European Journal of Plant Pathology **104:** 653–665, 1998.

Ultrastructural and cytochemical investigations of pathogen development and host reactions in susceptible and partially-resistant carrot roots infected by *Pythium violae*, the major causal agent for cavity spot

L. Guérin[1], N. Benhamou[2] and F. Rouxel[3,*]

[1] *ENITA de Bordeaux, UERD Protection des Végétaux, BP 201, 33175 Gradignan cedex, France;* [2] *Recherche en Sciences de la vie et de la santé, Pavillon Charles-Eugène-Marchand, Université Laval, Sainte-Foy, Québec, Canada G1K 7P4;* [3] *INRA, Centre de Recherches de Rennes, Station de Pathologie Végétale, BP 29, 35650 Le Rheu, France;* * *Author for correspondence*

Accepted 9 June 1998

Key words: Daucus carota, Pythium violae, susceptibility, cytochemistry, pectin, cellulose

Abstract

Two carrot genotypes, cultivar Nanco and line 24, susceptible and partially- resistant respectively to cavity spot, were compared ultrastructurally and cytochemically 24 h, 48 h and 72 h after root inoculation with a virulent *Pythium violae* isolate. The extent of pathogen ingress and the response of the host differed markedly with the two genotypes. In cv Nanco, growth of fungal hyphae was predominantly intracellular and was accompanied by pronounced damage; by 48 h after inoculation, pericycle and the first cell layers of the phloem parenchyma were invaded, resulting in host wall dissolution and cytoplasm aggregation. The growth of *P. violae* in line 24 was limited to the pericycle, even up to 72 h after inoculation; fungal colonization was accompanied by retraction of cytoplasm and in the appearance of granular or fibrillar material in the host cell lumen. Some affected host cells were filled with structureless osmophilic material. In cultivar Nanco, invading fungal hyphae were unaffected; by contrast in line 24, the cytoplasm of invading hyphae, particularly those inside the cell host, was disorganised and structureless. Infection and host response in the two cultivars were studied with two specific labels: *Aplysia* gonad lectin (AGL), a polygalacturonic acid-binding lectin, and an exoglucanase complexed to colloidal gold were used to locate pectin and cellulosic β-(1,4)-glucans respectively in infected tissues. The decrease of cytochemical labeling beyong fungal penetration showed clearly hydrolysis of pectin and cellulose in cell walls of the cv Nanco. By contrast, the cell wall of line 24 remained largely intact, although unlabeled amorphous and electron-dense material was observed inside the wall. Fibrillar or electron dense material commonly observed in infected tissue of line 24 apparently did not contain pectic or cellulosic substances. Moreover, material observed in host cells or fungal hyphae was also free of labeling. The origin and the chemical composition of these compounds as well as their possible role in the defence mechanisms of carrot against *P. violae* are discussed.

Introduction

Cavity spot of carrot (*Daucus carota* L.), a disease mainly characterized by the formation of elliptical lesions on all root parts (Perry and Harrison, 1979), has been reported in every country where carrots are grown commercially. The disease is highly destructive, resulting in severe economic losses for carrot growers throughout the world (Gladders and McPherson, 1986; Montfort and Rouxel, 1986). Since the first demonstration that cavity spot was associated with infection by *Pythium* spp. (White, 1984), several lines of evidence have shown that among the different causal species (Guérin et al., 1994; El-Tarabily et al., 1996), *Pythium violae* Chesters & Hickman remained the principal species involved in disease establishment (Groom and Perry, 1985; White, 1986; Montfort and

Rouxel, 1988; Vivoda et al., 1991; Benard and Punja, 1995).

Understandably, most efforts relating to cavity spot have been directed towards developing strategies for reducing disease losses. Among those, the use of resistant carrot cultivars could afford enhanced protection against *P. violae* attack. In this context, an interesting approach that has been proposed in several countries and especially in France has been the screening for genetic variability among carrot lines of various geographic origins (Guérin, 1993). According to the rate and extent of root lesion development, this has led to classification of lines into several groups ranging from moderately to highly susceptible. Partially-resistant and tolerant carrot lines were selected for further analysis of the inheritance of resistance and for cytological investigations of the infection process in comparison to that occurring in susceptible plants.

Evidence from a number of histological studies has shown that *P. violae* has the ability to colonize rapidly the outermost root tissues in susceptible cultivars (Groom and Perry, 1985; Briard, 1990). More recently, Zamski and Peretz (1995) and Campion (1997) provided evidence that ingress of *P. violae* towards the vascular stele coincided with host cell wall degradation. This was taken as an indication, later confirmed *in situ* by Zamski and Peretz (1996), and *in vitro* by Campion et al. (1997), that *P. violae*, like other *Pythium* spp. (Endo and Colt, 1974; Chérif et al., 1991; Rey et al. , 1996), displayed the ability to produce cell wall degrading enzymes as one of the major pathogenicity factors.

In spite of renewed interest in exploring the interaction between carrot roots and *P. violae*, the exact mechanisms by which the infection process in cavity spot operates remains a matter of speculation, and no studies on the different degrees of resistance among carrot varieties have been reported. There was no correlation between phytoalexin production and resistance to *P. violae* in carrot cultivars with different degrees of susceptibility (Briard, 1990), thus it was of interest to determine whether the resistance of some cultivars was associated with the formation of structural barriers in response to pathogen invasion. The present study was undertaken to compare the extent of tissue damage and host reactions in susceptible and tolerant carrot genotypes at an early stage of the infection. To gain a better insight into the biological significance of the carrot cell walls in restricting pathogen ingress, the localization of pectin and cellulose, two of the main carrot cell wall components (Massiot, 1988), was carried out using gold-conjugated probes.

Material and methods

Fungal culture and growth conditions

The isolate of *Pythium violae* Chesters & Hickman used in this study was isolated from an infected carrot root and maintained at 4 °C at INRA, Station de Pathologie Végétale, Le Rheu, France (Ref.: Pv. 20). It was grown on carrot juice-agar medium at 20 °C and was periodically inoculated on and reisolated from susceptible carrot roots.

Plant material and fungal inoculation

Five month-old carrot roots (*Daucus carota* L., cv Nanco, highly susceptible to cavity spot, and line 24, partially-resistant to cavity spot) were grown in sterilized soil and inoculated with *P. violae* as previously described by Guérin et al. (1994). Healthy, freshly collected roots were washed carefully with distilled water and the top and the bottom were removed. Root segments about 10 cm long were surface inoculated with three 4 mm-diameter agar plugs taken from the margin of a 7-day old culture of *P. violae*. Fungus-free agar plugs were used as controls. Roots were incubated at 20 °C in the dark, in a moist chamber with a RH of 95-98% maintained by daily spraying sterile water. A disease index (lesion area in mm^2) was calculated from two right-angle diameters before collecting samples for microscopic analysis, at various time intervals (24, 48 and 72 h) after inoculation. The experiment was repeated 3 times on 10 roots per treatment.

Tissue processing for light and electron microscope studies

Root samples ($1mm^3$) were collected by 24, 48 and 72 h after inoculation with *P. violae*, at the margin of the visible lesions for the inoculated roots, and by cutting off superficial tissues for the controls. Root samples were fixed by immersion in 3% (v/v) glutaraldehyde buffered with 0.1 m sodium cacodylate pH 7.2 for 3 h at room temperature. After thorough washing in the buffer, samples were post-fixed with 1% (w/v) osmium tetroxide in the same buffer for 2 h at 4 °C and dehydrated in a graded ethanol series prior to be embedded in EPON 812.

