Neth. J. Pl. Path. 85(1979) 1-17

Electron microscopic studies on the root hairs and cortex of a susceptible and a resistant variety of Brassica campestris infected with Plasmodiophora brassicae

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Accepted 20 April 1978

Keywords

Brassica campestris, *Plasmodiophora brassicae*, resistance.

Abstract

The cortex of the roots of a susceptible and a resistant variety of *Brassica campestris* var. *rapa* infected with sterile resting spores of *Plasmodiophora brassicae* from senescent callus was studied at a stage prior to disease symptom development. Electron micrographs show the presence of amoeboid structures within the cortical cells of the susceptible variety 10 days after inoculation. Cell wall perforations, hypertrophied host cell nuclei, nucleoli and broken tonoplasts were frequently found in the susceptible variety. It has been concluded that amoeboid structures of the parasite penetrate the cell wall and disrupt the cortical cells.

Electron micrographs of the resistant variety show the presence of zoosporangia with secondary zoospores in the root hairs nine days after inoculation. Two to four days later a large number of dead host cells can be observed in the outer cortical layer of the resistant variety, whereas no apparent changes are found in the inner cortex. The results suggest the occurrence of a hypersensitive host reaction which terminates further growth of *Plasmodiophora brassicae*.

Introduction

The life cycle of *Plasmodiophora brassicae* Woron. consists of two main phases: one occurring in the root hairs and one in the living cells of the cortex and the stele of the root. During the latter phase multinucleate plasmodia incite hypertrophy of the invaded host cells and of the neigbouring cells of the medullary rays. Ultrastructural studies revealed many details of the sporogenesis of *P. brassicae* in the medullary rays (Williams and McNabola, 1967).

The first phase of the life cycle has been studied in detail by Aist and Williams (1971) showing the penetration by the primary zoospore with the aid of a bullet-like Stachel into the root hair. The penetration of the root hair is followed by the formation of zoosporangia and the release of secondary zoospores (Karling, 1968). Little is known of what happens after the secondary zoospores enter the epidermis and there are still many unanswered questions about the migration of the fungus from the epidermis through the cortex into the medullary rays.

In a previous paper evidence was given regarding the presence of amoeboid structures in the cortex a few days before the fungus reached the medullary rays. Electron micrographs showed the presence of perforations in the cortical cells indicating that the fungus migrates through the cortical host cells before reaching the medullary rays (Dekhuijzen, 1976).

The present paper gives further evidence in support of this view. Furthermore, the fine-structural changes in the root hairs and in the cortex of a resistant variety have been compared with those of a susceptible variety of *Brassica campestris*. In order to prevent any possible infection of the cortex by organisms other than *P. brassicae* the host was inoculated with sterile resting spores from senescent infected callus callus tissue.

Material and methods

Resting spores of physiological race c. Varieties susceptible (differential AABBcc) and resistant (differential AABBCC) to physiological race c were obtained from the Foundation of Agricultural Plant Breeding. The virulence pattern of the pathogen population can be summarized as 20/31/31 using the European Clubroot Differential Set (ECD) according to the rules as published by Buczacki et al. (1975).

Callus tissue was grown from young clubs 14 days after inoculation with phy-

Fig. 1. Material of the parasite within a vacuole (HV) of a cortical cell of the main root of the susceptible variety 10 days after inoculation with resting spores of *Plasmodiophora brassicae*. Within the parasitical material lipid droplets (li), mitochondria (mt), tubules (tu), Golgi apparatus (g) are visible. Host cytoplasm (hc), cell wall (cw) and tonoplast (T) are intact.

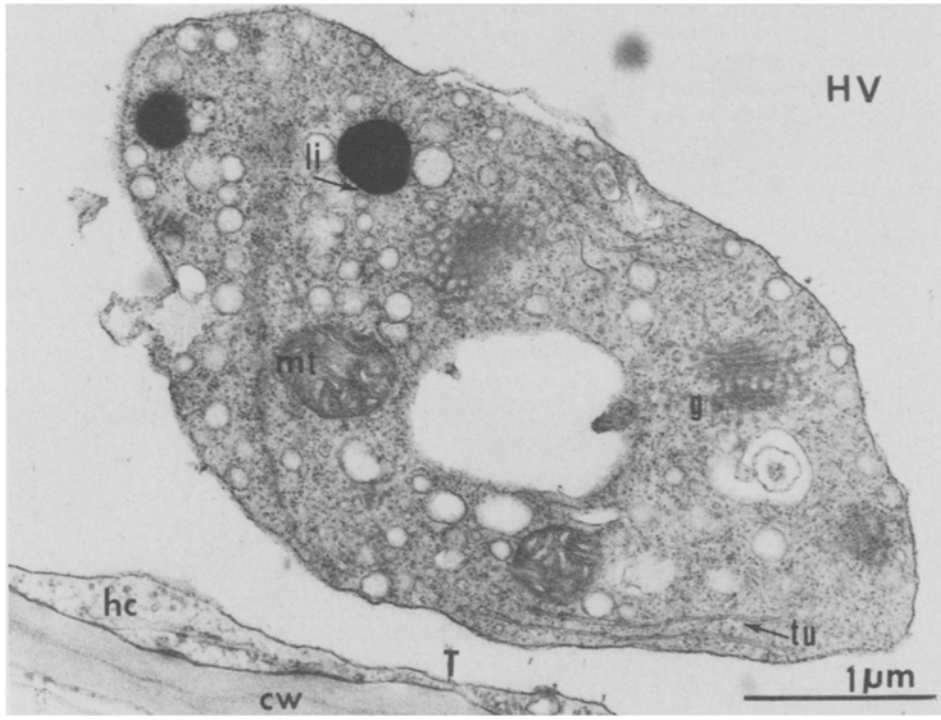


Fig. 1. Parasitair materiaal in een vacuole (HV) van een cortex-cel in de hoofdwortel van de vatbare variëteit 10 dagen na inoculatie met rustsporen van Plasmodiophora brassicae. In het parasitaire materiaal bevinden zich vetdruppels (li), mitochondriën (mt), tubulae (tu), Golgi-apparaat (g). Het waardplantcytoplasma (hc), de celwand (cw) en de tonoplast (T) zijn intact.

siological race c according to the method described by Dekhuijzen and Overeem (1971). Callus with multinucleate secondary plasmodia of physiological race c was transplanted every 3 weeks to a fresh medium supplemented with only 0.3 mg/l α-napthylene acetic acid. Callus was transplanted about 75 times during a 5 years period. By keeping the callus tissue on the same medium for 7 weeks the plasmodia transited into resting spores.

Inoculation of the susceptible and resistant variety. Seeds of turnip *Brassica campestris* L. var. *rapa* 'Gelria' were sown in steamed soil and grown for nine days at 18 °C in a growth chamber. Plants received 82 W/m² (Philips HPL fluorescent lamps and incandescent lamps) for 16 h per day. Nine days after sowing the temperature was raised from 18 °C to 23 °C in order to obtain optimal infection. One day later plants were inoculated by pipetting 1 ml of a suspension of 10^8 resting spores to each plant.

Fig. 2. As Fig. 1. Spore-like structures within a vacuole of a cortical cell.

