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Immunogold Localization of β-1,3-Glucanases in Two Plants Infected by Vascular Wilt Fungi

Nicole Benhamou,[a,1] Jean Grenier,[a] Alain Asselin,[a] and Michel Legrand[b]

[a] Département de Phytologie, Faculté des Sciences de L'Agriculture et de L'Alimentation, Université Laval, Sainte-Foy, Québec, G1K 7P4, Canada

[b] Institut de Biologie Moléculaire des Plantes, Centre National de la Recherche Scientifique, 12 rue du Général Zimmer, 67 000 Strasbourg, France

An antiserum raised against a purified tobacco β-1,3-glucanase (PR-N) was used to study the subcellular localization of enzyme in fungus-infected plant tissues by means of post-embedding immunogold labeling. In susceptible tomato plants, the enzyme accumulation was found to occur as a result of successful tissue colonization, whereas it appeared to be an early event associated with limited spread of the fungus in resistant tissues. Although marked differences between susceptible and resistant tomato cultivars were observed in the rate of production of β-1,3-glucanase, the pattern of enzyme distribution was similar. The enzyme was found to accumulate predominantly in host cell walls and secondary thickenings of xylem vessels. By contrast, a very low amount of enzyme was associated with compound middle lamellae. The occurrence of β-1,3-glucanase at the cell surface of invading fungi was an indication of their possible antifungal activity. A low enzyme concentration was detected in vacuoles of both healthy and infected tissues. In infected eggplant tissue, the pattern of β-1,3-glucanase distribution was similar to that observed with tomato. Whether these hydrolases accumulate first in vacuoles and are subsequently conveyed toward the outside to participate in fungal wall lysis remains to be determined.

INTRODUCTION

In recent years, attention has been paid to the possible implication of plant hydrolases in defense against fungal pathogens (Pegg and Young, 1981; Young and Pegg, 1982; Boller, 1985). Among these, β-1,3-glucanases and chitinases have been the focus of extensive studies dealing with their purification, induction, and characterization (Kauffmann et al., 1987; Legrand et al., 1987; Côté et al., 1989; Trudel, Audy, and Asselin, 1989). The rationale for such an interest was that these enzymes could not only degrade the main cell wall components of fungi (Young and Pegg, 1982) but also produce glycosidic fragments acting as elicitors of host stress metabolite biosynthesis (Keen and Yoshikawa, 1983; Darvill and Albersheim, 1984).

Since the initial discovery that β-1,3-glucanases and chitinases increased in tomato plants in response to fungal infection (Pegg, 1976, 1977), several reports have suggested that these hydrolases were likely involved in defense reactions in this plant (Ferraris, Abbattista Gentile, and Matta, 1987; Joosten and De Wit, 1989), as well as in tobacco (Kauffmann et al., 1987; Legrand et al., 1987), potato (Kombrick, Schröder, and Hahlbrock, 1988), cucumber (Boller and Métraux, 1988), muskmelon (Roby, Toppan, and Esquerré-Tugayé, 1988), bean (Vögeli, Meins, and Boller, 1988; Mauch and Staehelin, 1989), and pea (Mauch, Hadwiger, and Boller, 1988a; Mauch, Mauch-Mani, and Boller, 1988b). Support for implication of both enzymes in plant defense was drawn by the demonstration that they displayed the ability to attack and degrade isolated fungal cell walls (Young and Pegg, 1982; Schlumbaum et al., 1986; Mauch et al., 1988b). An additional line of evidence came from the recent study of Joosten and De Wit (1989), who reported that the enhancement of β-1,3-glucanases and chitinases in tomato plants infected by *Cladosporium fulvum* took place earlier and to a higher extent in resistant than in susceptible cultivars. In light of these results, it was concluded that the accumulation of plant hydrolases was an early event associated with resistance of tomato to *C. fulvum*. Although these observations provided strong support for the assumption that plant hydrolases were active determinants in defense reactions, conclusive evidence could not be reached in the absence of data concerning precise in situ distribution of these enzymes in fungus-infected plant tissues.

By combining both immunocytochemical and cell fractionation experiments, Mauch and Staehelin (1989) found that chitinases and most β-1,3-glucanases induced in eth-

[1] To whom correspondence should be addressed.

ylene-stressed bean leaves accumulated in large intravacuolar aggregates. These results, together with the observation that a low amount of β-1,3-glucanases was associated with the plant cell wall, mainly the middle lamella, led the authors to propose a model for the roles of chitinases and β-1,3-glucanases in defense responses against fungal infection. They postulated that fungal cells growing intercellularly were met by β-1,3-glucanases located in the middle lamella along the air spaces and that, in turn, oligosaccharides released from the fungal wall were probably elicitors of phytoalexins. In addition, they assumed that the invacuolar accumulation of both enzymes corresponded to lethal concentrations directed against the invading fungus. This model supports and extends the hypothesis that β-1,3-glucanases and chitinases are key proteins in the antimicrobial arsenal of plants. However, it remains to be investigated whether or not the induction of hydrolases in infected tissue is related to the resistance of a plant against fungal pathogens.

