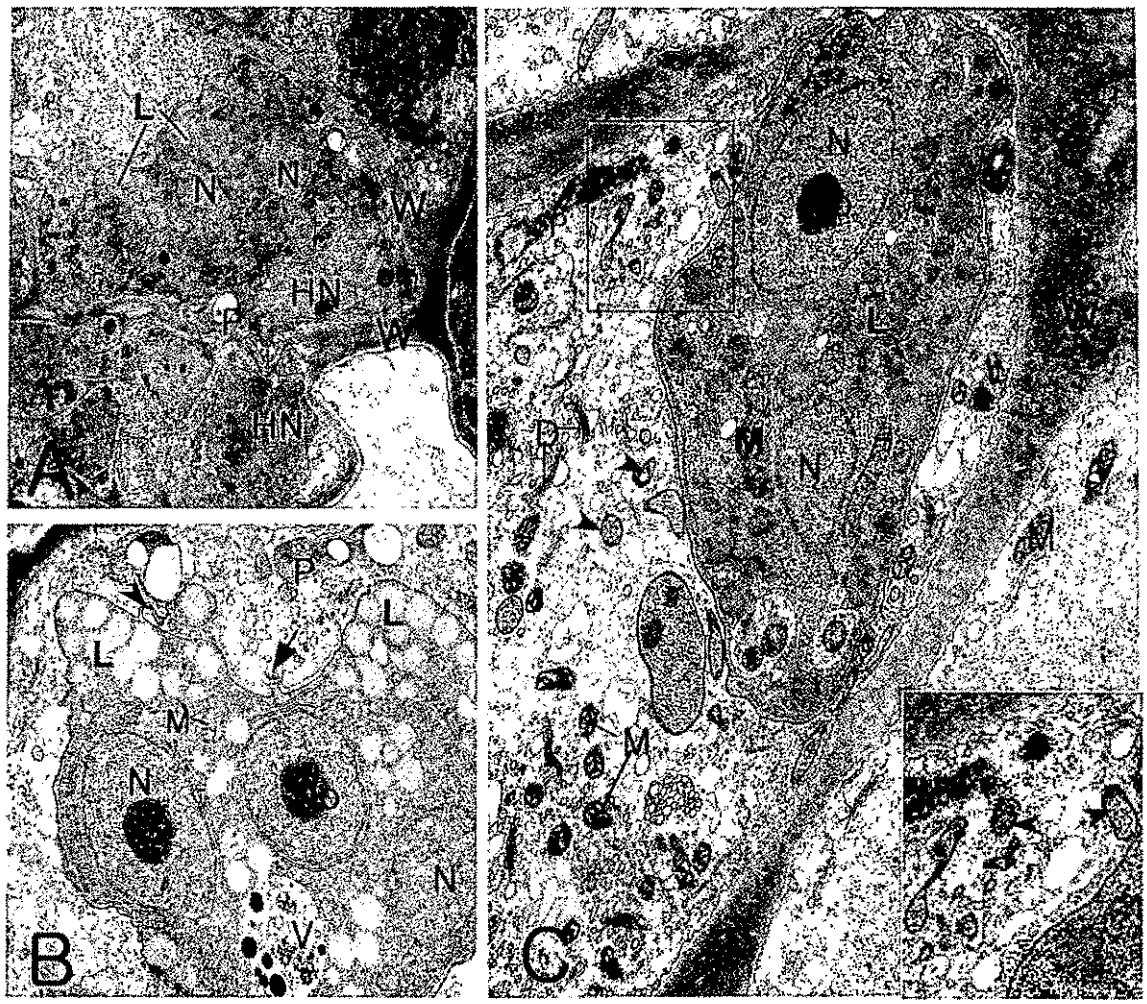

Fig. 3. Electron microscopy of the multinucleate state of plasmodia and Chinese cabbage root infected with *Plasmodiophora brassicae* at the state of gall index 2. **(A)** Multinucleate plasmodia in hypertrophied cells containing three nuclei (N) and numerous lipid droplets (L). Surrounding uninfected cells are much smaller than the infected one. Note lipid droplets are not conspicuously electron-dense in the multinucleate plasmodium. W: cell wall. HN: host nucleus. P: plastid. M: mitochondria. x 4,000. **(B)** Higher magnification of a multinucleate plasmodium, showing three nuclei (N) and numerous lipid droplets (L). Note the outgrowth of plasmodial cytoplasm (arrowhead) and broken plasmodial envelope (arrow) that appears to be discharging some materials into the infected cytoplasm. No: nuleolus. x 10,000. **(C)** Infected cell containing a binucleate (N) plasmodium has increased cellular organelles such as mitochondria (M) and dictyosomes (D), and plasmodial fragments (arrowheads). P: plastid, M: mitochondria, W: cell wall. x 10,000. **Insert**: higher magnification (x 15,000) of the rectangle region of **(C)** showing plasmodial fragmentation (arrowheads).

fragments were mostly larger than those of uninucleate plasmodia, and no chain of tiny spherical vesicles was found. Like the uninucleate plasmodia, vacuoles were sometimes found in the multinucleate plasmodia.