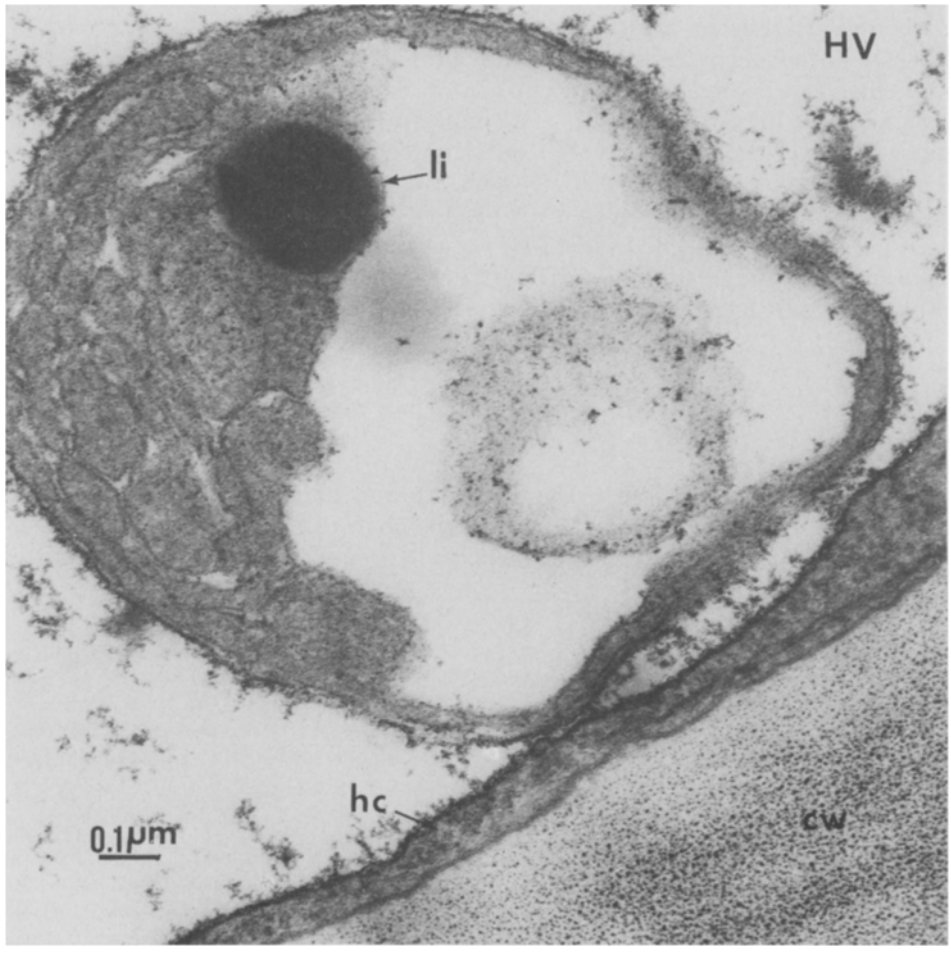


Fig. 2. Als Fig. 1. Op sporen lijkend materiaal ligt in een cel van de cortex.

Six days after inoculation the temperature was lowered from 23° to 20°C and the light was reduced to 58 W/m² to minimize premature wilting of the leaves.

Sampling of plant material. The first visible disease symptoms (swelling of the hypocotyl) of the susceptible variety became visible after 14 days. After 5 weeks the susceptible variety showed the characteristic clubroot symptoms, the resistant variety showed no disease symptoms or only very small swellings on the lateral roots (grade 1 according to the scale of Buczacki et al. 1975). The main roots with a length of 5 cm and a diameter of 0.15–0.20 cm from susceptible plants were collected 10–13 days after inoculation. The main roots of the resistant variety were collected 10, 11 and 24 days after inoculation. The lateral roots with root hairs from both varieties were collected 7 to 10 days after inoculation.

Light and electron microscopy. Sections of roots of resistant plants were mounted under rectangular no. 1 cover slips in water. They were observed live and unstained with Zeiss interference contrast optics. Specimens of 0.5 cm long of root segments of susceptible and resistant plants were prefixed at 5°C in 2.5% glutaraldehyde in 0.1 M Sörensen phosphate buffer (pH 7.2) for 2 hours and washed for 30 min in phosphate buffer (pH 6.8). The material was postfixed with 2% osmium tetroxide in 0.1 M of the same buffer at pH 6.8 for 2 h at room temperature stained with 2% uranylacetate for 1.5 h, dehydrated in an ethanol-propanol series, and embedded in Epon Araldite. Plastic was polymerized at 70 °C for 24–48 h. Sections of about 0.07 µm were stained with lead citrate for 5 min (Reynold, 1963).

Electron microscopy (Philips EM 300) was carried out by the Electron Microscope Department of the Technical and Physical Engineering Research Service of the Ministry of Agriculture, Wageningen, the Netherlands.

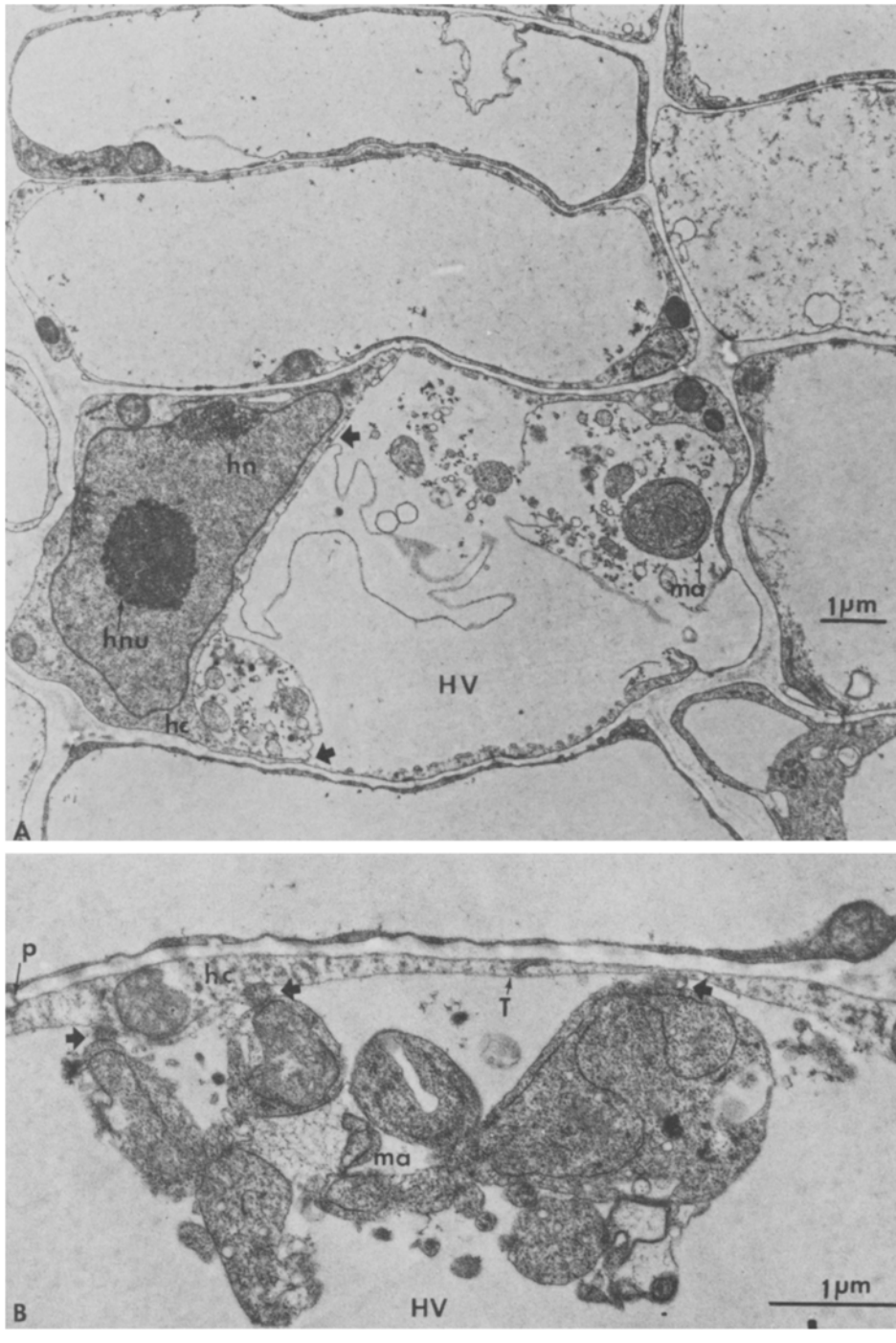
Results

The infected cortex of the susceptible variety

Ten days after inoculation. To determine how the fungus migrated through the cortex sections were made of the roots of symtomless plants at a stage prior to the occurrence of a hypertrophic reaction of cells of the medullary rays (13–15 days). Fig. 1 shows