In an attempt to address this question, we investigated the subcellular localization of β-1,3-glucanases in plant tissues infected by pathogenic fungi. We present evidence that β-1,3-glucanases are predominantly concentrated in cell walls and vacuoles of infected plant tissues and accumulate over fungal cell walls and septa, likely on the basis of an enzyme-substrate interaction.

RESULTS

Specificity of the Antiserum Raised against One Tobacco β-1,3-Glucanase (PR-N)

Electrophoresis of tobacco and tomato intercellular fluid (IF) extracts on denaturing SDS-polyacrylamide gels resulted in protein profiles illustrated in Figure 1, lanes A, C, E, and G.

The specificity of the antiserum raised against a β-1,3-glucanase (PR-N) from tobacco was verified on immunoblots after SDS-PAGE. As previously reported (Kauffmann et al., 1987), this antiserum reacted specifically with at least three β-1,3-glucanases occurring in tobacco IF extracts containing PR proteins (Figure 1, lane B). With healthy tomato IF extracts, no significant cross-reactivity could be detected (Figure 1, lane D). By contrast, the antiserum was found to cross-react specifically with one β-1,3-glucanase of about 33 kD present in tomato IF extracts from susceptible tomato plants (compatible interaction) and from resistant tomato (Figure 1, lane F) plants (incompatible interaction) (Figure 1, lane H). These results are in agreement with those recently reported by Joosten and De Wit (1989), who concluded that β-1,3-glucanases and chitinases within the Solanaceae and even between different plant families are closely related serologically. The use of rabbit pre-immune serum instead of the β-1,3-glucanase antiserum did not reveal any polypeptide on immunoblots (not illustrated).

Immunogold Localization of β-1,3-Glucanase in Tomato Root Cells Infected by *Fusarium oxysporum* f. sp. *radicis-lycopersici* (FORL)

Satisfactory ultrastructural preservation and antigen immobilization were obtained when tissues were fixed with either glutaraldehyde only or with a combination of glutaraldehyde and osmium tetroxide. Pre-incubation of ultrathin sections with normal goat serum prior to treatment with the antiserum was found to be essential for preventing nonspecific deposition of gold particles over the entire sections. In addition, the use of Tris-HCl buffer with a high salt concentration and enriched with BSA as an inert protein reduced significantly the background staining over both tissue sections and embedding resin.

Compatible Interaction: Tomato cv Bonny Best/FORL

In the susceptible tomato cultivar Bonny Best, root colonization started to appear in the epidermis within 48 hr

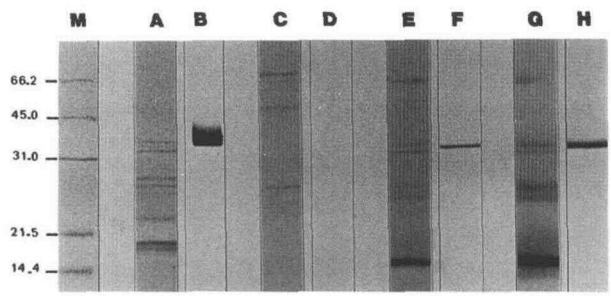

Figure 1. Protein Profiles of Fractions from Tobacco and Tomato Plants and Specificity of the Antiserum Raised against One β-1,3-Glucanase (PR-N) from Tobacco.

Coomassie-stained SDS-PAGE gels of tobacco IF extract containing PR proteins (lane A); healthy tomato IF extract (lane C); IF extract from susceptible tomato plants infected with FORL (compatible interaction) (lane E); and IF extract from resistant tomato plants infected with FORL (incompatible interaction) (lane G); and their corresponding immunoblots (lanes B, D, F, and H, respectively).

The antiserum reacts specifically with β-1,3-glucanases of tobacco IF extract (lane B) and cross-reacts with β-1,3-glucanase at about 33 kD in both IF extracts from susceptible (lane F) and resistant (lane H) tomato plants. No significant cross-reactivity is detected with healthy tomato IF extract (no PR proteins) (lane D). Lane M corresponds to proteins of known molecular masses (expressed in kilodaltons).

after inoculation. Fungal hyphae were frequently observed within intercellular spaces of the epidermis. By 72 hr to 96 hr after inoculation, fungal cells were found to ramify through much of the hypodermis and cortex. Colonization of such tissues resulted in pronounced cell alterations, as judged by the extensive cytoplasm breakdown, as illustrated in Figure 2A. Fungal growth occurred both inter- and intracellularly and, in some cases, intramurally. Penetration of the endodermis and the vascular stele was seldom observed. However, by 120 hr after inoculation, fungal colonization reached the vascular elements via infection of the pericycle and paratracheal parenchyma cells. Invasion of protoxylem as well as early and late metaxylem proceeded through the middle lamella and pit membranes. At this time after inoculation, tissue colonization was associated with marked disorders such as cytoplasm breakdown, papilla formation, swelling, and partial host wall disintegration, as well as localized cell necrosis (Charest, Ouellette, and Pauzé, 1984; Brammall and Higgins, 1988; Benhamou et al., 1989b).

