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Histological and Ultrastructural Study of Susceptible and Age-related Resistance Responses of Pepper Leaves to *Colletotrichum coccodes* Infection

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Infection of pepper leaves by *Colletotrichum coccodes* at the two- and eight-leaf stages caused susceptible and resistant lesions 96 h after inoculation, respectively. At the two-leaf stage, progressive symptom development occurred on the infected leaves. In contrast, localized necrotic spots were characteristic symptoms at the eight-leaf stage. Infected leaves at the two-leaf stage exhibited cell death accompanied by the accumulation of autofluorescent compounds. At the eight-leaf stage, pepper leaves infected by the anthracnose fungus displayed localized autofluorescence from the symptoms. Infection of pepper leaves by *C. coccodes* at the two-leaf stage resulted in its rapid and massive colonization of all the leaf tissues including the vascular tissue, together with cytoplasmic collapse, distortion of chloroplasts, and disruption of host cell walls. However, penetration of *C. coccodes* was very limited in the older leaf tissues of pepper plants at the eight-leaf stage. Fungal hyphae grew only in the intramural spaces of the epidermal cell walls at this stage. Occlusion of amorphous material in xylem vessels, aggregation of fibrillar material in intercellular spaces, and deposition of protein bodies were found as resistance responses to *C. coccodes*.

Keywords **:** age-related resistance, autofluorescence, *Capsicum annuum*, *Colletotrichum coccodes*, infection process.

Pepper leaf tissues of different plant ages respond to *Colletotrichum coccodes* infection by forming lesions of different types (Hong and Hwang, 1998). Expression of these different lesions was found to be influenced by wetness duration and inoculum concentration. In most cases, necrotic, resistant lesions were expressed in plants in a resistance gene-dependent manner. Several studies on differential lesion formation have been done on susceptible and resistant responses of host plants based on the gene-for-gene principle (Skipp and Deverall, 1972; Churchill et al., 1988). Hypersensitive cell death induced by biotic or abiotic elicitors has also been known as a marker of resistance (Ostazeski and Elgin, 1981; Ostazeski and Elgin, 1984; Kováts et al., 1991). Recently, studies of 'disease lesion mimic mutants' which produced spontaneous lesions similar to the lesions caused by pathogen infection have suggested the significance of lesion types for the understanding of plant resistance mechanisms in rice, barley, maize, and *Arabidopsis* (Hoisongton et al., 1982; Wolter et al., 1993; Dietrich et al., 1994; Weymann et al., 1995; Takahashi et al., 1999).

The formation of different types of lesions was found to depend on host developmental status in some plants infected by *Colleotrichum* spp. (Griffey and Leach, 1965; Marks et al., 1965; Landes and Hoffmann, 1979; Pring et al., 1995). Several histological studies have been conducted to help to understand host age-dependent resistance to the anthracnose fungus (Griffey and Leach, 1965; Marks et al., 1965; Landes and Hoffmann, 1979; Pring et al., 1995). Small necrotic lesions on the old hypocotyl tissues of susceptible bean cultivars, which were produced by *C. linthemuthianum* infection, resembled the small hypersensitive lesions on the young inoculated tissues of resistant cultivars (Griffey and Leach, 1965). Marks et al. (1965) found direct penetration of young susceptible leaves of *Populus tremuloides* by *C. gloeosporioides*, as compared with abortive penetration attempts on mature resistant leaves. Reduced penetration of *C. linthemuthianum* was markedly influenced by the age of the epidermal cells of *Phaseolus vulgaris* (Landes and Hoffmann, 1979). Disease development on younger cowpea hypocotyls infected by *C. capsici* was more extensive than that on older ones (Pring et al., 1995). These studies indicate that symptoms developed in old host tissue resembled ones in resistant host plants. In our earlier observations, age-related resistance in pepper plants was a distinct phenomenon against *Phytophthora* and *Colletotrichum* diseases (Kim et al., 1989; Hwang and Kim, 1990; Hong and Hwang, 1998). The susceptibility of pepper plants to these diseases was affected by plant growth stage. Interestingly, resistance factors against *C. coccodes* infec-

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tion seemed to induce lesions of different types on the infected leaves of mature pepper plants.

Accumulation of autofluorescent material in the host tissue has been demonstrated as a cytological hallmark of cell death by pathogen attack (Koga et al., 1988; Lummerzheim et al., 1993; Koga, 1994; Hückelhoven et al., 1999). Whether the autofluorescence may be related to the early events leading to host cell death or a result of cell death remained to be elucidated. A higher amount of autofluorescence, however, has often been detected in a 'resistant host', 'accelerated cell death mutant' or 'disease lesion mimic mutant' (Lummerzheim et al., 1993; Dietrich et al., 1994; Greenberg et al., 1994; Dai et al., 1995). Autofluorescence observed by UV excitation has been known to originate from phenolic compounds in plants (Lummerzheim et al., 1993). These findings indicated that the host cell death could be visualized by autofluorescence of the phenolics in leaf tissues infected by *Colletotrichum* spp.

Pepper plants, which were severely damaged by *C. coccodes* infection at the seedling stage became resistant as they matured (Hong and Hwang, 1998). In the present study, we initiated the observation of the cellular aspects of pathogenesis following the development of *C. coccodes* in pepper leaf tissues at different growth stages. The histological and ultrastructural study would provide a clue for the understanding of the age-related resistance of pepper plants to the anthracnose fungus.

Materials and Methods

Plant, fungus and inoculation. Pepper seedlings (*Capsicum annuum* L., cv. Hanbyul) were grown in plastic pots (5 × 15 × 10 cm) containing a steam-sterilized compost soil mixture, sand, and loam soil (1 : 1 : 1, v/v/v). Pepper plants at the two- and eight-leaf stages were raised in a growth room at 27 ± 2°C, with 4,000 lux illumination for 16 h per day.

Isolate 2-25 of *C. coccodes* was maintained on potato-dextrose agar (PDA). Conidial suspension was obtained from the 5 day-old cultures on oatmeal agar plates, as described previously (Hong and Hwang, 1998). The inoculum concentration was adjusted to 1 × 10^6 conidia ml^{-1} with sterile water. Pepper leaves were inoculated by pipetting two 8-μl droplets of the conidial suspension containing 0.05% Tween 20 on the adaxial surface. Inoculated pepper plants were then placed in a moist chamber at 98% relative humidity for 36 h, followed by further incubation at 28 ± 2°C. At 96 h after inoculation, pieces of infected leaf tissue were sliced with a razor blade and immediately fixed in a solution containing formaldehyde and glutaraldehyde.

Light and fluorescence microscopy. To observe the infection processes of *C. coccodes* in pepper leaf tissue, semi-thin sections (1.0-1.5 μm) of the prepared LR-White resin-embedded material were mounted on poly-L-lysine coated glass slides. Sections were stained with 0.0005% (w/v) 4,6-diamidine-2-phenylindole dihydrochloride (DAPI, Boehringer Mannheim) in phosphate-buffered saline (PBS) for 5 min. After rinsing twice with PBS, the sections were mounted in 50% glycerol.

To detect autofluorescent materials in the leaf tissues, pepper leaves infected by *C. coccodes* were fixed and cleared following the modified procedures described previously (Celio and Hausbeck, 1998). Leaf tissues were fixed with 37% formaldehyde : ethanol : acetic acid (1 : 18 : 1, v/v/v) for 2 h, cleared with chloral hydrate-containing ethanol for 48 h, and then mounted in glycerol.

A fluorescence microscope (BH2-RFC, Olympus, Japan) equipped with a 'U' exciter cube/filter was used to observe the infected leaf cells by ultraviolet epifluorescence (excitation at 334 to 365 nm). Photographs were taken with Kodak 400 ASA film.

Electron microscopy. Pepper leaf tissues infected by *C. coccodes* were cut into small pieces (5 mm in length). The leaf segments were fixed in a mixture of 1% (v/v) formaldehyde and 0.025% glutaraldehyde in 0.01 M phosphate-buffered saline (PBS) (pH 7.2) overnight at 4°C, after vacuum infiltration for 10 min. After rinsing several times with PBS for 5 h, the tissues were dehydrated in a graded ethanol series. The tissues were infiltrated with LR-White acrylic resin (The London Resin Co., UK) containing 0.5%(w/v) benzoin methyl ether (Fluka) in a series of ethanol : LR White mixtures of 2 : 1, 1 : 1, 1 : 2, and pure LR White resin, for 12 h each (Pain et al., 1994). The tissues were then polymerized in the fresh resin-containing gelatin capsules by UV light irradiation (360 nm) at -20°C for 48 h. Ultrathin sections (< 90 nm) were prepared from various pepper leaf tissues using a Diatome diamond knife on an ultramicrotome (LKB 2088 ultratome® V, Sweden). The sections were mounted on 75- or 150-mesh copper grids coated with piloform. The grids were stained with 8% uranyl acetate for 10 min, followed by treatment with Reynold's lead citrate for 10 min (Reynolds, 1963) The stained sections were examined with a JEOL 1200 EX transmission electron microscope operated at 80 kV.