Discussion

The pathogenesis of clubroot is well documented by Ingram and Tommerup (1972), showing that the second stage in the pathogenesis starts in root cortex with the secondary zoospores. Our study also showed that the pathogen localized primarily the cortex; however, as revealed in the LM of infected roots, the plasmodia were located in the stele as well. Since the stellar cells have a different origin from the cortical cells, the locality of the plasmodia suggests that their invasion into inner root tissues may not be solely dependent upon the spread of infected cells by stimulated cell division as hypothesized by Dekhuijzen (1975, 1981). Nonetheless, penetration of cell wall by plasmodia as means of pathogen spreading in cortex was not observed in our study, which coincides with their studies. In our study, the stimulated cell division was noted as neighboring infected cells had thinner cell walls than other surrounding cell walls. This indicates the spread of the pathogen may be

mostly by cell division.

Gall tissues, which were less compact in tissue organization than normal or initial infected tissues, may be formed following or simultaneously with the initial infection. As in root-knot nematode infection (Kim and Ohh, 1990), xylem vessels were differentiated in the club gall. The xylem differentiation may be one way to support water and minerals for the metabolism of infected cells as suggested in their study.

The electron microscopy of infected root cells and plasmodia was similar to that described by Williams and Yukawa (1967). Infected cells contained one or many plasmodia, and had no other cytopathological changes including degeneration of cell organelles and necrosis. But infected cells were much hypertrophied, compared to neighboring uninfected cells.

There were two kinds of plasmodial nuclear states, uninucleate and multinucleate. The multinucleate plasmodia and infected cells containing them seemed to be more enlarged than those of the uninucleate, indicating the multinucleate plasmodia may be at the more advanced stages. The aspect that uninucleate plasmodia were mostly observed in tiny galls in our study may support this. These results suggest the nuclear state of initial plasmodial invasion into the cortex may be uninucleate. In the generalized plasmodiophorid life cycle (Dylewski, 1990), no uninucleate stage was indicated after invasion into root cortex. Our study clearly shows that plasmodial divisions occur (Fig. 2C) before becoming multinucleate. No plasmodial divisions of the multinucleate plasmodia were observed in our study, supporting further the aspect mentioned above. Each nucleus may or may not be diploid, because fusion of plasmodia is needed for infection of the root cortex (Ingram and Tommerup, 1972) or not necessary (Voorrips, 1995). Structural differences between the two nuclear types were not noticed except electron density of lipid droplets; more electron-dense in the uninucleate than the multinucleate plasmodia. However, this may be an exclusive result because the electron density of a structure may vary depending on the procedures of sample preparation, and on the cytological environments of infected cells. Also there may be physiological variations of even the same nuclear state of plasmodium. Williams and Yukawa (1967) reported only the ultrastructural features of multinucleate plasmodia, and showed the lipid droplets were much electron-dense.

Cytoplasmic features on infected cells were also similar between the two nuclear states. Infected cells were somewhat filled with cytoplasm with inconspicuous central vacuoles, and increased with cell organelles such as mitochondria and dictyosomes. Plasmodial fragmentation was ubiquitous in both nuclear states. The plasmodial fragments were formed from outgrowth of plasmodial cytoplasm or from regional compartmentalization of plasmodium, containing plasmodial cytoplasm and bounded with plasmodial envelope. Sometimes large fragments involved lipid droplets. They degenerated with the rupture or degradation of the envelop, which seemed to discharge cellular components of plasmodium into host cytoplasm. This is probably one way for the pathogen to modify host cell metabolism for its survival and successful growth.

One thing unique to the plasmodial infection was the formation of chains of spherical vesicles surrounded by a single membrane and associated with a smooth osmophilic double membrane of vacuolar structures. The vacuolar structures seemed to be originated from plasmodial fragments and their typical features were visualized at the time of their degeneration, forming the chains of spherical vesicles. Williams and Yukawa (1967) also found the formation of vesicle chain structures in cortical cells infected with *P. brassicae*, and they mentioned their function was not known. These vesicles were structurally similar to vesicles of Golgi apparatus, which carry secretion material, mainly polysaccharides, to its destination such as cell wall (Esau, 1977). In our study, the vesicle chains were untied and some spherical vesicles appeared to be incorporated into cell wall (Fig. 2C). Therefore, these structural aspects may be involved in the cell wall synthesis that is required for the division and hypertrophy of infected cells. Contrary to Williams and Yukawa (1967), the structural characteristics were mostly found in infected cells with uninucleate plasmodia in our study. Considering the electron density of lipid droplets of plasmodium in their and our studies, however, the increase of vesicle chain structures may be related to the physiological state of plasmodium or cellular environments rather than the plasmodial nuclear states.

In conclusion, no cellular degeneration and hypersensitive reaction were noted in cells infected with *P. brassicae*. Nuclei adjacent to plasmodia in infected cells or in uninfected cells were intact. This peaceful coexistence may be a typical structural feature of a biotrophic parasite such as powdery mildew and rust in compatible relationships. Cellular hypertrophy of infected cells resembles that of giant cells and syncytial cells induced by root-knot and cyst nematodes (Kim and Ohh, 1990; Kim et al., 1999), which are also obligate parasites.

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