Table 1. Disease and invaded tissue observed in susceptible or partially-resistant cultivar roots at various times after inoculation with *Pythium violae*

Time after inoculation (h)	Susceptible cv			Partially-resistant line		
	Disease index (mm2)[a]	Depth of mycelium ingress [b]	Invaded tissue	Disease index (mm2)[a]	Depth of mycelium ingress [b]	Invaded tissue
24	31.2 (± 5.5)	6.7 (±4.3)	Pericycle (Phloem parenchyma)[c]	14.4 (±2.4)	2.0 (±3.0)	Periderm (Pericycle)[c]
48	46.1 (±18.2)	12.5 (±3.2)	Pericycle Phloem parenchyma	14.9 (±3.8)	4.2 (±4.6)	Periderm (Pericycle)[c]
72	53.5 (±17.6)	12.1 (±3.1)	Pericycle Phloem parenchyma	14.4 (±2.4)	5.6 (±7.3)	Pericycle Phloem parenchyma

[a] Mean of 10 lesions (SD).
[b] Number of cell layers invaded by the pathogen. Mean of 30 sections (SD).
[c] non-systematic tissue ingress.

Thin sections (1–1.5 μm) were mounted on glass slides and stained with 1% (w/v) toluidine blue. Three sections per sample were observed using a Leitz Dialux 22/22 EB optic microscope for cell wall modifications and pathogen growth in the tissues by counting the number of infected cell layers. Ultrathin sections (0.7 μm) collected on formvar-coated nickel grids were contrasted with uranyl acetate and lead citrate before examination with a JEOL 1200 EX transmission electron microscope at 80 kV or further processed for cytochemical labeling prior to examination.

Cytochemical labeling

Sections were labeled with gold-complexed probes prepared as follows: Colloidal gold with a mean particle diameter averaging 15 nm was prepared according to Frens (1973) by reducing an aqueous tetrachloroauric acid solution to 0.01% (w/v) with 1% (w/v) sodium citrate.

The *Aplysia* gonad lectin (AGL), a lectin with binding affinity for galacturonic acids (Gilboa-Galber et al., 1985), and the β-(1,4)-exoglucanase, an enzyme with binding affinity for cellulosic β-(1,4)-glucans, were complexed to colloidal gold, as previously described (Benhamou et al., 1987, 1988) and used for the detection of pectic compounds and the localization of cellulosic β-(1,4)-glucans.

Ultrathin sections from samples of inoculated or control carrot roots were floated for 5 min on a drop of phosphate-buffered saline (PBS) containing 0.02% (w/v) of PEG 20 000 at the pH corresponding to the optimal activity of the lectin (AGL, pH 8.0) or the enzyme (exoglucanase, pH 6.0). They were then transferred to a drop of each complex and incubated in a moist chamber at room temperature for 30 min. Grids were thoroughly washed with PBS, pH 7.4, rinsed with distilled water, and contrasted with uranyl acetate and lead citrate before examination.

Specificity of the two labelings was assessed by the following control tests: 1) incubation with the exoglucanase-gold complex to which was previously added 1 mg ml^{-1} of β-(1,4)-D-glucans from barley; 2) incubation with the AGL-gold complex to which 100 μg ml^{-1} of polygalacturonic acids was previously added; 3) incubation with the uncomplexed protein followed by incubation with the gold complex; 4) incubation with colloidal gold alone.

Results

Ultrastructural features of the infection process in carrot root tissues

Susceptible cultivar

By 24 h after inoculation with *P. violae*, lesions averaging 31.2 mm^2 in diameter were visible at the root surface. Lesion size increased gradually to reach 53.5 mm^2 by 72 h after inoculation (Table 1).

Ultrastructural examination of infected carrot root samples showed that fungal growth was mainly intracellular in underperidermic cells (Figures 1B-D). By 24 h after inoculation, pathogen colonization was observed within an average of 6,7 cell layers (Table 1). At that time, pericycle colonization was accompanied by striking wall modifications including primary wall alteration and middle lamella dissolution, as judged by