Fig. 3. Cortical cells of the susceptible variety 12 days after inoculation. A) One of the cells contains an enlarged host nucleus (hn), nucleolus (hnu) and host cytoplasm (hc). The tonoplast is broken (arrows). Material of the parasite (ma) lies within the vacuole (HV). B) Material of the parasite (ma) within a vacuole (HV) of another cortical cell of the susceptible variety 12 days after inoculation. The material is in close contact with the tonoplast of the host vacuole (broad arrows). A plasmodesma (p) is visible in the host cell wall.

Fig. 3. Een aantal cellen van de cortex van de vatbare variëteit 12 dagen na inoculatie. A) Eén van de cellen bevat een vergrote waardplantkern (hn), kernlichaampje (hnu) en waardplant cytoplasma (hc). De tonoplast is op verschillende plaatsen kapot (pijlen). Het parasitaire materiaal ligt in de waardplant vacuole (HV). B) Parasitair materiaal in een vacuole (HV) van een andere cel in de cortex van de vatbare variëteit 12 dagen na inoculatie. Het parasitaire materiaal ligt dicht tegen de tonoplast van de waardplant vacuole (vette pijlen). Een plasmodesma (p) is zichtbaar in de celwand van de waardplant.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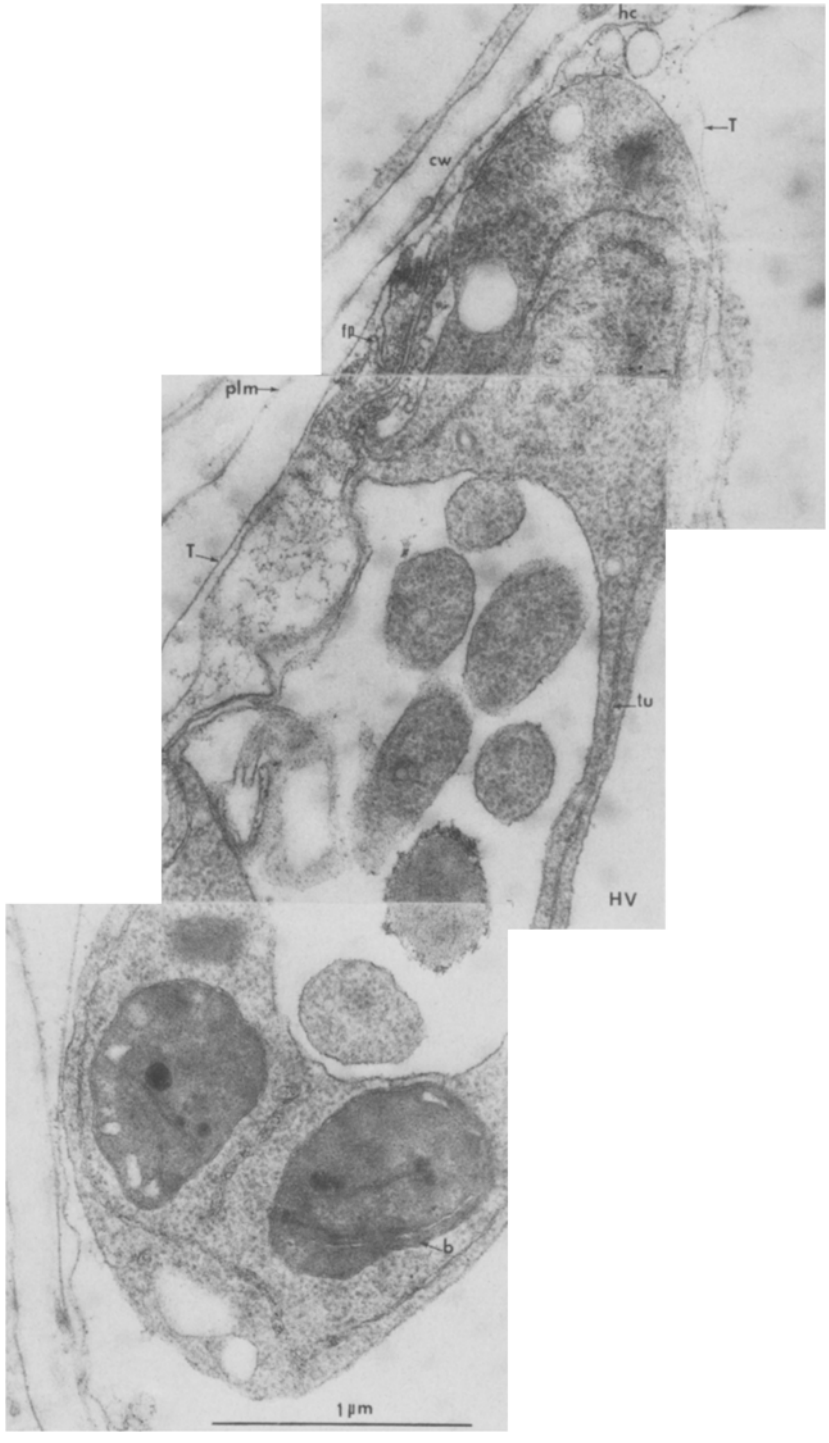
hc
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Fig. 4. Mounted electron micrographs of material of the parasite within a vacuole (HV) of a cortical cell of the susceptible variety 12 days after inoculation. Fingerlike protrusions (fp) of the fungus are in close contact with the tonoplast (T). Tubules (tu) may be linked with the protrusions. The bodies (b) may represent mitochondria. Host cytoplasm (hc) lies against the plasmalemma (plm) and host cell wall (cw).

Fig. 4. Een montage van de elektronenmicroscopische foto's van parasitair materiaal in een vacuole (HV) van een cortex-cel van de vatbare variëteit 12 dagen na inoculatie. Vingerachtige uitstulpingen (fp) van de schimmel zijn in nauw contact met de tonoplast (T). Tubulae (tu) zouden in contact met de vingerachtige uitstulpingen kunnen staan. De lichaampjes (b) zouden mitochondriën kunnen zijn. Het waardplant-cytoplasma ligt tegen de plasmalemma (plm) en de celwand van de waardplant (cw).

Fig. 5. Electron micrograph of an opening (3.3 μm) in a cortical cell wall (cw) of the susceptible host 12 days after inoculation. Remnants of host cytoplasm are visible within the opening.