Results

Differential responses of pepper plants to *C. coccodes* infection. Pepper leaves at different growth stages responded to *C. coccodes* infection by forming different lesion types. Different responses occurred in pepper leaves after inoculation of *C. coccodes* at the two-and eight-leaf stages (Fig. 1). At 96 h after inoculation, leaf tissues at the two-leaf stage were severely damaged and blighted (Fig. 1A). Cell walls of the epidermis and mesophyll were disrupted by *C. coccodes* infection. Xylem vessels and phloem cells in the main or minor leaf veins were severely collapsed. In contrast to the response of younger pepper plants to the fungal infection, limited necrotic lesions appeared on old leaves at the eight-leaf stage. Sites infected by the fungus were collapsed and withered, which formed irregular lesions of 1 to 4 mm in diameter. The localized area of spot lesion accompanying restricted margin at the symptom was observed in the transverse section of the leaf tissue at the

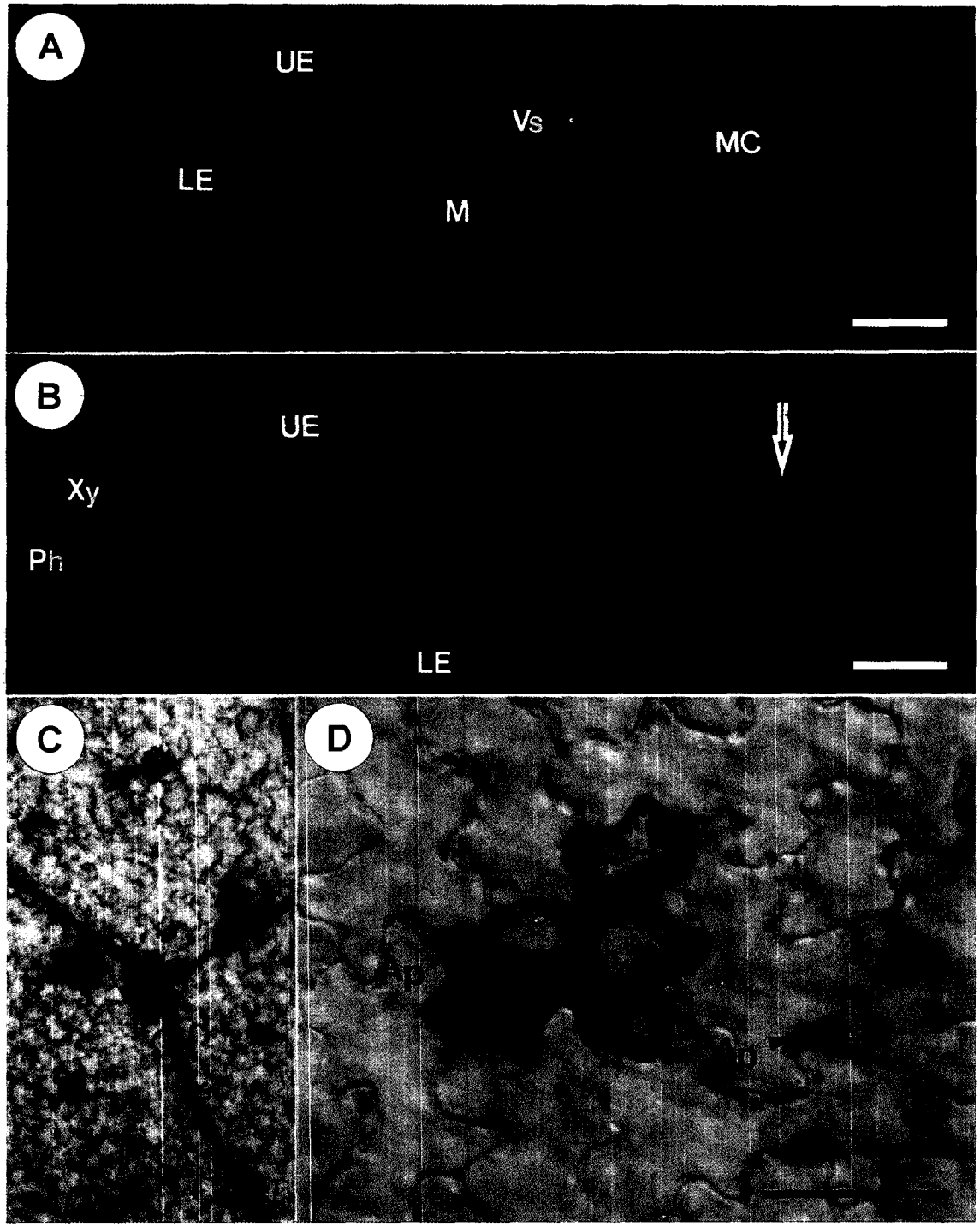

Fig. 1. Fluorescence and light micrographs of transverse sections of pepper (cv. Hanbyul) leaves infected by *Colletotrichum coccodes* 96 h after inoculation at the two- and eight-leaf stages. (**A**) At the two-leaf stage, systemically spreading lesions of the anthracnose disease occurred on the infected pepper leaves. Upper and lower epidermal and mesophyll cells were damaged. The cytoplasm was aggregated, and vascular bundles collapsed. (**B**) At the eight-leaf stage, necrotic leaf spots were sparsely localized on some leaf tissues. The collapsed areas are seen in the restricted necrotic spot lesions (arrow), but other leaf tissues remained in normal structure. 4,6-Diamidine-2-phenylindole dihydrochloride (DAPI) was used for staining plant nucleus and structures. (**C**) Localized single dead cells caused by *C. coccodes* infection on the pepper leaves at the eight-leaf stage are visible with a dark brown color. (**D**) High magnification of a single necrotic epidermal cell invaded by the pigmented mature appressoria. Some appressoria attached themselves to the host epidermal cells (arrowheads). Ap=appressorium, LE=lower epidermis, M=mid vein, MC=mesophyll cell, Ph=phloem, UE=upper epidermis, Vs=vascular bundle, Xy=xylem. Bar=50 μm.