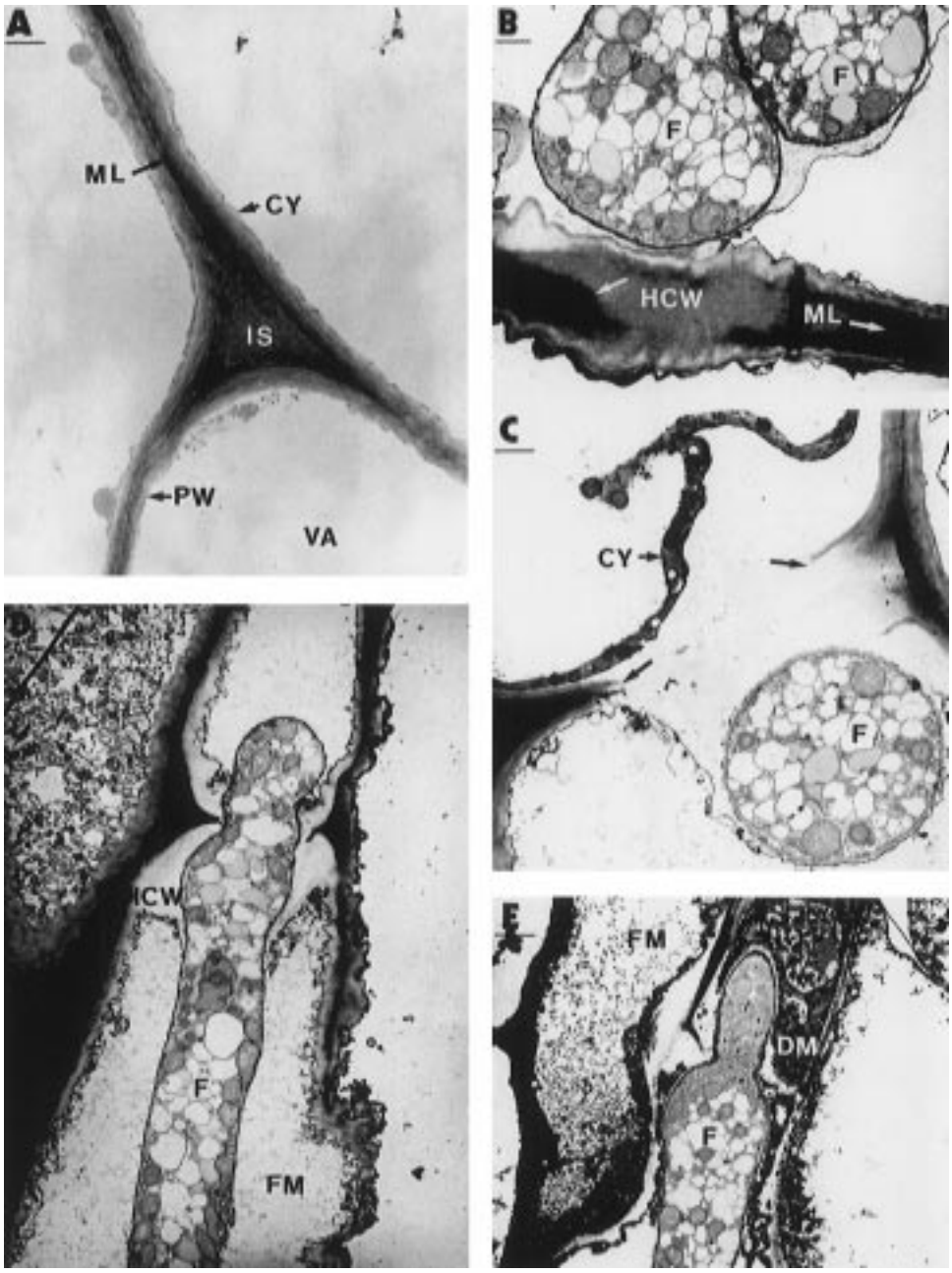

Figure 1. Transmission electron micrographs of carrot root tissues (susceptible cv) non-inoculated or 24, 48 and 72 h after inoculation with *Pythium violae*. Bar = 1 μm. A: In non-infected tissues, primary cell walls (PW) are joined closely together by a middle lamella (ML). A thin cytoplasm layer (CY) is joined to the cell wall and an enlarged vacuole (VA) takes up the cell; electron-dense material is clearly visible in the intercellular space (IS). B: By 24 h after inoculation, fungal penetration is accompanied by host cell wall (HCW) swelling and a decrease in electron density in the middle lamella (ML) (arrow). C: By 48 h after inoculation, intracellular hyphae (F), apparently undamaged, are visible in phloem parenchyma cells which show a wall degradation (arrow) and an electron-dense, retracted cytoplasm (CY). D: By 72 h after inoculation, pathogen ingress is associated with host cell-wall (HCW) widening. Colonized and adjacent cells show cytoplasm alteration and accumulation of fibrillar material (FM) in the cell lumen. E: At the same time, cellular space is plugged with electron-dense (DM) and fibrillar material (FM). In contrast, a fungal hypha(F) is apparently undamaged.

the marked decrease in the respective electron densities of these structures (Figure 1B) and as compared to the preserved cell wall architecture in control, non-inoculated carrot tissues (Figure 1A). In addition, host cells underwent major disorganization characterized by cytoplasm aggregation and organelle disruption (Figure 1B). By contrast, pathogen hyphae did not exhibit any apparent disorganization.

From 24 to 72 h after inoculation, 10-15 host cell layers were colonized (Table 1). By 48 h after inoculation, colonized phloem parenchyma cells were severely affected as shown by frequent disruption of primary walls and middle lamella matrices (Figure 1C). In infected cells as well as in adjacent non-colonized cells, typical retraction of the plasmalemma and aggregation of the cytoplasm were observed.

At a more advanced stage of infection (72 h after inoculation), strong cytoplasmic alterations were observed in host cells (Figure 1D). Penetration of pericycle tissues by fungal hyphae occurred directly through wall penetration. At sites of fungal entry, host cell walls were usually disrupted, even at a distance from the hyphae. Adjacent cells often showed marked disorganization of the cytoplasm and contained abnormal fibrillar material deposits.

In some cases, the pericycle was directly penetrated through the tangential wall by means of constricted fungal hyphae (Figure 1E). Massive accumulation of an amorphous, electron-dense material was detected in infected cells as well as in neighbouring cells which were also found to undergo considerable cytoplasmic change.

Partially-resistant line

From 24 to 72 h after inoculation, host colonization by *P. violae* resulted in a limited lesion at the root surface. The lesion sizes varied between 14.0 and 15 mm^2, corresponding approximatively to the inoculum plug diameter (Table 1). By 24 h after inoculation, a maximum of 3 cell layers in the suberized periderm was colonized by the pathogen. At the surface of the peridermic cells surrounded by suberized walls, hyphal colonization correlated with marked alterations of the host walls (Figure 2B). An unusual circumvoluted membrane system embedded in an osmiophilic material was frequently observed at the interface between the fungus and the host cell wall. The fungus appeared to suffer from some damage, as evidenced by the formation of numerous vesicles in the disorganized cytoplasm (Figure 2B).

From 24 to 72 h after inoculation, hyphal growth extended to the pericycle and occasionally to the vascular parenchyma cells (Table 1). An average of 5,6 cell layers was colonized by *P. violae* by 72 h after inoculation.

By 48 h after inoculation, host cells in the pericycle exhibited marked structural alterations, as compared to non-inoculated root tissues (Figure 2A). The plasma membrane was usually retracted and distorted, and the cytoplasm was reduced to a fine layer of electron-opaque material (Figures 2C and D). The host vacuolar space was plugged with deposits of various textures and electron densities and was often colonized by hyphae of the pathogen. By contrast, the host cell walls were apparently preserved (Figure 2C). Invading hyphae were often damaged, with accumulation of amorphous compounds in the cytoplasm and incrustation of electron-dense compounds in the walls (Figure 2D, arrow).

By 72 h after inoculation, pathogen ingress in host cells was associated with major disorders and host reactions. The host plasma membrane was retracted from the cell wall (Figures 3A and B) and was associated with a cytoplasmic disorganization, characterized by the accumulation of electron-opaque deposits along the tonoplast. Some host cells were plugged by an amorphous osmiophilic material. The fungus invasion was associated with less strong wall alteration than in the susceptible cells (Figures 3A and B). Invading hyphae were greatly damaged inside cells, as shown by the accumulation of amorphous compounds in the cytoplasm where organelles were hardly discernible (Figure 3A).

Cytochemical labeling of host wall modifications during the infection process

Susceptible cultivar

Following incubation with the AGL-gold complex for localization of pectic compounds, labeling was associated with primary walls and middle lamella matrices in non-inoculated carrot root tissues (Figure 4A). Following pathogen inoculation, the middle lamella was no longer visible at fungal penetration sites and labeling was markedly reduced (Figure 4D). The primary host walls were also frequently degraded and poorly labeled, both in the areas adjacent or penetrated by *P. violae* hyphae (Figure 4C). Labeling still occurred in the more electron-opaque compounds in host wall areas. No labeling was observed in the fungus wall. Incubation of ultrathin sections of non-