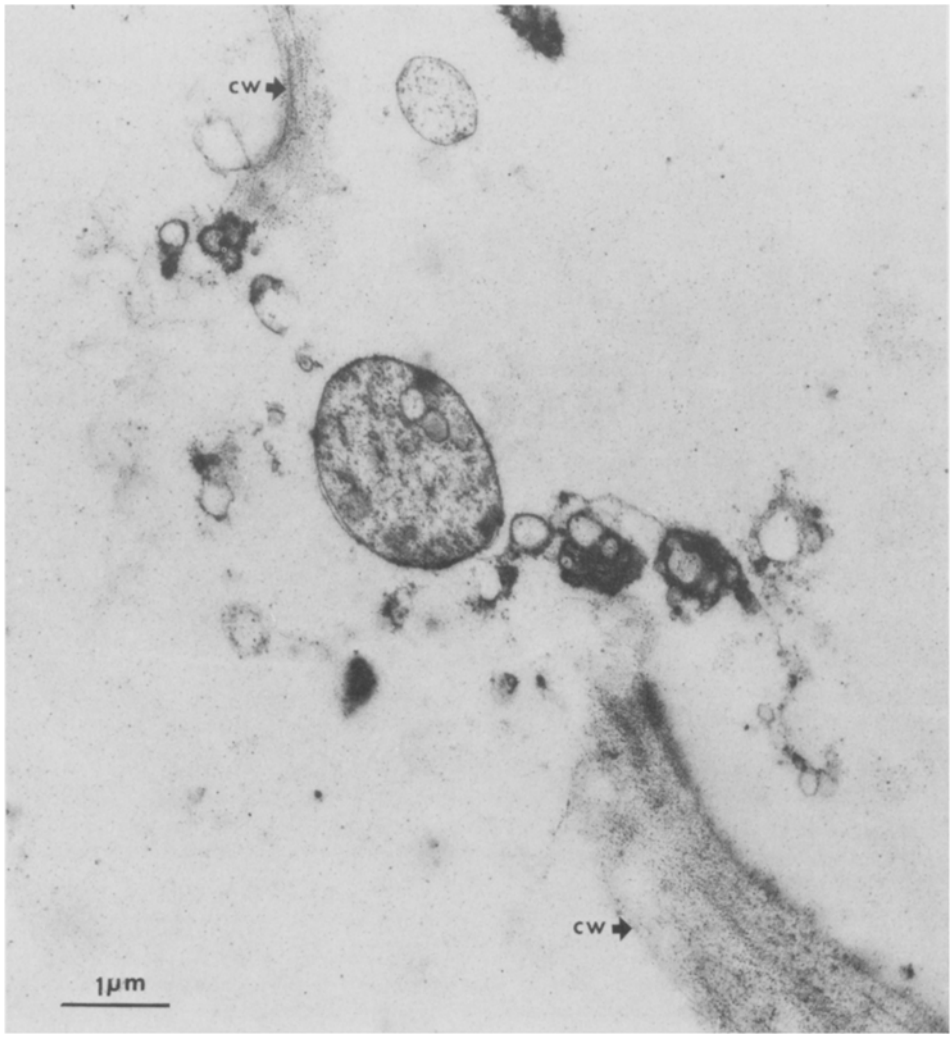


Fig. 5. Elektronenmicroscopische foto van een gat (3,3 μm) in de celwand van de cortex van de vatbare waardplant 12 dagen na inoculatie. Resten van waardplant cytoplasma zijn zichtbaar in het gat.

parasitical material within a host vacuole of a cortical cell 10 days after inoculation. Particularly striking is the presence of lipid droplets which is characteristic of this fungus (Dekhuijzen, 1976). The number of droplets is, however, much smaller than those at the plasmodial phase of the life cycle (see Fig. 6). The host cytoplasm and tonoplast were not disrupted. The diameter of the parasitical material varied between 1.4–5 μm (Fig. 1 and 2).

Twelve days after inoculation. Fig. 3A shows three cortical cells, one of which contains an enlarged host nucleus and nucleolus. The tonoplast is disrupted at several points and parasitical material is present in the vacuole. Remnants of host cytoplasm are visible. In another cortical cell of the same section the parasitical material was found to be in close contact with the not yet disrupted tonoplast (Fig. 3B).

Fig. 4 shows parasitical material within a vacuole. Protrusions and lobes of the parasite are in close contact with the broken tonoplast. It is possible that the tubules within the parasitical body are linked to the protrusions. These observations together with those published in a previous paper (Dekhuijzen, 1976) suggest the presence of amoeboid structures penetrating the cortical cells. Much attention was therefore paid to the cortical cell walls and indeed frequently large openings of about 3 μm were found in the walls (Fig. 5).

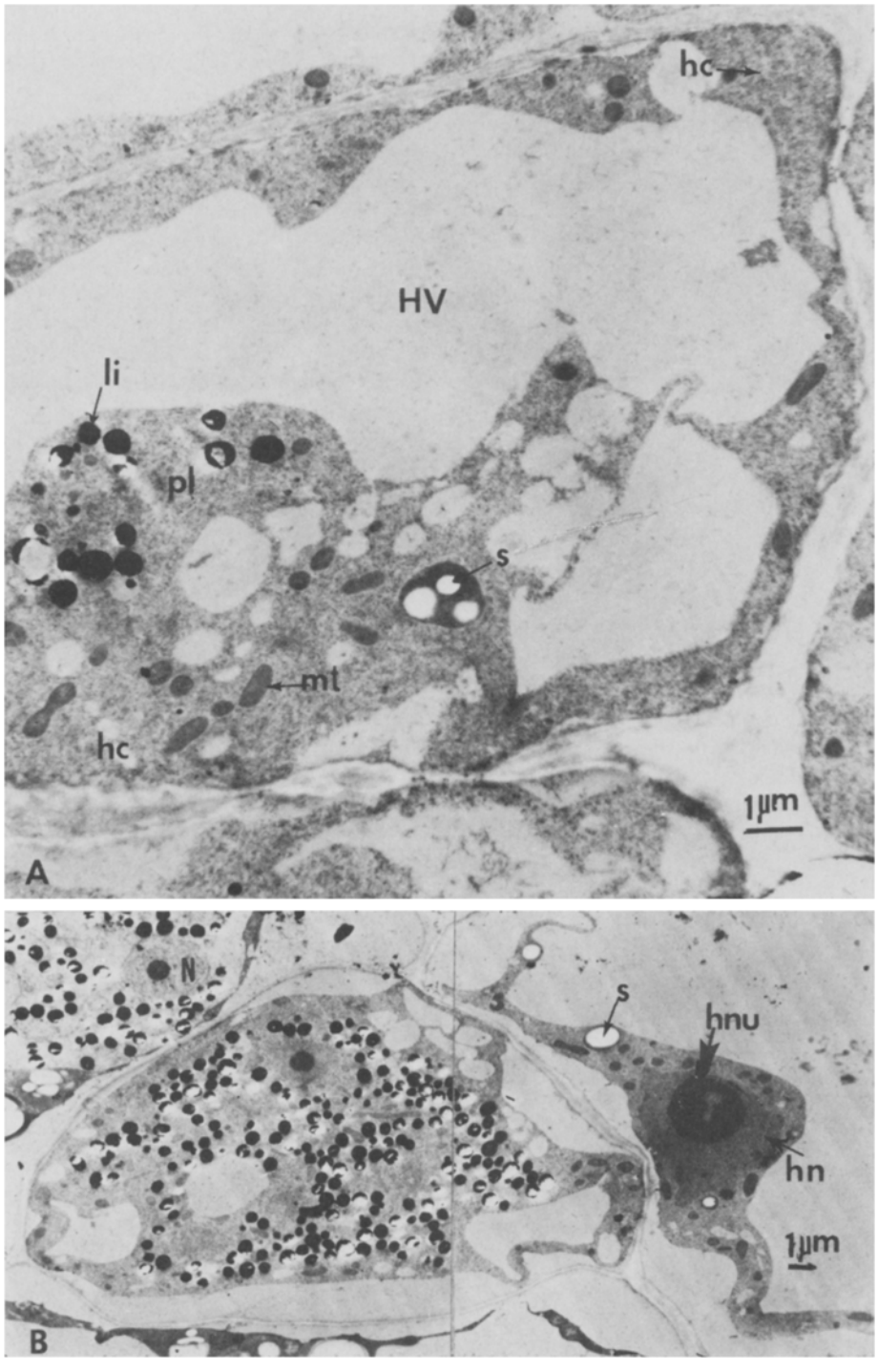
Thirteen days after inoculation. At this time the first multinucleate plasmodia became visible in the main root of the susceptible variety (Fig. 6). The plasmodia show a large number of nuclei, nucleoli and mitochondria as described by others (Williams and McNabola, 1967). Characteristic for this phase of the life cycle is the presence of abundant lipid droplets within the multinucleate plasmodia, the presence of starch within the host cytoplasm and an intact tonoplast. Furthermore the cytoplasm seems not as seriously disrupted as during the amoeboid phase of the life cycle (Fig. 3).