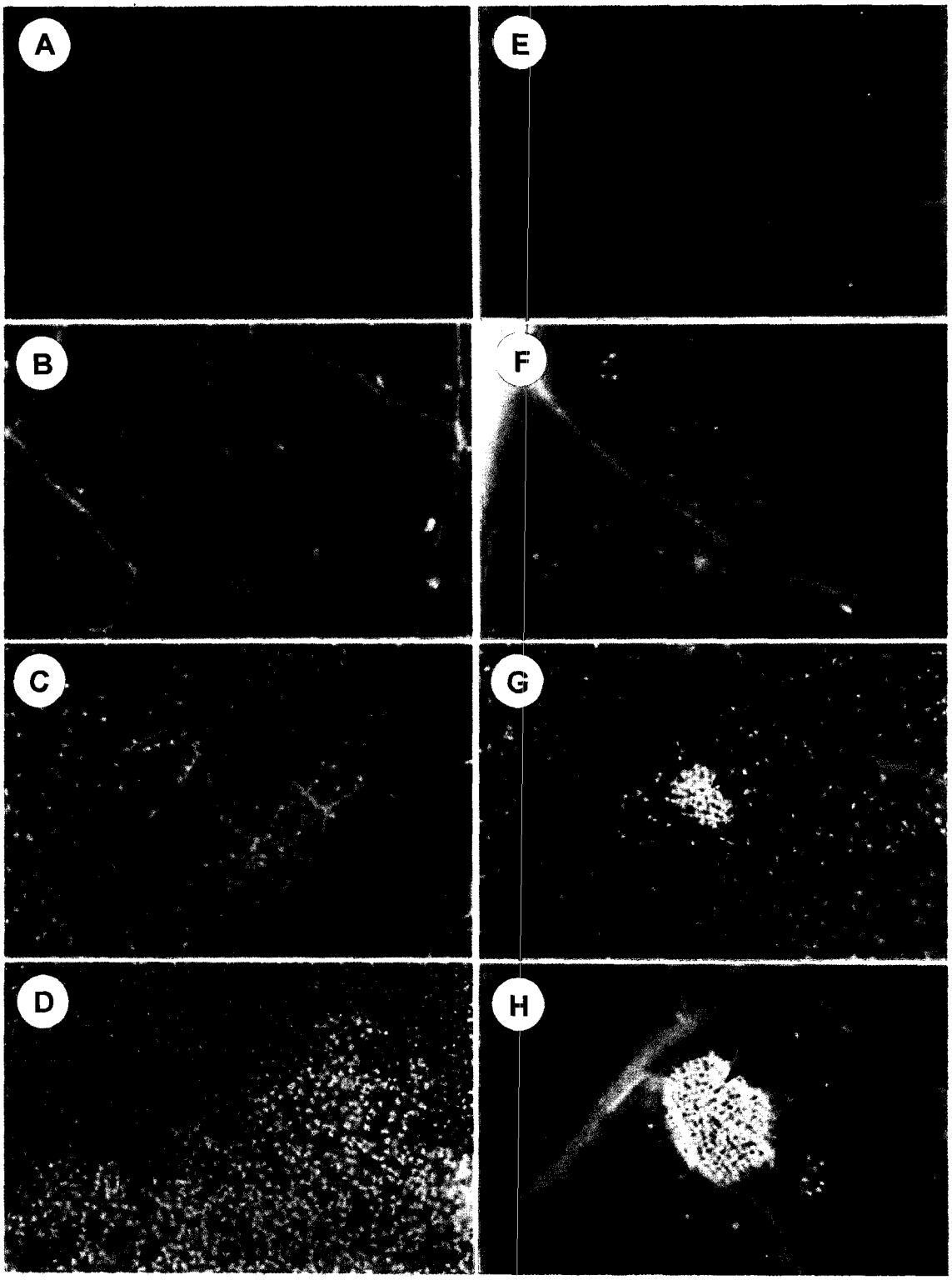

Fig. 2. Accumulation of autofluorescent materials in the leaf tissues of pepper plants at different time intervals after inoculation with *Colletotrichum coccodes* at the two- and eight-leaf stages. (**A**) to (**D**), leaf tissues at the two-leaf stage. (**E**) to (**H**), leaf tissues at the eight-leaf stage. (**A**) and (**E**), healthy leaf tissues. (**B**) and (**F**), leaf tissues 24 h after inoculation. (**C**) and (**G**), leaf tissues 48 h after inoculation. (**D**) and (**H**), leaf tissues 72 h after inoculation, Autofluorescent materials in infected leaf tissue are seen by illumination with UV light. Bright yellow fluorescence was visualized by the UV-illumination. Autofluorescence was emitted from the visible symptoms (arrows) or single dead cells (arrowheads) on pepper leaves. Bar=100 μm.

eight-leaf stage (Fig. 1B). Chloroplasts and cell walls within the tissues of symptomless areas have well conserved structure. Xylem vessels and phloem cells of leaf tissues remained in normal form. A necrotic spot surrounded by a dark brown margin were found on the infected leaves at 72 h after inoculation at the eight-leaf stage. Single cell death in the epidermis was observed near the localized spot lesions. Pigmented mature appressoria were produced over the junctions of the anticlinal cell walls between adjacent epidermal cells. Some appressoria penetrated into the host tissue and the infected cells became necrotic, whereas some epidermal cells were not invaded by the mature appressoria (Fig. 1C and 1D). Infection by appressorium and necrosis of host tissues were more frequently observed in young leaf tissues at two-leaf stage (figure not shown).

Differential responses to *C. coccodes* infection were analyzed by fluorescence microscopy. Cleared whole leaf tissues at the two- and eight-leaf stages were observed using ultraviolet epifluorescence (Fig. 2). Specific yellow autofluorescnece materials, which were not detected in healthy leaves (Fig. 2A), were found in the leaf tissues infected by *C. coccodes* at the two-leaf stage. At this stage, death of single cells emitting autofluorescence was observed at 24 h after inoculation (Fig. 2B). The number of autofluorescent cells had drastically increased 48 h after inoculation (Fig. 2C). Lesions were enlarged and coalesced 72 h after inoculation, while most of the cells emitted autofluorescence (Fig. 2D). At the eight-leaf stage, differential host reactions were expressed in terms of different types of autofluorescence. At 24 h after inoculation, a few autofluorescent cells were detected at this stage, which was similar to that at the two-leaf stage (Fig. 2F). Some aggregates of autofluorescence were more or less enlarged at 48 h after inoculation (Fig. 2G). Most of the autofluorescence from single cell death were scattered near the aggregates of autofluorescence 72 h after inoculation (Fig. 2H).

Ultrastructure of healthy leaf tissues. Electron micrographs of transverse sections of healthy leaf tissues of pep-

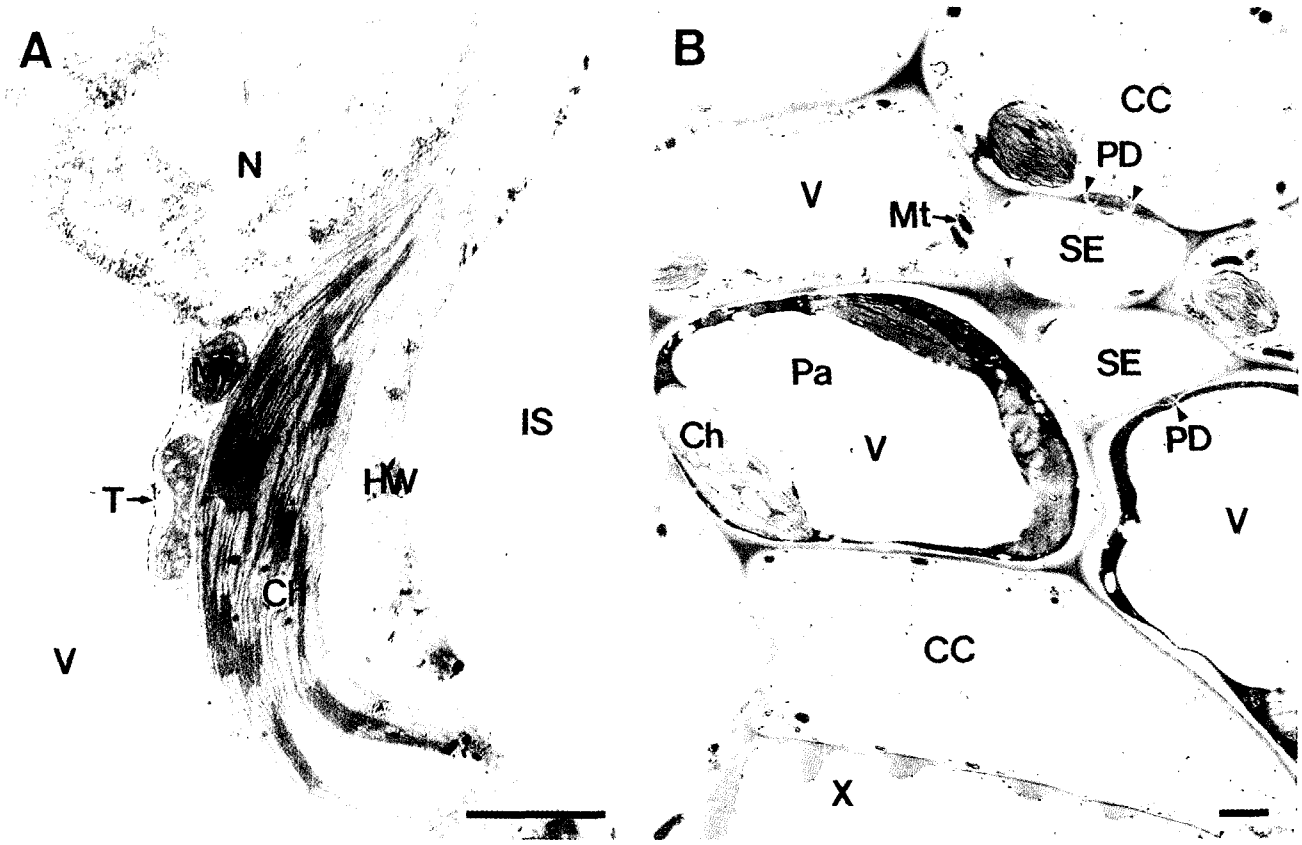

Fig. 3. Transmission electron micrographs of healthy pepper (cv. Hanbyul) leaf tissues at the two- and eight-leaf stages. (**A**) Ultrastructure of leaf mesophyll cell at the two-leaf stage. Plant cell wall is seen in normal structure. Note the nucleus, chloroplasts, and mitochondria in the cytoplasm surrounded by plasma membrane. (**B**) Ultrastructure of vascular bundles of leaf tissues at the eight-leaf stage. Xylem vessels are surrounded by parenchyma cells and companion cells. Note the plamodesmata (arrowheads) at the cell wall between companion cell and sieve element. CC=companion cell, Ch=chloroplast, Cy=host cytoplasm, Ep=epidermal cell wall. FC=fungal cell, FW=fungal cell wall, HW=host cell wall, IS=intercellular space, Mt=mitochondria, N=nucleous, Pa=parenchyma cell, PD=plasmodesmata, S=fungal septum, SE=sieve element, T=tonoplast, V=vacuole, X=xylem vessel. Bar=1 μm.

per plants at the two- and eight-leaf stages are presented in Figure 3. Nucleus, mitochondria and chloroplast were present in normal structures within the cytoplasm of parenchyma cell which was well surrounded by the plasma membrane (Fig. 3A). The sieve elements, and companion cells adjacent to the parenchyma cells were observed in the phloem structure of vascular bundles of leaf tissues at eight-leaf stage (Fig. 3B). Xylem vessel was also shown in close contact with a companion cell. Plasmodesmata were observed at the upper wall of sieve element.