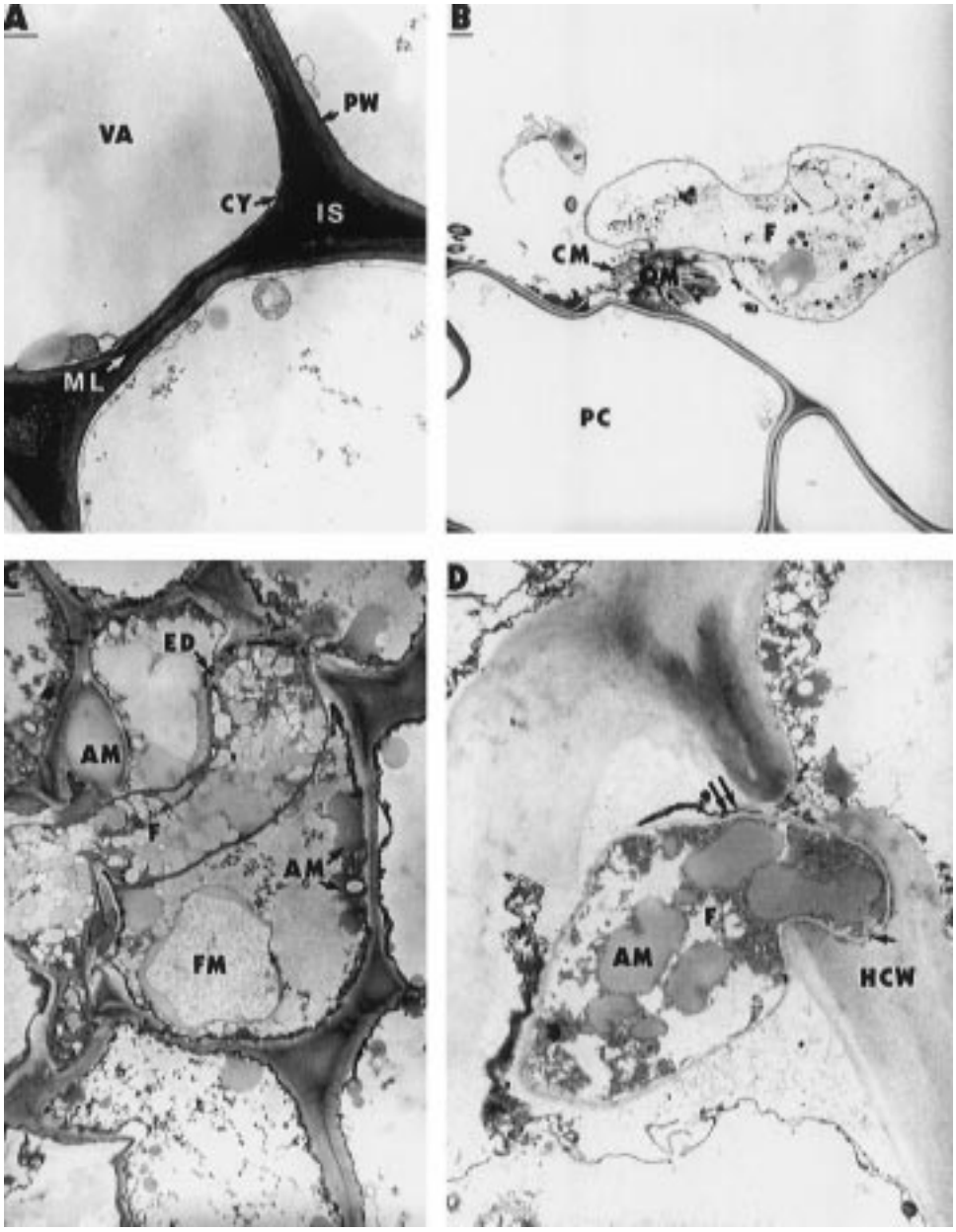

Figure 2. Transmission electron micrographs of carrot root tissues (partially-resistant line) non-inoculated or 24 and 48 h after inoculation with *Pythium violae*. Bar = 1 μm. A: In non-infected tissues, primary wall (PW) and middle lamella (ML) are apparently undamaged. Intercellular space (IS) is filled with electron-dense material. Vacuoles (VA) fill in cell spaces and thin cytoplasm (CY) layers are joined to the cell wall. B: By 24 h after inoculation, a *P. violae* hypha (F) is visualized in contact with a peridermic cell (PC). A circumvoluted membrane system (Cm) embedded in osmiophilic material (OM) is observed in near contact with the hypha showing a disorganized cytoplasm. C: By 48 h after inoculation, fungal hyphae (F) are observed in strongly altered host cells with amorphous (AM) and fibrillar material (FM); cell walls are slightly damaged; electron-dense deposits (ED) are incrusted in fungal wall (arrow). D: At the same time, a fungal hypha (F) penetrating a host cell wall (HCW) shows a marked cytoplasm alteration with accumulation of amorphous material (AM). Electron-dense material (DM) is incrusted in (arrow) or joined to (double arrow) the wall.

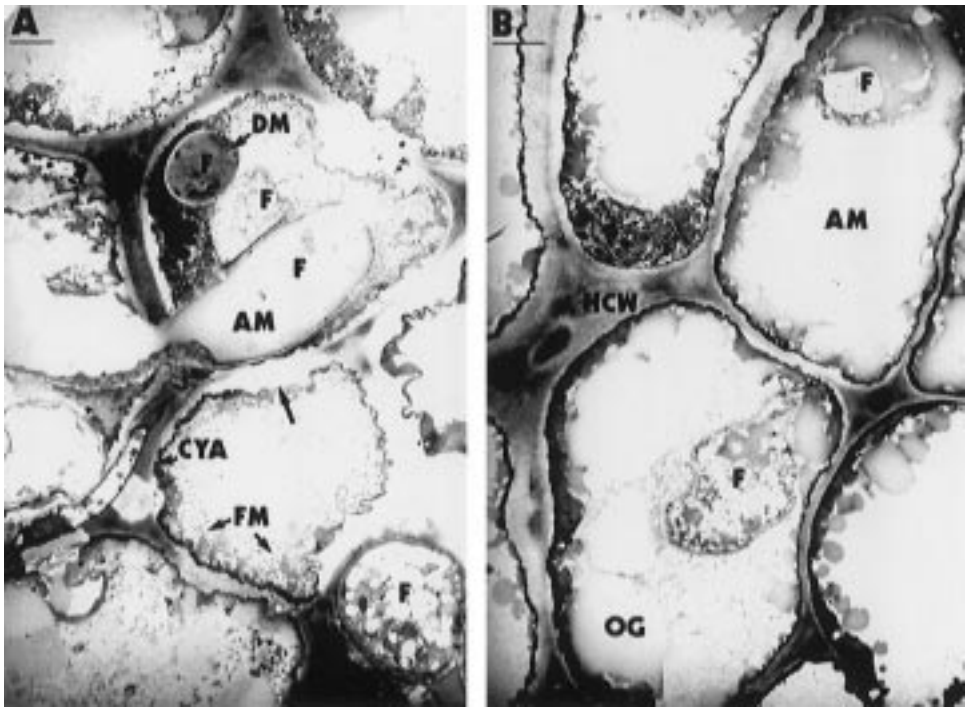

Figure 3. Transmission electron micrographs of carrot root tissues (partially-resistant line) 72 h after inoculation with *Pythium violae*. Bar = 1 μm. A: Infected host cells by *P. violae* show pronounced alterations. Notice the strongly damaged aspect of fungal hyphae (F) inside the host cell, characterized by an accumulation of electron-dense (DM) or amorphous (AM) material in the cytoplasmic space. Hyphae growing in intercellular spaces seem to be less damaged. Host cell content is significantly altered: notice the retraction of plasmalemma (arrow), aggregation of cytoplasm (CYA) and accumulation of fibrillar material (FM) in the cell lumen. B: Ovoid globules (OG) of amorphous material occur along the host cell wall (HCW). The same type of amorphous, structureless material (AM) is spread all over in an other cell infected with *P. violae* (F). In contrast, host cell walls (HCW) are apparently undamaged.

inoculated root with the exoglucanase-gold complex resulted in a regular distribution of gold particles over the primary walls (Figure 4B). In *Pythium* infected tissues, pronounced changes in host cell wall organization were observed at penetration sites. Strong label of cellulose appeared loosened within swollen host cell walls, which were less electron-dense than normal (Figure 5A). In the wall areas neighbouring fungal hyphae, labeling was irregularly distributed. By contrast, an intense labeling was observed at a distance from hyphae (Figure 5B). In cases of strong wall degradation, alteration was often associated with a disorganization of the fibrillar structure, as judged by few gold particles in the area of residual microfibrils (Figure 5C). Moreover, fungal walls were always labeled (Figure 5A). All control tests performed to assess the labeling specificity resulted in a near absence of gold particles in the sections (not illustrated).