The infected root hairs and cortex of the resistant variety

Seven to nine days after inoculation zoosporangia were clearly visible in the root hairs of both the susceptible and resistant varieties (Fig. 7 and 8). Occasionally dark abnormal plasmodia were found but most often zoosporangia with zoospores were

Fig. 6. Plasmodia in close contact with the host cytoplasm of a cell of the inner cortex or a medullary ray of the main root 13 days after inoculation of the susceptible variety. A) The plasmodium (pl) shows abundant lipid droplets (li). The host cytoplasm (hc) contains starch (s) and mitochondria (mt). A large host vacuole (HV) is visible in the centre of the host cell. B) Plasmodium almost filling a host cell. An enlarged host nucleus (hn) and nucleolus (hnu) are visible in an adjacent host cell. Nuclei (N) and nucleoli are visible within the plasmodia.

Fig. 6. Plasmodiën liggen in nauw contact met het waardplant-cytoplasma van een cel in het binnenste gedeelte van de cortex of van een mergstraalcel van de hoofdwortel 13 dagen na inoculatie van de vatbare variëteit. A) Het plasmodium (pl) bevat een groot aantal vetdruppels (li). Het waardplant-cytoplasma (hc) bevat zetmeel (s) en mitochondriën (mt). Het centrum van de waardplantcel bevat een grote vacuole (HV). B) Een plasmodium vult vrijwel de gehele waardplantcel. Een vergrote waardplantkern (hn) en kernlichaampje (hnu) zijn zichtbaar in een naburige waardplantcel. Kernen (N) en kernlichaampjes zijn zichtbaar in de plasmodiën.


hc
HV
li
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s
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B

Fig. 7. Phase contrast micrographs of root hairs of the resistant variety A) normal zoosporangia (ZP) with secondary zoospores (Z) 10 days after inoculation. B) Disorganized primary plasmodia 13 days after inoculation. In both pictures the epidermis of the host root is situated at the left hand side (not photographed).

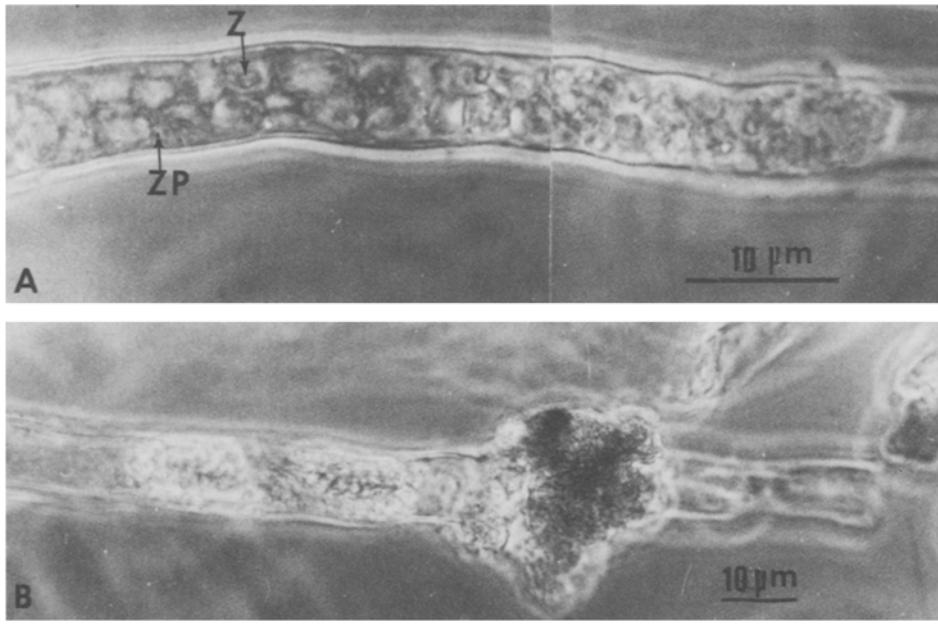


Fig. 7. Fasecontrast foto's van wortelharen van de resistente variëteit. A) Normale zoosporangiën (ZP) met secondaire zoosporen (Z) 10 dagen na inoculatie. B) Gedesorganiseerde primaire plasmodiën 13 dagen na inoculatie. In beide foto's ligt de epidermis van de wortel aan de linkerzijde (niet gefotografeerd).

present in the root hairs of the resistant variety (Fig. 7). Electron microscopic photographs of zoosporangia with secondary zoospores are shown in Fig. 8. Openings of about 0.37–1.0 µm between two adjacent zoosporangia are visible. The zoospores show a remarkable resemblance to the primary zoospores as observed by Aist and Williams (1971). They contain flagellae which have the usual (9 + 2) arrangement of microtubules (Fig. 9).

Eleven days after inoculation a large number of dead host cells were observed in the outer cortical layer of the main root of the resistant variety, whereas no apparent changes were found in the inner cortex (Fig. 10). Electron microscopic observation showed the presence of disorganized material within the cortical host cell. Often fungal and host material could not be distinguished from each other (Fig. 11A). In some cases the shape and size suggested structures of the parasite (Compare Fig. 11B with Fig. 1). These results suggest the occurrence of a hypersensitive reaction within the outer cortex which terminated further growth of *P. brassicae*.

Fig. 8. Zoosporangia with secondary zoospores in the basal end of a root hair of a lateral root of the resistant variety 9 days after inoculation. Each zoosporangium (ZP) contains 2–4 or more secondary zoospores (Z). Remnants of host cytoplasm with unidentified bodies are present in the cortical cells (CC) of the lateral root.