Ultrastructure of fungal hyphae and infection sites at the two-leaf stage. At 96 h after the inoculation of *C. coccodes*, fungal hyphae were examined in leaf tissues at the two-leaf stage (Fig. 4). The early events of *C. coccodes* infection from 24 to 72 h after inoculation were not observed under the electron microscope. Fungal colonization rapidly progressed in leaf tissues at this stage, and long hypha was found in the infected leaf tissues (Fig. 4A). Fungal cells, which were highly vacuolated, were compartmentalized by septa between adjacent fungal cells. Epidermal and mesophyll cells of the leaf tissues were severely damaged and distorted. Distorted chloroplasts which had small

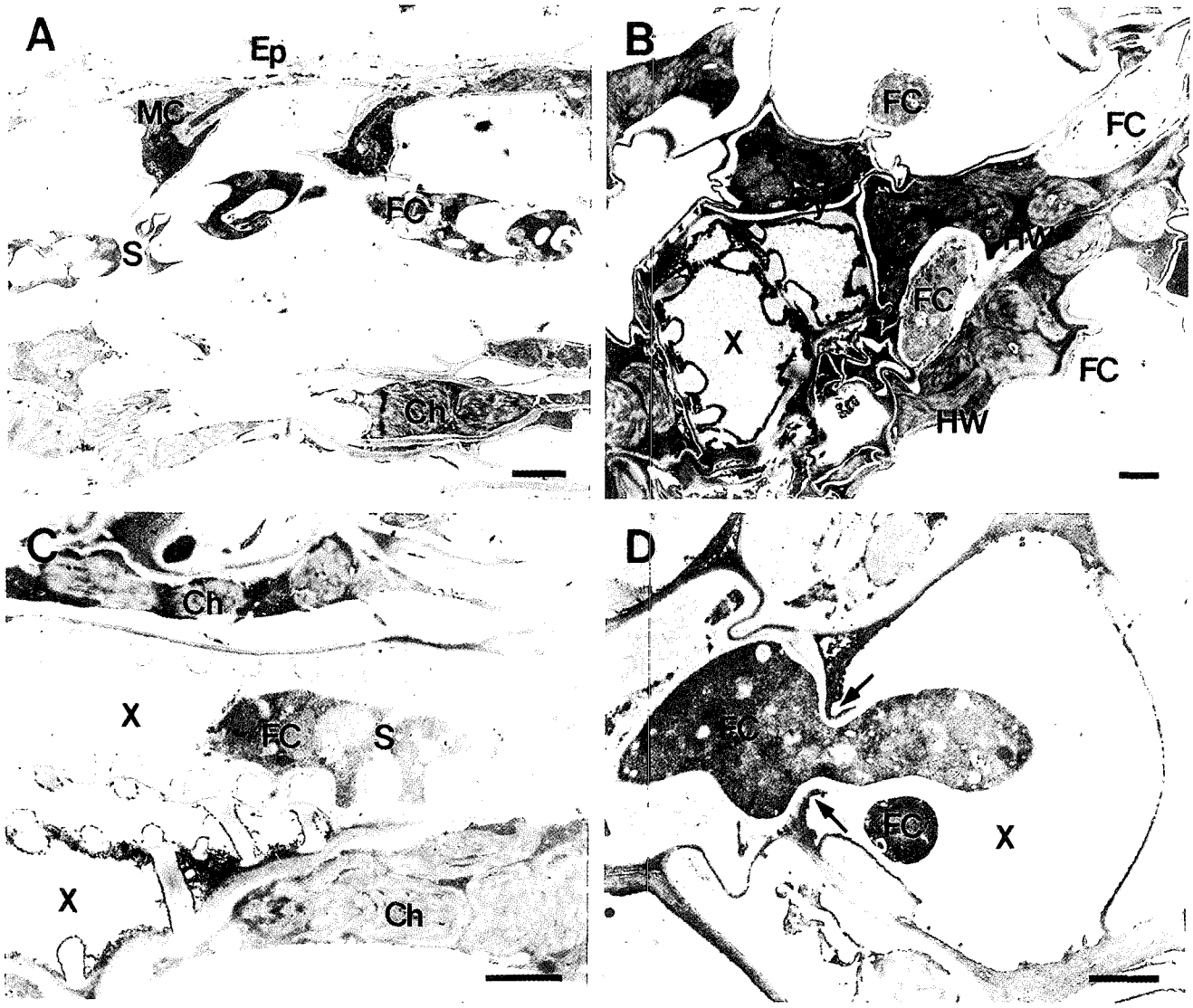

Fig. 4. Transmission electron micrographs of fungal structures in pepper (cv. Hanbyul) leaf tissues 96 h after inoculation with *Colletotrichum coccodes* at the two-leaf stage. (**A**) Extensive fungal growth in the mesophyll cells of the leaf tissue. Note the vacuolization of the fungal hyphae with the septum in the leaf tissues. (**B**) Fungal cells colonized near the xylem cells. Fibrous materials accumulated within the xylem vessel. Malformed chloroplasts were found in the dense cytoplasm. (**C**) Fungal hypha growing within xylem vessel. (**D**) Fungal hypha penetrating the xylem vessels. Note constrictions (arrows) of hypha passing from the host cell into xylem vessel. Ch=chloroplast, Cy=host cytoplasm, Ep=epidermal cell wall. FC=fungal cell, FW=fungal cell wall, HW=host cell wall, S=fungal septum, X=xylem vessel. Bar=2 μm.

starch granules were present within the collapsed mesophyll cell. Fungal cells were found near the xylem vessel, and the collapsed chloroplasts were located in an electron-dense cytoplasm (Fig. 4B). Fungal cells were present in the intercellular spaces of the leaf tissues. Host cell walls in contact with the fungal cells were degraded and thinned. Some fibrous materials occurred faintly in the inner side of the xylem vessel. Septated fungal hypha growing in the xylem vessel became dark-stained with some small vacuoles (Fig. 4C). Host cells were heavily damaged by fungal infection. Some disorganized chloroplasts aggregated around the host cell wall in contact with the xylem vessels. Fungal hypha penetrated into the xylem vessel. The remnants caused by the mechanical burst of xylem vessel wall were found on both sides of the fungal penetrating channel (Fig. 4D). Constriction of the penetrating hypha occurred at the point of intimate contact with the xylem vessel wall.

Various fungal structures of *C. coccodes* penetrating into the leaf tissues were observed 96 h after inoculation at the two-leaf stage (Fig. 5). The fungal cell intimately in contact with the host cell wall is shown in the intercellular space of the host cells. The cytoplasm and cell organelles of the host