Partially-resistant line

The labeling pattern obtained after application of the AGL-gold complex to inoculated carrot root tissues was mainly associated with host cell walls. Wall areas neighbouring degraded fungal hyphae were substantially labeled (Figure 6A). Some gold particles were found to be deposited in fibrillar fragments accumulating in the host cell lumen (Figure 6B). However, they were absent in the amorphous and electron-dense material infiltrated in the host cell walls or incrustated in the walls of fungal hyphae (Figure 6A).

When ultrathin sections were treated with the exoglucanase-gold complex, an irregular labeling occurred in the host cell walls. The deposition of gold particles was preferentially associated with the electron-dense areas (Figure 6C). In the host cell lumen, the electron-dense, amorphous material formed at the interface between the host cell wall and the pathogen was unlabeled (Figure 6D).

Fungal growth in the peridermic tissues of the partially-resistant cultivar induced pronounced disor-

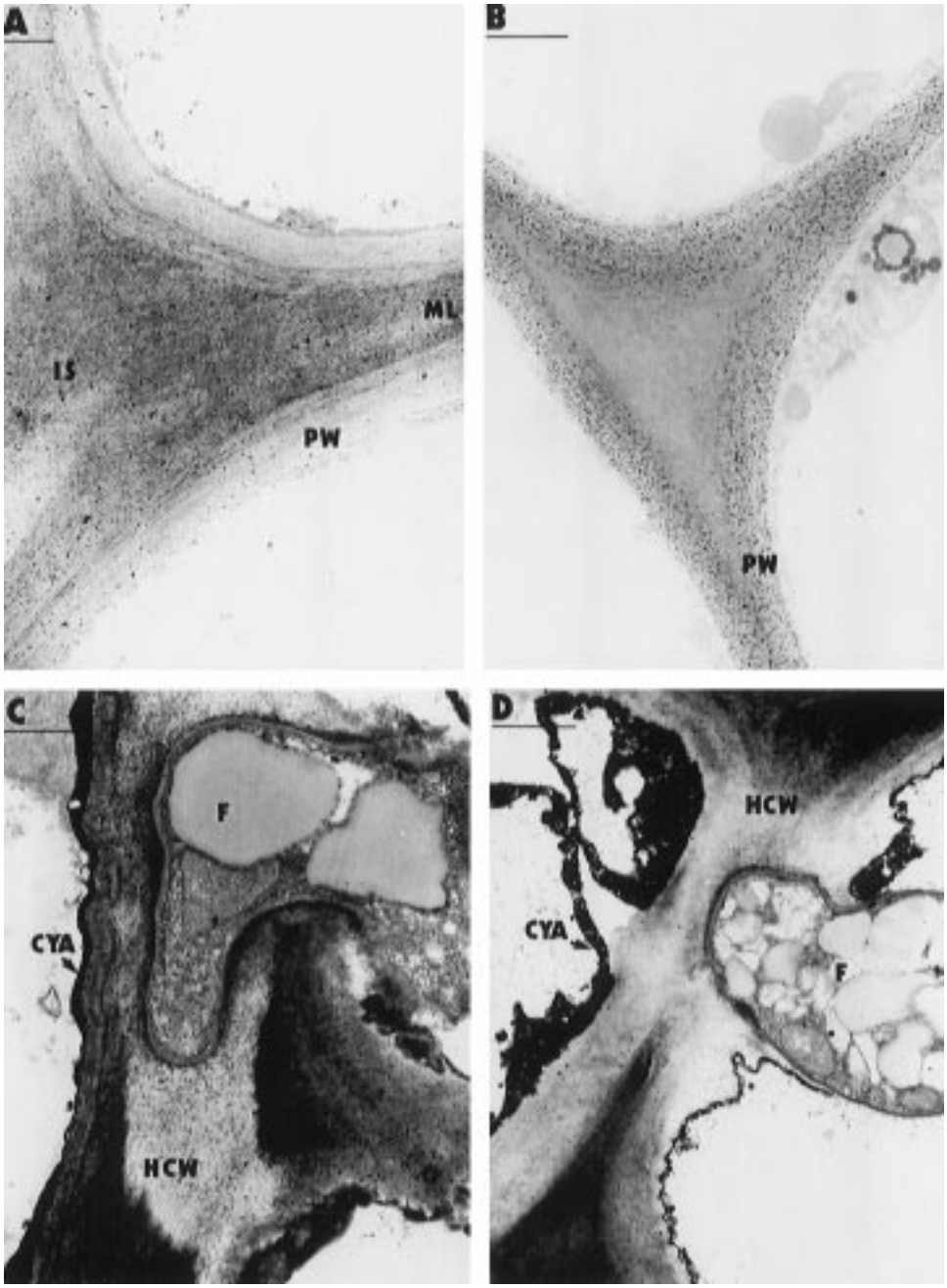

Figure 4. Transmission electron micrographs of carrot root tissues (susceptible cv.) inoculated or not with *Pythium violae*. Sections were treated with gold complexed probes. A: After incubation of non-inoculated sections with the *Aplysia* gonad lectin-gold complex, which labels pectic substances, gold particles accumulate in the primary cell wall, middle lamella (ML) and intercellular space (IS). Bar = 1 μm. B: In non-inoculated sections incubated with exoglucanase gold complex, which reveals cellulosic β-(1,4)-glucans, only primary cell walls (PW) are labeled. Bar = 1 μm. C and D: Labeling of pectic substances with the *Aplysia* gonad lectin-gold complex in tissues infected by *P. violae* (F). Bar = 0.5 μm. C: In parietal area (HCW) around and in advance of the front of fungal penetration, gold particle density correlates with electron density. Labeling is absent in the fungal hyphae (F) and the agregated host cytoplasm (CYA). D: Pectic substances are degraded in the host cell wall (HCW) near a site of hyphal penetration, as judged by the limited labeling observed. Labeling is absent in the dense aggregated cytoplasm (CYA).