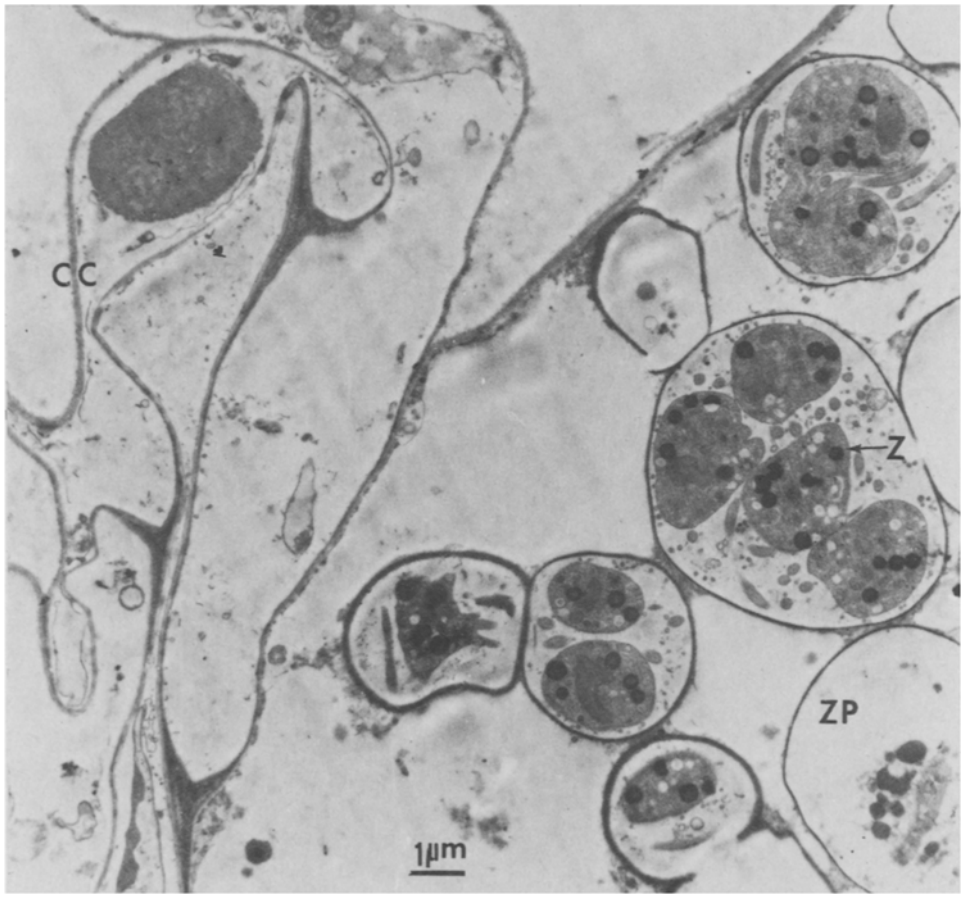


Fig. 8. Zoösporangiën met secondaire zoosporen in het basale gedeelte van een wortelhaar aan een zijwortel van de resistente variëteit 9 dagen na inoculatie. Ieder zoösporangium (ZP) bevat 2–4 of meer zoosporen (Z). Resten van waardplant-cytoplasma met niet-geïdentificeerde lichaampjes liggen in de cortex (CC) van een zijwortel.

Discussion

The susceptible variety. The electron micrographs in this paper show structures in the cortex of the susceptible variety at a stage of infection prior to disease symptom development which differ markedly from secondary plasmodia (Fig. 6 and Dekhuijzen, 1976). In contrast to secondary plasmodia they contain only very few lipid droplets. Aist and Williams (1971) found that uninucleate amoebae (1.3–2.7 µm) in the root hairs lacked lipid droplets.

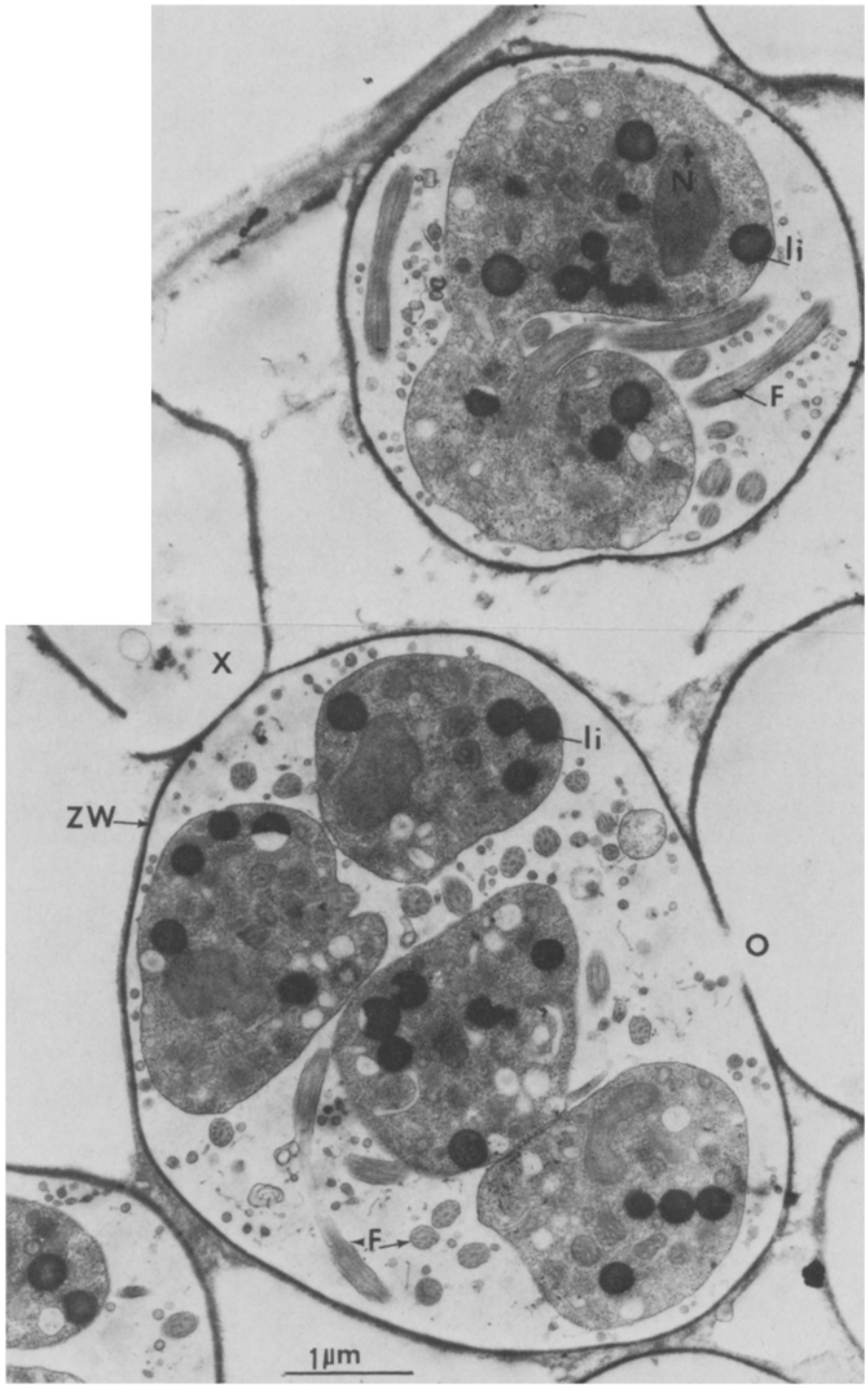
Another important difference is the number of nuclei. Although nuclei have been sectioned only in a few cases it is remarkable that the parasitical material contains only one nucleus, whereas the secondary plasmodia are multinucleate (Fig. 6 and Dekhuijzen, 1976 - Fig. 3). The size of the irregularly shaped parasitical material ranges from 1–5 μm which is smaller than the smallest spherical shaped secondary plasmodia (5–30 μm) (Dekhuijzen, 1975, 1976). Definite proof is lacking but the presence of amoeboid material and of perforations in the cortical cell wall (Fig. 5) support the earlier view that the amoebae penetrate the cortical cells and disrupt the tonoplast and host cytoplasm (Dekhuijzen, 1976). This phase differs markedly from the secondary phase of the life cycle in which the host cytoplasm remains intact for a long time (Fig. 6, Dekhuijzen, 1976; Williams and McNabola, 1967). The numerous strand-like tubules within the amoebae are very characteristic of the cortical phase of the life cycle. They seem to be connected with the protrusions and lobes of the amoebae (Fig. 4). Similar tubular systems have been found in free living slime molds (Rhea, 1966). It has been suggested that their function is related to phagocytosis or to the contractile movement of the amoebae.