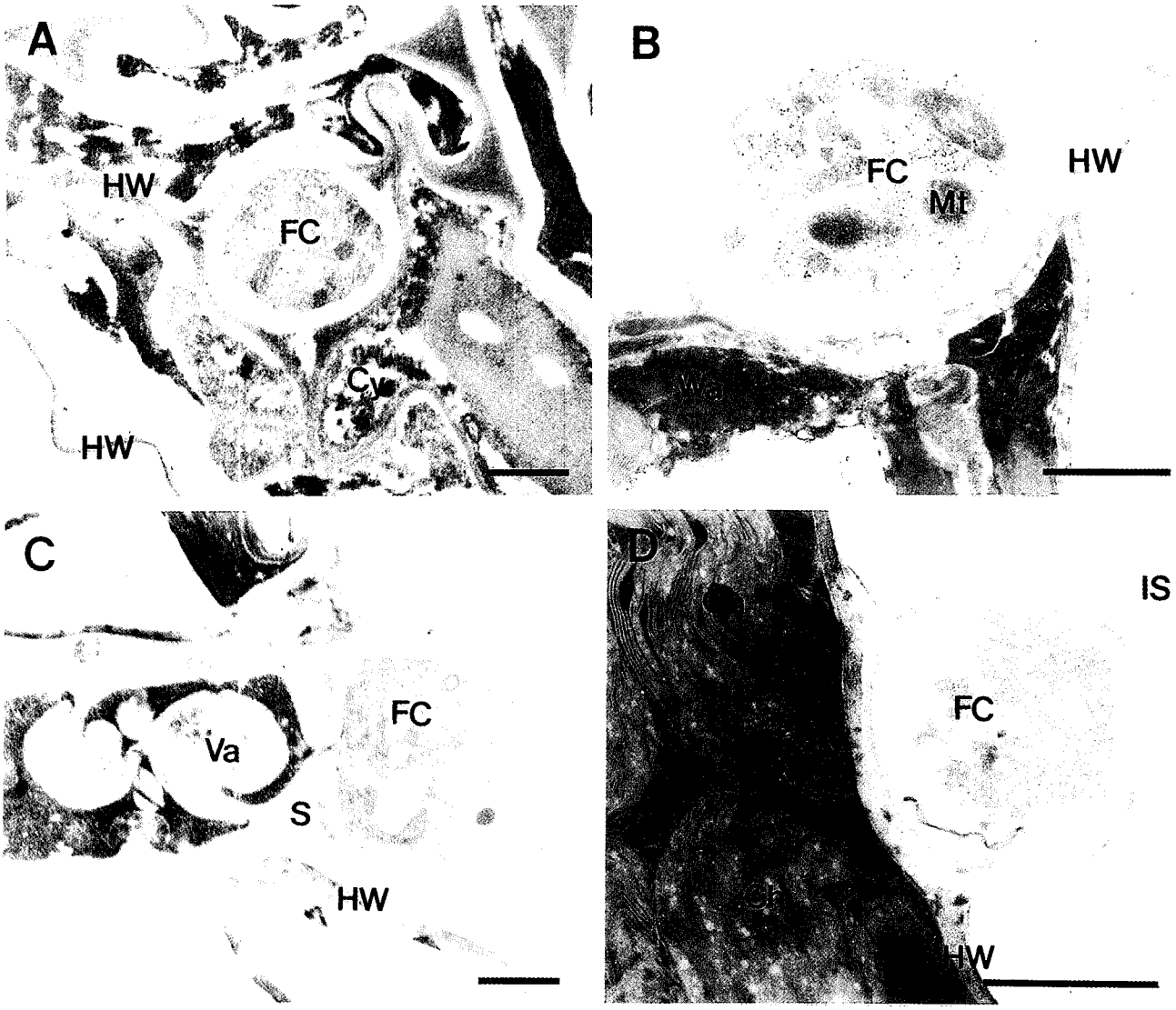

Fig. 5. Transmission electron micrographs of fungal structures penetrating into pepper (cv. Hanbyul) leaf tissues 96 h after inoculation with *Colletotrichum coccodes* at the two-leaf stage (**A**) The intercellular fungal cell intimately contacted with the host walls. Alterations of the host cytoplasm is seen. (**B**) The fungal cell formed a penetration peg-like structure within the leaf tissue for the penetration to another host cell. (**C**) Septum formation in a fungal cell in leaf tissue. A septal pore is seen. Note the vacuolization in the fungal cell. (**D**) The electron-lucent host cell wall in intimate contact with a fungal cell. Malformed chloroplasts are seen in the host cells. Ch=chloroplast, Cy=host cytoplasm, FC=fungal cell, HW=host cell wall, IS=intercellular spaces, Mt=fungal mitochondria, S=septum, Va=fungal vacuole. Bar=1 μm.

parenchyma cells were disorganized, usually with irregular and indistinct structures. A cell wall apposition occurred below the host cell walls intimately in contact with the fungal cell (Fig. 5B). The peg-like penetration structure was surrounded by the electron-dense wall apposition. In particular, numerous vacuolated areas and a distinct septum were visible in the fungal hypha growing in the damaged host cells (Fig. 5C). Host cell walls in close contact with the intercellular fungal hypha became electron-lucent, and many malformed chloroplasts developed in host cells (Fig. 5D).

Ultrastructure of fungal hyphae and infection sites at the eight-leaf stage. At 72 h after inoculation of *C. coccodes* at the eight-leaf stage, visible symptoms began to occur on the leaves. Distinct and restricted necrotic small lesions of 1 to 4 mm in diameter appeared at 96 h after inoculation. At the margin of the limited lesion, fungal cells were arrested and grew in the intramural space of the epidermal cell wall (Fig. 6A). In the infected epidermal host cell, central vacuoles contained amorphous aggregates. Intercellular spaces of epidermal and mesophyll cells were filled with fibrillar materials. After 96 h of inoculation, some hyphae were found in the intercellular spaces. However, most of the fungal cells were arrested at the epidermal

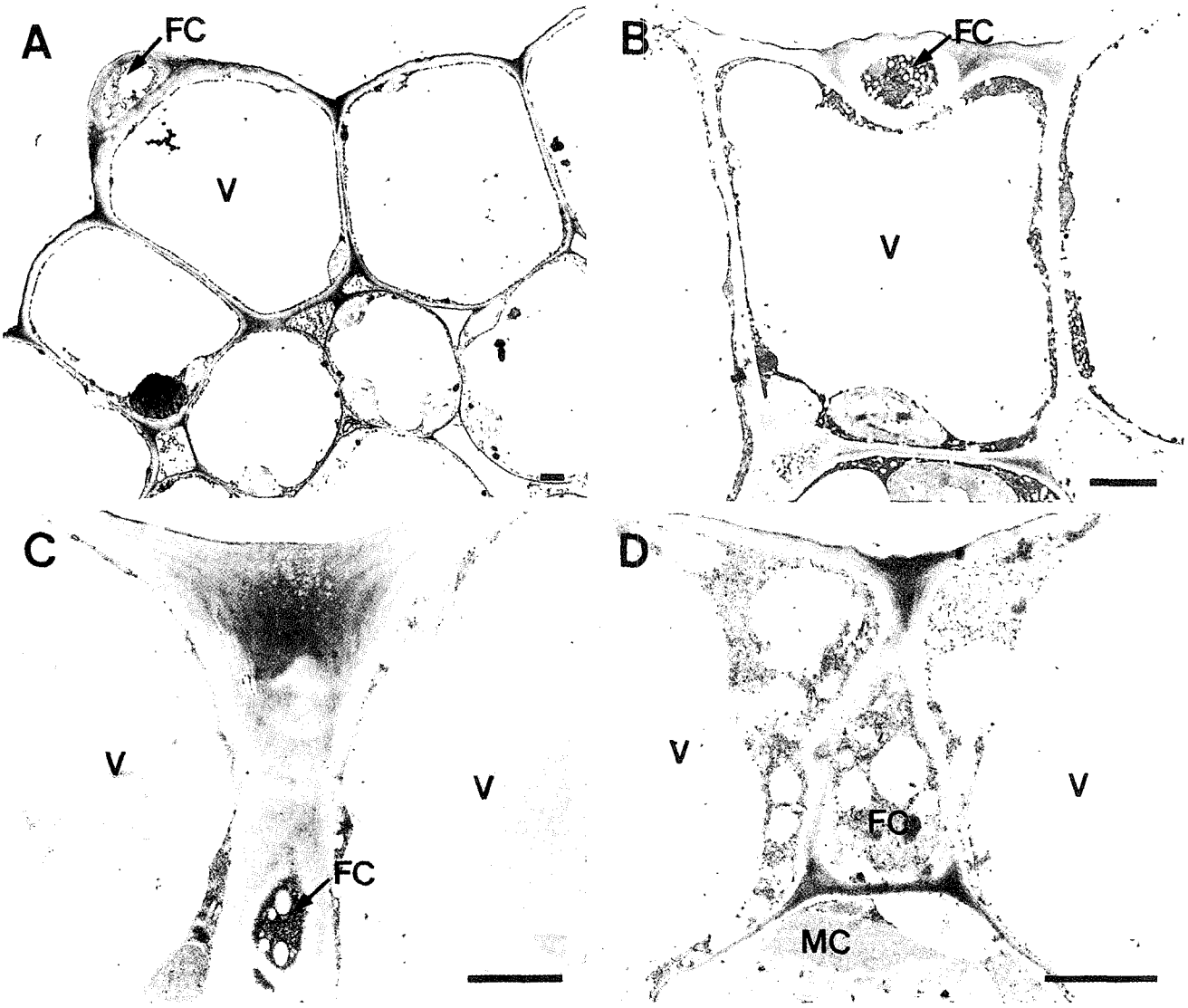

Fig. 6. Transmission electron micrographs of fungal hypha growing within an epidermal cell wall in pepper (cv. Hanbyul) leaf tissue 96 h after inoculation with *Colletotrichum coccodes* at the eight-leaf-stage. (**A**) and (**B**) Fungal hypha growing within the epidermal cell wall. (**C**) Fungal hypha penetrating through the intermural space of the host cell wall. (**D**) A fungal cell within the host cell wall. Numerous small vacuoles are present in the cytoplasm of epidermal cell. EC=epidermal cell, EW=epidermal cell wall, MC=mesophyll cell, V=plant vacuole. Bar=2 μm.

layer (Fig. 6B). Fungal hypha grew in the epidermal cell wall (Fig. 6C). Central and small vacuoles were located in the epidermal cells (Fig. 6D). The penetrating fungal hypha was inserted into the epidermal cell wall.