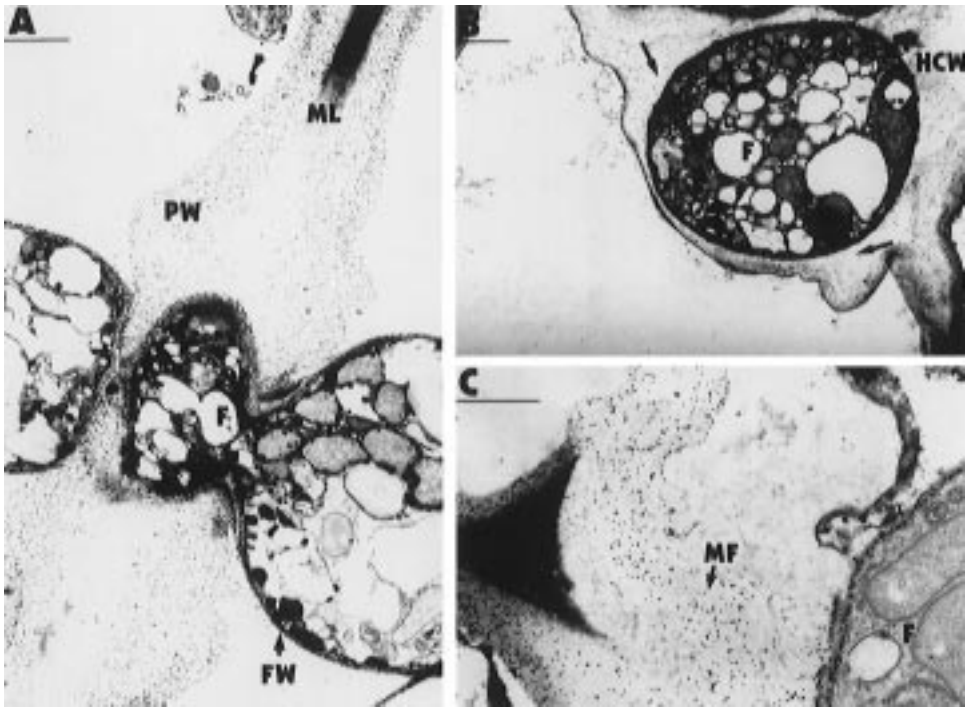

Figure 5. Transmission electron micrographs of carrot root tissues (susceptible cv) inoculated with *Pythium violae*. Sections were treated with exoglucanase-gold complex. A: Gold particles are associated with a swollen primary cell wall (PW) at the site of attempted penetration by a *P. violae* hypha (F). The fungal wall (FW) is uniformly labeled; ML = Middle lamella. Bar = 1 μm. B: Weak labeling (arrow) is observed in host wall (HCW) areas adjacent to the fungal wall (FW). Bar = 1 μm. C: Near the fungus (F) , strong host parietal degradation is characterized by disorganization of the fibrillar structure; gold particles are associated with some microfibrils (MF). The intercellular space (IS) is not labeled. Bar = 0,5 μm.

ganization of host cells, and masses of electron-dense material were found to accumulate in the lumen of the fungal hyphae (Figure 6C). Incubation of sections with the exoglucanase-gold complex resulted in a regular deposition of gold particles in the fungal walls, whereas no accumulation of gold particles was detected in the material accumulating in the lumen of the fungus cell (Figures 6C and D).

Discussion

The present study dealing with the pattern of *P. violae* colonization in carrot root tissues brings new insights into the infection process leading to the formation of cavity spot. Our observations demonstrate that in a susceptible carrot cultivar Nanco, *P. violae* is able to colonize the periderm *via* inter- and intracellular growth through direct and rapid hyphal ingress, as indicated by the substantial number of cell layers that were invaded as soon as 24 h after infection. This property, together with the ability to induce plasmolysis as well as cytoplasm retraction, confirms the necrotrophic nature of *P. violae* as previously reported (Briard, 1990).

Changes in the staining density of the host cell walls as early as 24 h after inoculation, as well as alteration of pectic macromolecules in the middle lamella matrices, revealed after labeling with the AGL-gold complex, indicate that pectin degrading enzymes are produced *in situ*. These observations are in agreement with recent data of Benard and Punja (1995) and Campion (1997) who showed a relationship between the degree of virulence of some *Pythium* strains on carrot and the pectolytic enzyme activity in *in vitro* test conditions. Alteration of the pectin component of the host cell wall, occurring at some distance from the point of fungal ingress, supports the idea that pectin-degrading enzymes may diffuse extracellularly. In addition, fungal infection was frequently associated with swelling of the primary wall. This phenomenon, also observed by Campion et al. (1998) in carrot root tissues infected by *P. violae*, suggests a loosening of the cellulosic framework after degradation of the pectic component and cellulase activity.