The resistant variety. Up to about 10 days after inoculation the development of the fungus in the resistant variety does not differ very much from that in a susceptible variety. In most cases zoosporangia with zoospores developed normally although occasionally abnormal primary plasmodia were found within the root hairs of the resistant variety (Fig. 7–9). Williams et al. (1973) and Butcher et al. (1976) also found the root hair stages of the life cycle of the pathogen in resistant varieties. How the secondary zoospores enter the cortex is not known. Aist and Williams (1971) assumed that the secondary zoospores, similarly to the primary zoospores, penetrate the host cell wall with the aid of a Stachel. The origin of the amoebae in the cortex of the susceptible and resistant plant is not known, but it may be speculated that secondary zoospores released from the zoosporangia give rise to an amoeboid phase of the life cycle. The occurrence of an amoeboid phase in the life cycle has been mentioned before (Fedorintschik, 1936).

The formation of the amoebae and the possible formation of spore-like material (Fig. 2; Dekhuijzen, 1976–Fig. 2B) with a diameter of about 1 μm by the amoebae needs further research. The amoebae may provide a mechanism for the migration of *P. brassicae* within the host. Another mechanism for migration of this fungus has been suggested by Ingram and Tommerup (1972). They observed zoosporangia in

Fig. 9. Magnification of a part of Fig. 8. The cytoplasm within the upper zoosporangium may be in the process of cleavage and the formation of 2 zoospores. The zoospores contain one nucleus (N), lipid droplets (li) and flagellae (F). The zoosporangial wall (ZW) is 30 nm thick. Openings (O) of 0.37-1.0 μm are visible between 2 zoosporangia. Some zoosporangia are empty (X). The lower zoosporangium contains four zoospores. Cross sections of the flagellae (F) show the (9 + 2) arrangement of the microtubules.

Fig. 9. Vergroting van een gedeelte van Fig. 8. Het cytoplasma in het bovenste zoösporangium zou zich in het stadium van deling kunnen bevinden, wat leidt tot de vorming van 2 zoösporen. De zoösporen bevatten een kern (N), vetdruppels (li) en zweepharen (F). De diameter van de wand van het zoosporangium is 30 nm. Tussen 2 naburige zoösporangiën bevinden zich openingen (O) van 0,37 - 1 μm. Sommige zoösporangiën zijn leeg (X). Het onderste zoösporangium bevat 4 zoösporen. Dwarsdoorsneden van de zweepharen (F) vertonen de (9 + 2) structuur van de microtubulae.


N
li
F
X
li
ZW
O
F
1µm

squashes made from deep-seated host cells of six week old club-root galls, the zoospores of which could also provide a mechanism for internal spread of *P. brassicae* and the consequent development of secondary clubs. Obviously more research has to be carried out before the role of secondary zoospores and of the amoebae in the migration of *P. brassicae* within the host is fully understood.

Resistance became most clearly manifest in the cortex of the main root 10–24 days after inoculation. At that time necrotic host cells were found (Fig. 10 and 11). Most probably a hypersensitive type of resistance occurred in the cortical cells and growth of the amoebae became arrested.

Samenvatting

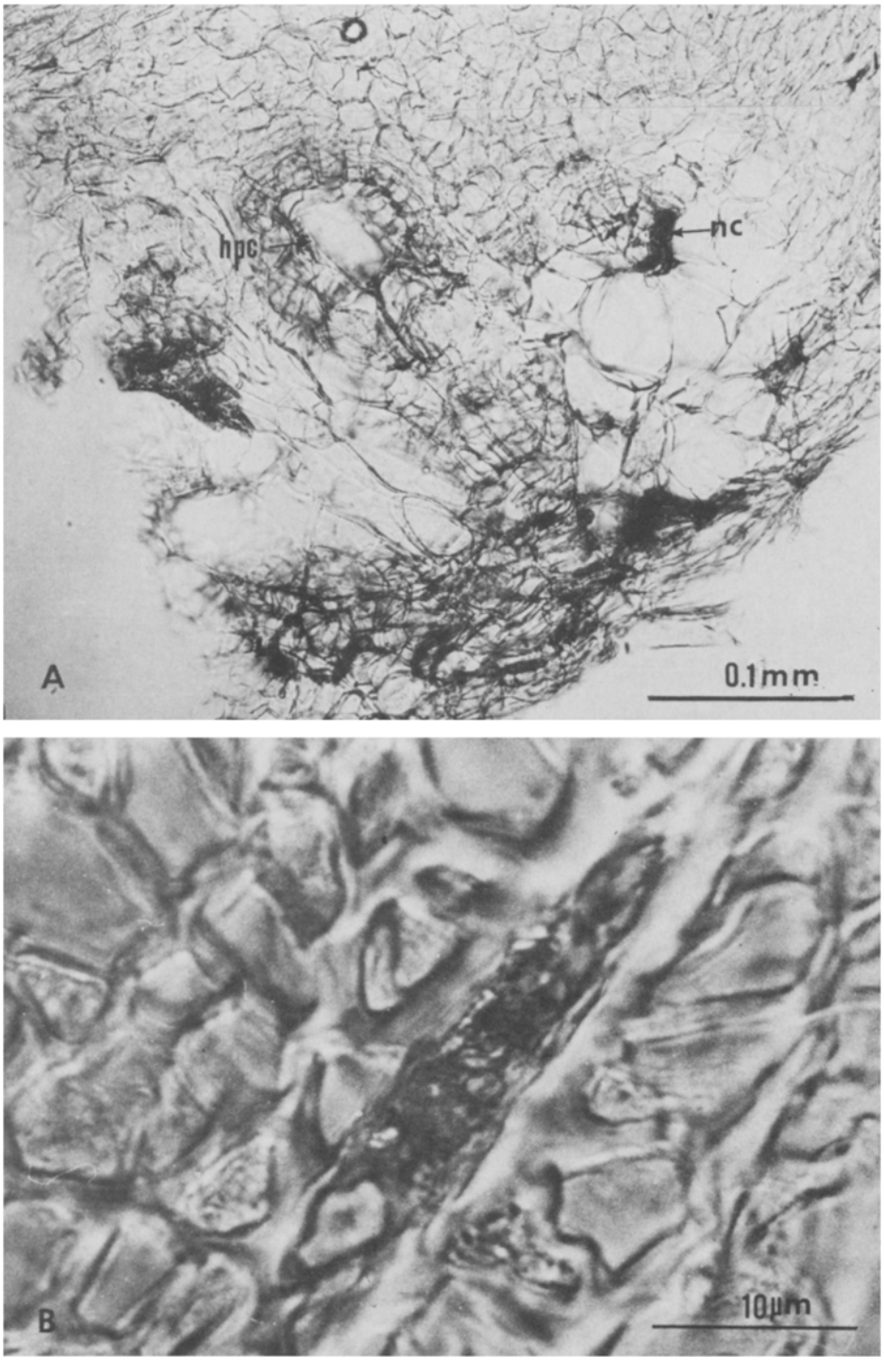
Elektronenmicroscopische onderzoekingen aan de wortelharen en de cortex van een vatbare en resistente variëteit van Brassica campestris geïnfecteerd met Plasmodiophora brassicae