Distinct host responses to *C. coccodes* infection were found in the leaf tissues of pepper plants at the eight-leaf stage (Fig. 7). However, undamaged nuclei, chloroplasts, and cytoplasm in the mesophyll cells remained intact. Electron-dense, amorphous deposits were visible in the xylem vessel (Fig. 7A). Fibrillar materials occurred in the intercellular spaces near host mesophyll cells (Fig. 7B), and protein bodies aggregated in the host vacuoles (Fig. 7B). Xylem vessels were partially filled with amorphous materials (Fig. 7B). Fibrillar materials were found in intercellular spaces surrounded by electron-dense middle lamella (Fig. 7C). Protein bodies of various forms were detected in the vacuoles of epidermal, mesophyll and phloem cells (Fig. 7 D).

Discussion

Pepper plants at different growth stages differentially responded to *C. coccodes* infection. At the two-leaf stage, true leaves, cotyledons, and stems inoculated with *C. coc-*

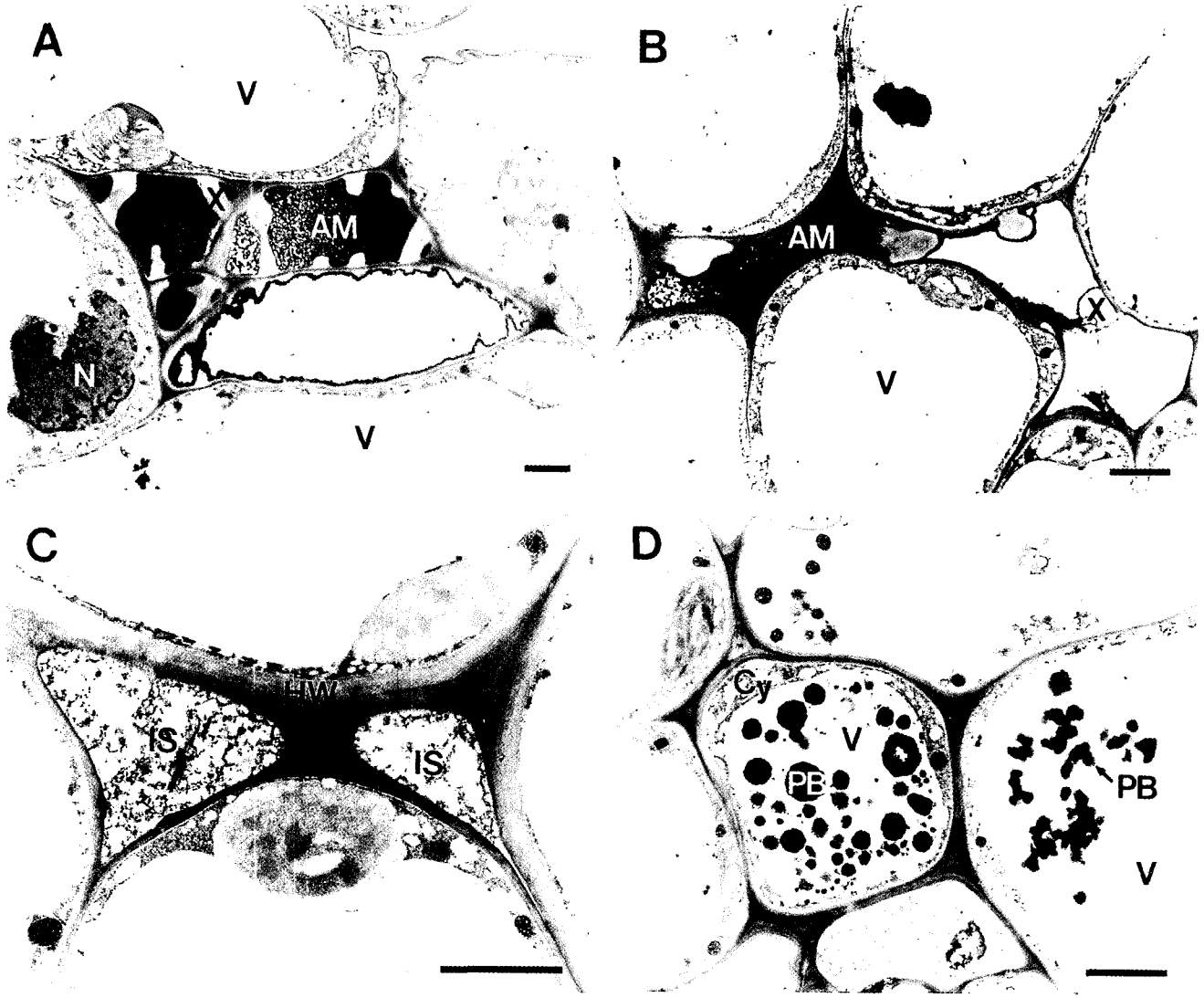

Fig. 7. Transmission electron micrographs of host reactions in pepper (cv. Hanbyul) leaf tissue 96 h after inoculation of *Colletotrichum coccodes* at the eight-leaf stage. The fungal cell is not seen where host defense reactions occur. (**A**) Accumulation of electron-dense amorphous materials in the xylem vessels. A large amount of amorphos materials is seen in the xylem cells. (**B**) The amorphous material is partially filling the xylem. (**C**) Intercellular spaces are filled with fibrillar materials. (**D**) Protein bodies are found mainly in the vacuoles of host cells. AM=amorphous material, Cy=cytoplasm. HW=host cell wall, IS=intercellular space, N=nucleous, PB=protein body, V=plant vacuole, X=xylem vessel. Bar=2 μm.

codes were severely infected and eventually blighted. *C. coccodes* grew well in leaf tissue at the two-leaf stage. Visible symptoms developed as water-soaked lesions resulted in the collapse of leaf tissues. In contrast, small necrotic and localized lesions occurred on the pepper leaves at the eight-leaf stage. Moreover, further lesion enlargement was restricted with a brownish margin at 72 to 96 h after inoculation. Leaf tissues severely collapsed by fungal infection at the two-leaf stage were comparable to the necrotic resistant reactions of the pepper leaves at the eight-leaf stage, at which time, many mature appressoria were found to have penetrated the epidermal cell, through some failed to penetrate the host cells. Abortive appressoria have been considered a factor of incompatiblility between *Colletotrichum* and their host plants (Marks et al., 1965). The dark brown color of the host cells may be induced by cell death.

Accumulation of autofluorescent material was detected under UV excitation in pepper leaf tissues infected by *C. coccodes*. The occurrence of autofluorescence in the leaf tissues was paralleled with the types of lesion development at the different growth stages. The autofluorescence accumulation seems to be due to the cell necrosis. Phenolics have been regarded as a cause of autofluorescence in dead cells (Mayama and Shinshiyama, 1978; Koga et al., 1988; Lummerzheim et al., 1993; Von Röpenack et al., 1998). At the eight-leaf stage, accumulation of the phenolic compounds may be effective in inhibiting fungal growth. Localized autofluorescence in the necrotic spot lesions of pepper leaves at the eight-leaf stage reflected accumulation of autofluorescent material such as phenolics in the dead cells, which in turn a hypersensitive response of the plants to incompatible pathogens (Lummerzheim et al., 1993; Koga, 1994). The visible necrotic spots were scattered on the pepper leaves at the eight-leaf stage. These necrotic cells emitted a bright yellow autofluorescence.