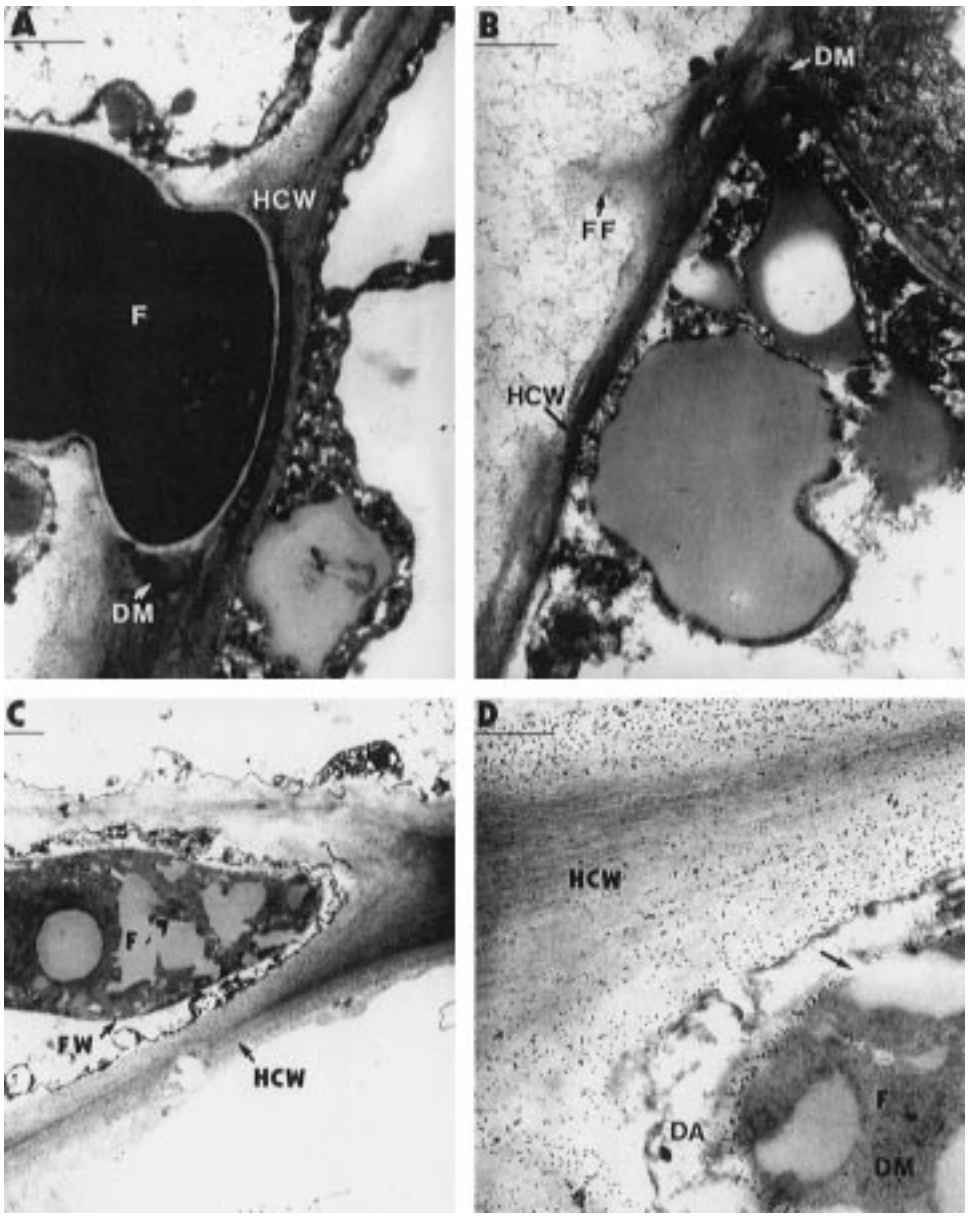

Figure 6. Transmission electron micrographs of carrot root tissues (partially-resistant line) inoculated with *Pythium violae*. Sections were treated with gold complexed probes. Bars = 1 μm. A and B: Labeling with the *Aplysia* gonad lectin-gold complex for localisation of pectic substances. A: Labeling occurs in the host cell walls (HCW), but amorphous and electron-dense material (DM) included in these walls, as well as in adjacent fungal hyphae (F), is unlabeled. B: In tissues heavily infected with fungus, gold particles accumulate mainly in walls (HCW) and poorly in fibrillar fragments (FF) in the cell lumen (arrow). Amorphous and electron-dense compounds (DM) included in the wall are not labeled. C and D: Labeling with the exoglucanase-gold complex. C: Host (HCW) and fungal (FW) cell walls show regular labeling. D: The electron-dense aggregates (DA) between labeled host cell walls (HCW) and modified hyphal content (F) are unlabeled. The gold particle distribution is irregular over the fungal cell wall (arrow), whereas electron-dense material (DM) accumulating in the lumen of the fungal hypha is unlabeled.

Cellulose breakdown was clearly visualized in the primary cell wall, near the *P. violae* hyphae, following incubation with the gold-complexed exoglucanase; moreover, some unlabeled wall areas were detected at a distance from the pathogen, thus indicating that cellulolytic enzymes secreted by *P. violae* were also diffusible, unlike those from *P. ultimum* which remained localized in the channel of penetration, according to Chérif et al. (1991).

Based on our observations, disease development in cultivar Nanco is associated with degradation of the host cell walls by pectin and cellulose-degrading enzymes during the process of pathogen invasion. This assumption confirms and complements previous data reported by Zamski and Peretz (1996) who detected significant increases of cellulase and polygalacturonase activities in carrots soon after inoculation by *P. violae*.

Considering that the structure of the carrot cell wall is composed of a cellulosic skeleton covered with pectic material (Massiot, 1988), it is reasonable to assume that enzymes are sequentially produced by *P. violae,* leading to the typical symptoms of cavity spot. Although studies on kinetics of cell wall degradation would be essential to delineate this sequence in a way similar to that carried out during the infection process of the fungal pathogen *Mycocentrospora acerina* on carrots (Le Cam et al., 1997), one may postulate, in line with the histological description reported by Benard and Punja (1995) and Campion (1997), that the pathogen secretes pectin degrading enzymes which alter the cell wall and contribute to expose the cellulosic matrix. Enzymes able to hydrolyse the cellulose component are then produced, resulting in complete wall breakdown. In the invaded, macerated tissues, only some intercellular spaces seem to remain undamaged, thus leading to formation of an underperidermic empty space ending to cavity spot, after collapse or tear of the superficial suberized tissues.

Macroscopically, the interaction between *P. violae* and the partially-resistant line 24 induced browner and more limited symptoms, compared to the ones occurring in cv Nanco. Histological observations showed that this difference was associated with a spatial restriction of mycelium growth in the superficial tissues. These data, and especially the presence of limited brown lesions, suggest a rapid oxydation of phenolic compounds associated with resistance to cavity spot.

In our study, striking differences in the intensity of cell wall degradation between cv Nanco and line 24 were clearly established. During ingress of *P. violae* in the partially-resistant host plants, the cell walls remained apparently preserved, even when perfored by fungal hyphae. This observation suggests that the enzymes produced by the fungus were not efficient enough to hydrolyse the main wall compounds, likely because the cell walls were reinforced by other substances.

Among the different hypotheses that can be raised to explain this phenomenon, one may suggest that resistance is conferred by a naturally occurring wall architecture, including for example high amounts of protopectin, as suspected for carrot cultivars tolerant to *Mycocentrospora acerina* by Le Cam et al. (1994), or insoluble pectin linked to calcium bridges. Such a possibility has been reported and discussed by Eckert and Raytrayake (1983), who showed that the activity of hydrolytic fungal enzymes decreased in immature fruits with cell walls rich in insoluble pectin associated with calcium. Another possible explanation is that a host response is induced upon pathogen attack resulting in a strenghtening of the host wall structure by the accumulation of specific structural molecules. Support for this hypothesis is provided by the observation that densely stained deposits accumulated around fungal hyphae in the partially-resistant carrot cultivar. These deposits were never observed in the cell walls of cv Nanco. The nature of this material remains to be elucidated, even though according to Scalet et al. (1989), such a material with high electron density may be enriched with phenolic compounds. A similar induced response has been observed by Benhamou (1995) in eggplant cell walls proximal to vessels colonized by *Verticillium albo-atrum* and by Schnitzler and Seitz (1989), who demonstrated that a parietal elicitor of *P. aphanidermatum* was able to trigger a *de novo* synthesis of phenolic compounds in carrot cell walls. Thus, we can hypothesize that the parietal accumulation of this material protects the wall polymers in line 24 from an hydrolytic action of enzymes produced by the pathogen. Similar electron-opaque deposits were frequently infiltrated in the fungal cell walls, or surrounded the invading hyphae that exhibited a noticeable alteration of their cell contents. Similar observations have been previously reported after cytochemical characterization of the defence reactions induced in tomato plants against *Fusarium oxysporum* f.sp. *radicis-lycopersici:* interaction of compounds with the cell walls of fungal hyphae apparently suffering from some damage was associated with a direct fungitoxic activity. These compounds were

identified as phenolic substances after labeling with a gold-complexed laccase (Benhamou et al., 1994).

Another typical feature of the infected line 24 was the occlusion of some infected cells with an amorphous material and the presence of necrotic fungal hyphae within these occluded cells. A similar type of cell occlusion has been observed in cucumber plants after inoculation with *Pythium* F (Rey et al., 1996) or *P. ultimum* (Chérif et al., 1991); but according to these last authors, this plant reaction was expressed too late (at least 3 days after inoculation) to prevent effectively pathogen invasion. In line 24, the role of these newly-formed occlusions remains unknown, although they could contribute in halting or at least in restricting pathogen ingress.

In conclusion, it appears that phenomena located in the plant periderm are preferentially associated with less susceptibility to the pathogen. Our data do not prove that these features, from a physical and/or chemical origin, are really involved in the resistance manifestation, but they allow to assume that they play a role in restricting fungal ingress and enzymatic cell wall degradation in the internal root tissues. These features, either constitutive or induced by the pathogen, appear to be characterized by a very early efficiency. This may explain their ability to halt disease development, in comparison with other *Pythium* expressing defence reactions later (Chérif et al., 1991).

The electron-dense deposits and amorphous material we observed only in the periderm of a partially-resistant carrot line have been noticed previously: after the conclusions of Zamski and Peretz (1995), such compounds may be lignin-like or other phenolic materials, as a consequence of infection by *P. violae*, and able to have a protective function halting the spread of the invader (Vance et al., 1980). Thus, a large field of investigations is open up for elucidating the exact origin and chemical composition of these materials, as well as for analysing their real role in the defence mechanisms of carrot against the pathogen.

Acknowledgements

This work was supported by a grant from the Ministère de la Recherche et de l'Enseignement Supérieur Français, and by the S & G Seeds Company (France). The authors thank S. NOEL (University Laval, Québec, Canada) for his excellent technical assistance and Dr D. C. Harris for correcting the English language of the manuscript.