Kiemplanten van een vatbare en een resistente variëteit van *Brassica campestris* var. *rapa* werden geïnoculeerd met een suspensie van rustsporen van *Plasmodiophora brassicae*. De rustsporen waren afkomstig uit verouderd ziek callusweefsel. Elektronenmicroscopisch onderzoek werd verricht aan de cortex van de hoofdwortel van vatbare planten in de periode die vooraf gaat aan de ontwikkeling van ziektesymptomen (10–13 dagen na inoculatie). Tien dagen na inoculatie werden amoebe-achtige structuren in de cortex gevonden (Fig. 1–4). Herhaaldelijk werden gaten in de celwand van de cortex gevonden (Fig. 5). De waardplantkern en kernlichaampje vertoonden een hypertrofische reactie terwijl de tonoplast vaak kapot was (Fig. 3). Deze resultaten vormen een aanwijzing dat de parasiet in de vorm van een amoebe door de celwand dringt en de celinhoud van de cortex verstoort. Dertien dagen na inoculatie werden de eerste secondaire plasmodiën in het binnenste gedeelte van de cortex of in de mergstraalcellen van de hoofdwortel gevonden (Fig. 6). Vanaf dat moment traden de typische knolvoetsymptomen op.

Uit elektronen-microscopisch onderzoek van de resistente variëteit bleek dat negen dagen na inoculatie zoösporangien en secondaire zoösporen aanwezig waren in de wortelharen van de zijwortels (Fig. 7–9). Twee tot vier dagen later werden een groot aantal dode waardplantcellen in de buitenste cortexcellen van de hoofdwortel gevonden, terwijl het binnenste gedeelte van de cortex geen veranderingen vertoonde (Fig. 10 en 11). Deze resultaten wijzen er op dat in de resistente variëteit een overgevoeligheidsreactie in de cortex opgetreden is waardoor verdere groei van *Plasmodiophora brassicae* verhinderd werd.

Fig. 10. Light microscopical pictures of cortical cells of the resistant variety. A) 24 days after inoculation. Many necrotic cells (nc) and many hyperplastic cells (hpc) are present in the cortex. B) Magnification of a necrotic spot 11 days after inoculation.

Fig. 10. Lichtmicroscopische foto's van cortex-cellen van de resistente variëteit. A) 24 dagen na inoculatie. In de cortex bevinden zich veel necrotische cellen (nc) en veel hyperplastische cellen (hpc). B) Een vergroting van een necrotische plek 11 dagen na inoculatie.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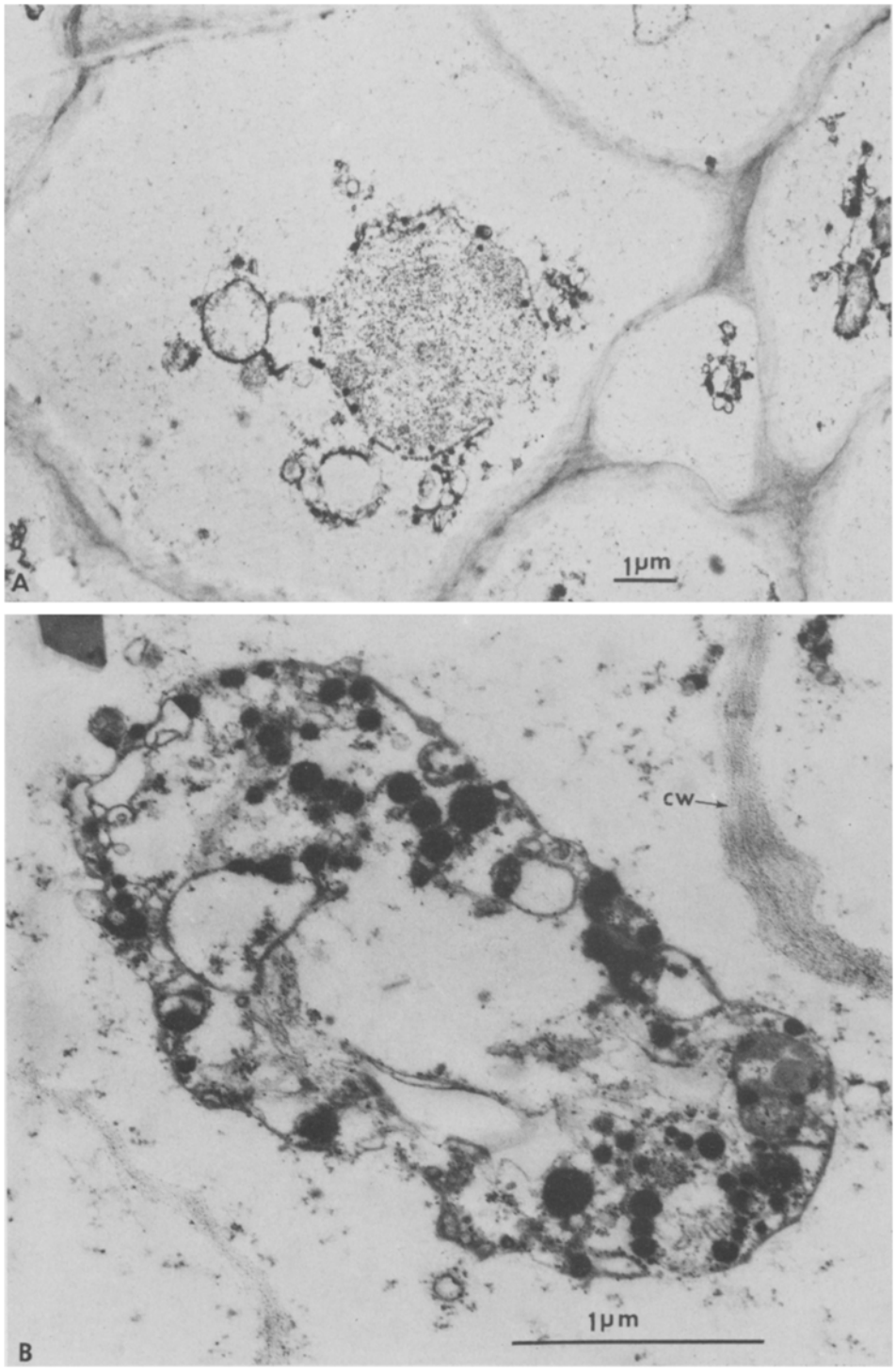
A
1µm
B
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1µm

Fig. 11. Electron micrographs of cortical cells of the resistant variety 10 days after inoculation. A) Disorganized structures in a cortical cell. Material of the parasite and host cytoplasm cannot be distinguished from each other. B) An oval particle, which according to its shape, size and osmiophilic globules, probably belongs to disintegrated material of the parasite (compare with Fig. 1). Cell wall (cw).

Fig. 11. Elektronenmicroscopische foto's van cortex-cellen van de resistente variëteit 10 dagen na inoculatie. A) Gedesorganiseerd materiaal in een cortex-cel. Parasitair materiaal en waardplant-cytoplasma kunnen niet van elkaar onderscheiden worden. B) Vorm, grootte en de aanwezigheid van elektronen verstrooiend materiaal doet de aanwezigheid van gedesintegreerd parasitair materiaal veronderstellen (vergelijk Fig. 1). Celwand (cw).

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