The ultrastructure of *C. coccodes* behavior in infected pepper leaf tissues showed that aggressive fungal growth at the two-leaf stage was characteristic of the vacuolization of fungal cell, formation of septum in the fungal hyphae, and condensation of host cytoplasm (Fig. 4A and 4C). These findings are well supported by the observations of O'Connell *et al.* (1985) that fungal vacuolization by extensive growth is typical in the compatible interaction of the French bean with *C. linthemuthianum*. Many hyphae of *C. coccodes* were shown near the xylem vessel (Fig. 4B). Many phytopathogenic fungi such as *Colletotrichum* spp. grew inter- and intracellularly toward the vascular bundle in host tissues (Anderson and Walker, 1962; Brammall and Higgins, 1988; Porto et al., 1988; Ghaouth et al., 1994). Effective water or nutrient uptake through vessel colonization may be a final goal of phytoparasitic fungi. A hypha of *C. coccodes* with a septum was observed within the xylem vessel 96 h after inoculation (Fig. 4C), indicating an establishment of stable nutritional relationship between *C. coccodes* and pepper leaf tissues at the two-leaf stage. We could detect the necrotrophic phase of the fungi in the young leaf tissues. At 96 h after inoculation, however, early infection events could not be detected. *Colletotrichum* spp. were found to be classified into several groups based on the nature of the infection processes in host tissues (Bailey et al., 1992).

Constriction of penetrating hypha at the point of intimate contact with the xylem vessel wall (Fig. 4D) suggests that penetration of *C. coccodes* into the xylem wall for the colonization of the xylem vessel may be dependent on the physical force of the fungal hyphae. Enzymatic degradation as well as physical pressures also seemed to meditate the penetration of *C. coccodes,* because the host cell wall in contact with the intercellular fungal cell was less intense than any other area of the cell walls (Fig. 4B and 5D). Many cell wall-degrading enzymes were isolated and characterized from the *Colletotrichum* spp. (Cervone et al., 1981; Prusky et al., 1989; Wijesundera et al., 1984; Wijesundera et al., 1989), but those of *C. coccodes* were not yet identified. The hyphal constriction was frequently accompanied by the formation of a septum at the constricted site (Porto et al., 1988; Mould et al., 1991a, b; Pring et al., 1995).

Leaf tissues colonized by the ramified hyphae of *C. coccodes* at the 2-leaf stage were severely damaged. Adaxial and abaxial epidermal cells were collapsed, and pallisade and spongy mesophyll cells were also distorted. The cytoplasm was highly condensed and electron-dense. Membrane structures of the chloroplast inside the mesophyll cells were disorganized (Fig. 5). Chloroplasts were broken down in advance of fungal hyphae. Chloroplasts have been known to be the most sensitive organelle to *Colletotrichum* infection (Anderson and Walker, 1962; Brown, 1977).

Figures 5A and 5B showed that intercellular fungal hypha formed the peg-like infection structure. Fungal hyphae in severely infected leaf tissues were highly vacuolated (Fig. 4A and 5C). In contrast, infection hyphae contained many fungal organelles, such as fungal mitochondria. Septa were observed in the fungal hyphae growing in the leaf tissues. In addition, a septum pore was revealed between the two fungal cells (Fig. 5C), whereas the pore was blocked in the serial sections (data not shown), indicating that the formation of a fungal septum occurred outside of the fungal cell wall.

Growth of *C. coccodes* was restricted in the epidermis of leaf tissues at the eight-leaf stage. Fungal invasion caused rapidly necrotic lesions in pepper leaves. Some fungal hyphae were detected in the intramural spaces of the epidermis at this stage (Fig. 6). The intramural hypha growing in the host cell wall has been demonstrated as characteristic of

some *Colletotrichum* spp., such as *C. capsici* and *C. gloeosporioides* (Chau and Alvarez, 1983; Pring et al., 1995). An age-related resistance of pepper plant to *C. coccodes* seemed to be expressed at the margin of the restricted lesions upon epidermal penetration. At 96 h after inoculation, *C. coccodes* colonized all the young leaf tissues at the two-leaf stage. A normal nutritional relationship between pepper plants and *C. coccodes* at the two-leaf stage, however, did not occur in the pepper tissues at the eight-leaf stage. The infection process of *Colletotrichum* spp. was widely different based on their pathosystem (Politis, 1976; O'Connell et al., 1985; O'Connell et al., 1993; Latundedada et al., 1996). Restriction of fungal development in host tissues and nutritional behavior were closely associated with the resistance mechanisms of the host plants on two *Colletotrichum*-host pathosystem (Politis and Wheeler, 1973; Mercer et al., 1974; Mercer et al., 1975; Politis, 1976; O'Connell et al., 1985). Further research for the *C. coccodes* infection process and host responses to their infection may provide a clue for the understanding of resistance mechanisms.

Several distinct host resistance responses to *C. coccodes* infection were shown at the eight-leaf stage (Fig. 7). These were amorphous materials in the xylem vessel, fibrillar material in the intercellular space, and protein aggregates in the vacuole. The distribution of protein bodies in the vacuole may imply the expression of defense-related proteins such as chitinase and β-1,3-glucanase (Mauch and Staehelin, 1989; Wubben et al., 1992). Much amounts of protein bodies detected in the vacuoles of infected leaf tissues at the eight-leaf stage might reflect increased defense reactions in pepper leaves at this stage. The fungal cell was arrested where the expression of host reactions may occur. Although no fungal hypha was found in the xylem vessel or intercellular spaces, electron-dense or fibrillar materials were revealed in these sites, possibly in relation to resistance mechanisms. It seems likely that host tissues may tranduce signals from a fungal infection site into adjacent cells. The components involved in the age-related resistance of pepper plants to the anthracnose fungus remains to be elucidated.

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References

Anderson, J. L. and Walker, J. C. 1962. Histology of watermelon anthracnose. *Phytopathology* 52:650-653.

Bailey, J. A., OConnell, R. J., Pring, R. J. and Nash, C. 1992. Infection strategies of *Colletotrichum* species. In: Bailey, J. A., Jeger, M. J. (eds) Colletotrichum biology, pathology, and control. CAB International, Wallingford, pp 88-120.

Brammall, R. A. and Higgins, V. J. 1988. A histological comparison of fungal colonization in tomato seedlings susceptible and resistant to Fusarium crown and root rot disease. *Can. J. Bot.* 66:915-925.

Brown, G. E. 1977. Ultrastructure of penetration of ethylene-degreened Robinson Tangerines by *Colletotrichum gloeosporioides*. *Phytopathology* 67:315-320.

Celio, G. J. and Hausbeck, M. K. 1998. Conidial germination, infection structure formation, and early colony development of powdery mildew on poinsettia. *Phytopathology* 88:105-113.

Cervone, F., Andebrhan, T., Coutts, R. H. A. and Woods, R. K. S. 1981. Effects of French bean tissue and leaf protoplasts on *Colletotrichum lindemuthianum* polygalacturonase. *Phytopathol. Z.* 102:238-246.

Chau, K. F. and Alvarez, A. M. 1983. A histological study of anthracnose on *Carica papaya*. *Phytopathology* 73:1113-1116.

Churchill, A. C. L., Baker, C. J., O'Neill, N. R. and Elgin, J. H. Jr. 1988. Development of *Colletotrichum trifolii* races 1 and 2 on alfalfa clones resistant and susceptible to anthracnose. *Can. J. Bot.* 66:75-81.

Dai, G. H., Andary, C., Mondolot-Cosson, L. and Boubals, D. 1995. Histochemical studies on the interaction between three species of grape vine, *Vitis vinifera, V. rupestris* and *V. rotundifolia* and downy mildew fungus, *Plasmopara viticola*. *Physiol. Mol. Plant Pathol.* 46:177-188.

Dietrich, R. A., Delaney, T. P., Uknes, S. J., Ward, E. R., Ryals, J. A. and Dangle, J. L. 1994. Arabidopsis mutants simulating disease resistance response. *Cell* 77:565-577.

Ghaouth, A. E., Arul, J., Wilson, C. and Benhamou, N. 1994. Ultrastructural and cytochemical aspects of the effect of chitosan on decay of bell pepper fruit. *Physiol. Mol. Plant Pathol.* 44:417-432.

Greenberg, J. T., Guo, A., Klessig, D. F. and Ausubel, F. M. 1994. Programmed cell death in plants: A pathogen-triggered response activated coordinated with multiple defense functions. *Cell* 77:551-563.

Griffey, R. T. and Leach, J. G. 1965. The influence of age of tissue on the development of bean anthracnose lesions. *Phytopathology* 55:915-918.

Hoisington, D. A., Neuffer, M. G. and Walbot, V. 1982. Disease lesion mimic in maize. *Devel. Biol.* 93:381-388.

Hong, J. K. and Hwang, B. K. 1998. Influence of inoculum density, wetness duration, plant age, inoculation method, and cultivar resistance on infection of pepper plants by *Colletotrichum coccodes*. *Plant Dis.* 82:1097-1083.

Hwang, B. K. and Kim, Y. J. 1990. Capsidiol production in pepper plants associated with age-related resistance to *Phytophthora capsici*. *Kor. J. Plant Pathol.* 6:193-200.