References

Benard D and Punja ZK (1995) Role of *Pythium* species in cavity spot development on carrots in British Columbia. Can J Plant Pathol 17: 31–45

Benhamou N (1995) Ultrastructural and cytochemical aspects of the response of eggplant parenchyma cells in direct contact with *Verticillium*-infected xylem vessels. Physiol Mol Plant Pathol 46: 321–338

Benhamou N, Chamberland H, Ouellette GB and Pauze FJ (1987) Ultrastructural localization of β-(1,4)-D-glucans in two pathogenic fungi and in their host tissues by means of an exoglucanase-gold complex. Can J Microbiol 33: 405–417

Benhamou N, Gilboa-Garber N, Trudel J and Asselin A (1988) A new lectin-gold complex for ultrastructural localization of galacturonic acids. J Histochem Cytochem 36: 1403–1411

Benhamou N, Lafontaine PJ and Nicole M (1994) Induction of systemic resistance to *Fusarium* crown and root rot in tomato plants by seed treatment with chitosan. Phytopathology 84: 1432–1444

Breton D and Rouxel F (1993) Données récentes sur le cavity-spot de la carotte en France. Acta Horticulturae 354: 159–170

Briard M (1990) Etude comparée de *Phytophthora megasperma* et *Pythium violae* et de leurs relations avec la plante dans l'expression des symptômes de bague et de cavity-spot sur carotte (*Daucus carota*). PhD thesis, Université de Rennes I, 119 pp

Campion C (1997) Etude comparée des processus infectieux de *Pythium violae*, *Pythium sulcatum* et *Pythium ultimum* chez *Daucus carota* L.. Rôles des enzymes fongiques et de la paroi végétale dans l'expression du cavity spot. PhD thesis, Université de Rennes I, 142 pp

Campion C, Massiot P and Rouxel F (1997) Aggressiveness and production of cell-wall degrading enzymes by *Pythium violae*, *Pythium sulcatum* and *Pythium ultimum*, responsible for cavity spot on carrots. Eur J Plant Pathol 103: 725–735

Campion C, Vian B, Nicole M and Rouxel F (1998) A comparative study of carrot root tissue colonization and cell wall degradation by *Pythium violae* and *Pythium ultimum*, two pathogens responsible for cavity spot. Can J Microbiol 44: 7–16

Chérif M, Benhamou N and Bélanger RR (1991) Ultrastructural and cytochemical studies of fungal development and host reactions in cucumber plants infected by *Pythium ultimum*. Physiol Mol Plant Pathol 39: 353–375

Eckert WJ and Raytrayake M (1983) Host pathogen interactions in postharvest disease. Postharvest Physiology and Crop Preservation 46: 247–261

El-Tarabily KA, Hardy GE and Sivasithamparam K (1996) Association of *Pythium coloratum* and *Pythium sulcatum* with cavity spot disease of carrots in Western Australia. Plant Pathol 45: 727–735

Endo RM and Colt WM (1974) Anatomy, cytology and physiology of infection by *Pythium*. Proc Am Phytopathol Soc 1: 215–223

Frens G (1973) Controlled nucleation for regulation of the particle size in monodisperse gold solutions. Nature (London) Physical Sciences 241: 20–22

Gilboa-Garber N, Susswein AJ, Mirashi L and Avichezer D (1985) Purification and characterization of gonad lectin of *Aplysia depilans*. FEBS Letter: 181–276

Gladders P and McPherson GM (1986) Control of cavity spot in carrots with fungicides. Ann Appl Biol 12: 223–233

Groom MR and Perry DA (1985) Induction of 'cavity spot like' lesions on root of *Daucus carota* by *Pythium violae*. Trans Brit Mycol Soc 84: 755–757

Guérin L (1993) Analyse de la diversité des *Pythium* spp. impliqués dans le cavity spot de la carotte et étude des relations hôte-parasite en vue de la sélection variétale pour la résistance. PhD thesis, Université de Rennes I, 101pp

Guérin L, Briard M and Rouxel F (1994) Biochemical characterization of *Pythium* spp. involved in cavity spot of carrots in France. Ann Appl Biol 125: 255–265

Jordan CM, Jordan LS and Endo RM (1989) Association of phenol-containing structures with *Apium graveolens* and resistance to *Fusarium oxysporum* f. sp. *apii* race 2. Can J Bot 67: 3153–3163

Le Cam B, Massiot P, Campion C and Rouxel F (1994) Susceptibility of carrot cultivars to *Mycocentrospora acerina* and the structure of cell wall polysaccharides. Physiol Mol Plant Pathol 46: 321–338

Le Cam B, Lebreton L, Massiot P and Rouxel F (1997) Production of cell wall polysaccharide enzymes in carrot root tissues infected by *Mycocentrospora acerina*. Plant Pathol 46: 276–281

Massiot P (1988) Caractérisation structurale et dégradation enzymatique des polysaccharides de parois cellulaires de la racine de carotte (*Daucus carota* L.). PhD thesis, Université de Rennes, 142 pp

Montfort F and Rouxel F (1986) La maladie de la « Tache » de la carotte. Phytoma. Défense des cultures 376: 43–45

Montfort F and Rouxel F (1988) La maladie de la « Tache » de la carotte due à *Pythium violae* Chester & Hickman: Données symptomatologiques et étiologiques. Agronomie 8: 701–706

Perry DA and Harrison JG (1979) Cavity-spot of carrot. I. Symptomatology and calcium involvement. Ann Appl Biol 93: 101–108

Rey P, Benhamou N and Tirilly Y (1996) Ultrastructural and cytochemical studies of cucumber roots infected by two *Pythium* species with different modes of pathogenicity. Physiol Mol Plant Pathol 49: 213–231

Scalet M, Crivaletto E and Mallardi F (1989) Demonstration of phenolic compounds in plant tissues by an osmium-iodide post-fixation procedure. Stain Technol. 64: 273–290

Schnitzler JP and Seitz HU (1989) Rapid responses of cultured carrot cells and protoplasts to an elicitor from the cell wall of *Pythium aphanidermatum* (Edson) Fitzp Z Naturforsch Teil C 44: 1020–1028

Vance CP, Kirk TK and Sherwood RT (1980) Lignification as a mechanism of disease resistance. Ann Rev Phytopathol 18: 259–288

Vivoda E, Davis RM, Nunez JJ and Guerard JP (1991) Factors affecting the development of cavity-spot of carrot. Plant Dis 75: 519–522

White JG (1984) Association of *Pythium* and*Phytophthora* spp. with cavity spot. Annual Rep NVRS Wellesbourne: 80 and 81

White JG (1986) The association of *Pythium* spp. with cavity-spot and root dieback of carrots. Ann Appl Biol 108: 265–273

White JG (1988) Studies on the biology and control of cavity-spot of carrots. Ann Appl Biol 113: 259–268

Zamski E and Peretz I (1995) Cavity spot of carrots: Interactions between the host and pathogen, related to the cell wall. Ann Appl Biol 127: 23–32

Zamski E and Peretz I (1996) Cavity spot of carrots: Cell-wall-degrading enzymes secreted by *Pythium* and pathogen-related proteins produced by the root cells. Ann Appl Biol 128: 195–207