Hückelhoven, R., Fodor, J., Preis, C. and Kogel, K. H. 1999. Hypersensitive cell death and papilla formation in barley attacked by the powdery mildew fungus are associated with

hydrogen peroxide but not with salicylic acid accumulation. *Plant Physiol.* 119:1251-1260.

Kim, Y. J., Hwang, B. K. and Park, K. W. 1989. Expression of age-related resistance in pepper plants infected with *Phytophthora capsici*. *Plant Dis.* 73:745-747.

Koga, H. 1994. Hypersensitive death, autofluorescence, and ultrastructural changes in cells of leaf sheaths of susceptible and resistant near-isogenic lines of rice (*Pi-z*^{t}) in relation to penetration and growth of *Pyricularia oryzae*. *Can. J. Bot.* 72: 1463-1477.

Koga, H., Zeyen, R. J., Bushnell, W. R. and Ahlstrand, G. G. 1988. Hypersensitive cell death, autofluorescence, and silicon accumulation in barley leaf epidermal cells under attack by *Erysiphe graminis* f.sp. *hordei*. *Physiol. Mol. Plant Pathol.* 32:395-409.

Kováts, K., Binder, A. and Hohl, H. R. 1991. Cytology of induced systemic resistance of cucumber to *Colleotrichum lagenarium*. *Planta* 183:484-490.

Landes, M. and Hoffmann, G. M. 1979. Zum Keimungs- und Infektionsverlauf bei *Colletotrichum linthemuthianum* auf *Phaseolus vulgaris*. *Phytopathol. Z.* 95:259-273.

Latunde-dada, A. O., O'Connell, R. J., Nash, C., Pring, R. J., Lucas, J. A. and Bailey, J. A. 1996. Infection process and identity of the hemibiotrophic anthracnose fungus (*Colletotrichum destrctivum*) from cowpea (*Vigna unguiculata*). *Mycol. Res.* 100:1133-1141.

Lummerzheim, M., de Oliveira, D., Castresana, C., Miguens, F. C., Louzada, E., Roby, D., Van Montagu, M. and Timmerman, B. 1993. Identification of compatible and incompatible interactions between *Arabidopsis thaliana* and *Xanthomanas campestris* pv. *campestris* and characterization of the hypersensitive response. *Mol. Plant-Microbe Interact.* 6:532-544.

Marks, G. C., Berbee, J. G. and Riker, A. J. 1965. Direct penetration of leaves of *Populus tremuloides* by *Colletotrichum gloeosporioides*. *Phytopathology* 55:408-412.

Mauch, F. and Staehelin, L. A. 1989. Functional implications of the subcelluar localization of ethylene-induced chitinase and β-1,3-glucanase in bean leaves. *Plant Cell* 1:447-457.

Mayama, S. and Shishiyama J. 1978. Localized accumulation of fluorescent and U.V.-absorbing compounds at penetration sites in barley leaves infected with *Erysiphe graminis* f.sp. *hordei*. *Physiol. Plant Pathol.* 13:347-354.

Mercer, P. C., Wood, R. K. S. and Greenwood, A. D. 1974. Resistance to anthracnose of French bean. *Physiol. Plant Pathol.* 4:291-306.

Mercer, P. C., Wood, R. K. S. and Greenwood, A. D. 1975. Ultrastructure of the parasitism of *Phaseolus vulgaris* by *Colletotrichum linthemuthianum*. *Physiol. Plant Pathol.* 27:75-98.

Mould, M. J. R., Boland, G. J. and Robb, J. 1991a. Ultrastructure of the *Colletotrichum trifolii-Medicago sativa* pathosystem. I. Pre-penetration events. *Physiol. Mol. Plant Pathol.* 38:179-194.

Mould, M. J. R., Boland, G. J. and Robb, J. 1991b. Ultrastructure of the *Colletotrichum trifolii-Medicago sativa* pathosystem. II. Post-penetration events. *Physiol. Mol. Plant Pathol.* 38:195-210.

Ostazeski, S. A. and Elgin, J. H. Jr. 1981. Protection against race 2 of *Colletotrichum trifolii* in alfalfa hypodermically inoculated with mixtures of race 1 and race 2 conidia. *(Abstr.) Phytopathology* 71:770.

Ostazeski, S. A. and Elgin, J. H. Jr. 1984. Resistance induced by race 1 of *Colletotrichum trifolii* to race 2 in alfalfa resistant to race 1. *Plant Dis.* 68:285-288.

O'Connell, R. J., Bailey, J. A. and Richmond, D. V. 1985. Cytology and physiology of infecion of *Phaseolus vulgaris* by *Colletotrichum linthemuthianum*. *Physiol. Plant Pathol.* 27:75-98.

O'Connell, R. J., Uronu, A. B., Waksman, G., Nash, C., Keon, J. P. R. and Bailey, J. A. 1993. Hemibiotrophic infection of *Pisum sativum* by *Colletotrichum truncatum*. *Plant Pathol.* 42:774-783.

Pain, N., O'Connell, R. J., Mendgen, K. and Green, J. R. 1994. Identification of glycoproteins specific to biotrophic intracellular hyphae formed in the *Colletotrichum linthemuthianum*-bean interaction. *New Phytol.* 127:233-242.

Politis, D. J. 1976. Ultrastructure of penetration by *Colletotrichum graminicola* of highly resistant oat leaves. *Physiol. Plant Pathol.* 8:117-122.

Politis, D. J. and Wheeler, H. 1973. Ultrastructural study of penetration of maize leaves by *Colletotrichum graminicola*. *Physiol. Plant Pathol.* 3:465-471.

Porto, M. D. M., Grau, C. R., de Zoeten, G. A. and Gaard, G. 1988. Histopathology of *Colletotrichum trifolii* on alfalfa. *Phytopathology* 78:345-349.

Pring, R. J., Nash, C., Zakaria, M. and Bailey, J. A. 1995. Infection process and host range of *Colletotrichum capsici*. *Physiol. Mol. Plant Pathol.* 46:137-152.

Prusky, D., Gold, S. and Keen, N. T. 1989. Purification and characterization of an endopolygalacturonase produced by *Colletotrichum gloeosporioides*. *Physiol. Mol. Plant Pathol.* 35: 121-133.

Reynolds, E. S. 1963. The use of lead citrate at high pH as an electron opaque stain in electron microscopy. *J. Cell Biol.* 17:208-212.

Skipp, R. A. and Deverall, B. J. 1972. Relationships between fungal growth and host changes visible by light microscopy during infection of bean hypocotyls (*Phaseolus vulgaris*) susceptible and resistant to physiological races of *Colletotrichum linthemuthianum*. *Physiol. Plant Pathol.* 2:357-374.

Takahashi, A., Kawasaki, T., Henmi, K., Shii, K., Kodama, O., Satoh, H. and Shimamoto, K. 1999. Lesion mimic mutants of rice with alterations in early signaling events of defense. *Plant J.* 17:535-545.

Von Röpenack, E., Parr, A. and Schulze-Lefert, P. 1998. Structural analyses and dynamics of soluble and cell wall-bound phenolics in a broad spectrum resistance to the powdery mildew fungus in barley. *J. Biol. Chem.* 273:9013-9022.

Weymann, K., Hunt, M., Uknes, S., Neunschwander, U., Lawton, K., Steiner, H. Y. and Ryals, J. 1995. Suppression and restoration of lesion formation in *Arabidopsis lsd* mutants. *Plant Cell* 7:2013-2022.

Wijesundera, R. L. C., Bailey, J. A., Byrde, R. J. W. and Fielding, A. H. 1989. Cell wall degrading enzymes of *Colletotrichum*

lindemuthianum: their role in the development of bean anthracnose. *Physiol. Mol. Plant Pathol.* 34:403-413.

Wijesundera, R. L. C., Bailey, J. A. and Byrde, R. J. W. 1984. Production of pectin lyase by *Colletotrichhum lindemuthianum* in culture and in infected bean (*Phaseolus vulgaris*) tissue. *J. Gen. Microbiol.* 130:285-290.

Wolter, M., Hollricher, K., Francesco, S. and Schulze-Lefert, P. 1993. The *mlo* resistance alleles to powdery mildew infection in barley trigger a developmentally controlled defence mimic phenotype. *Mol. Gen. Genet.* 239:122-128.

Wubben, J. P., Joosten, M. H. A., van Kan, J. A. L. and de Wit, P. J. G. M. 1992. Subcellular localization of plant chitinases and β-1,3-glucanases in *Cladosporium fulvum* (syn. *Fulvia fulva*)-infected tomato leaves. *Physiol. Mol. Plant Pathol.* 41:23-32.