As soon as 72 hr after inoculation, inter- and intracellular colonization of cortical cells was intense (Figures 2A and 2B). Treatment of ultrathin sections with the antiserum raised against one tobacco β-1,3-glucanase (PR-N) and with gold-conjugated goat antiserum to rabbit immunoglobulins (GAR-gold antibodies) resulted in the labeling of specific host wall areas. Gold particles seemed to accumulate preferentially over host wall areas in the immediate vicinity of fungal cells (Figure 2B, arrow). Labeling was also associated with wall appositions, known to be papillae (Aist, 1976) and considered as typical host reactions (Figure 2B). However, gold deposition within these newly formed structures was irregular. Fungal cells were not intensely labeled but gold particles, when present, were mainly associated with the cell surface that included the cell wall and plasmalemma area (Figure 2B, double arrow).

Between 96 hr and 120 hr after inoculation, pericycle and paratracheal parenchyma cells were markedly invaded, as illustrated in Figure 3. Following treatment with the β-1,3-glucanase antiserum and GAR-gold antibodies, an intense and specific deposition of gold particles was found to occur over host wall areas that were closely neighboring fungal cells (Figure 3A). At a high magnification, it was easily noticeable that the labeling was evenly distributed over the entire host wall portion in contact with hyphae (Figure 3B). Hyphal penetration of paratracheal parenchyma cell walls was frequently observed (Figure 3D). A heavy deposition of gold particles occurred always along the fungus pathway as well as over host wall areas neighboring the channel of penetration. Interestingly, fungal wall alterations were observed during penetration (Figure 3D, arrow). Gold labeling accumulated at the periphery of fungal areas that were in close contact with the host wall (Figure 3D, double arrows). A considerable decrease in labeling intensity was noted over host wall areas devoid of an intimate contact with fungal cells (Figure 3C). In some invaded intercellular spaces, fungal cells were enclosed in an amorphous material enveloped by an electron-opaque membrane (Figure 3C). Gold particles observed as clusters were associated with the amorphous material surrounding fungal cells (Figure 3C, arrows).

By 120 hr after inoculation, fungal cells were found to colonize abundantly the vascular stele, as shown in Figure 4A. Secondary thickenings of invaded xylem vessels were heavily labeled, whereas only few scattered gold particles occurred over middle lamellae and pit membranes (Figure 4A). Observations at higher magnifications revealed that fungal cell walls and septa were significantly labeled (Figure 4B). Although unevenly distributed, gold particles accumulated specifically over some fungal wall areas, especially over septa (Figure 4B).

Figures 5A and 5B illustrate that in uninvaded parenchyma cells neighboring colonized ones, labeling with the β-1,3-glucanase antiserum was mainly associated with vacuoles and intracytoplasmic vesicles. Intravacuolar labeling was characterized by a scattered distribution of gold particles with predominant accumulation over electron-dense polymorphic inclusions (Figure 5A). In some areas, retraction of the cytoplasm led to the formation of free spaces where polymorphic vesicles accumulated subsequently (Figure 5B). Gold particles were not associated with these vesicles but occurred over the stroma surrounding them (Figure 5B).

Incompatible Interaction: Tomato cv Larma/FORL

From 24 hr to 48 hr following inoculation, fungal spores had germinated on the root surface of the resistant tomato cultivar (cv Larma) and started to penetrate the epidermis. By 72 hr after inoculation, fungal colonization remained localized in the epidermis and hypodermis and occurred occasionally in the outermost cortical cell layers. Later after inoculation, the pattern of tissue colonization was unchanged. Fungal growth was restricted to outer tissues, whereas inner areas, including the endodermis, pericycle, paratracheal parenchyma, and vascular stele, were not invaded.

Incubation of ultrathin sections with the β-1,3-glucanase antiserum and GAR-gold antibodies resulted in an irregular labeling of colonized epidermal and hypodermal cell walls (Figure 5C). This labeling pattern of outer invaded tissues was mostly similar to that observed over corresponding tissues in the susceptible cultivar. By contrast, marked differences were seen with inner tissues. Although uninvaded, cells of the endodermis, pericycle, and paratracheal parenchyma displayed an intense labeling of their walls. Similarly, secondary thickenings of uncolonized xylem vessels were heavily labeled (Figure 5D). This contrasted sharply with susceptible root tissues, where labeling occurred only over walls of invaded cells.

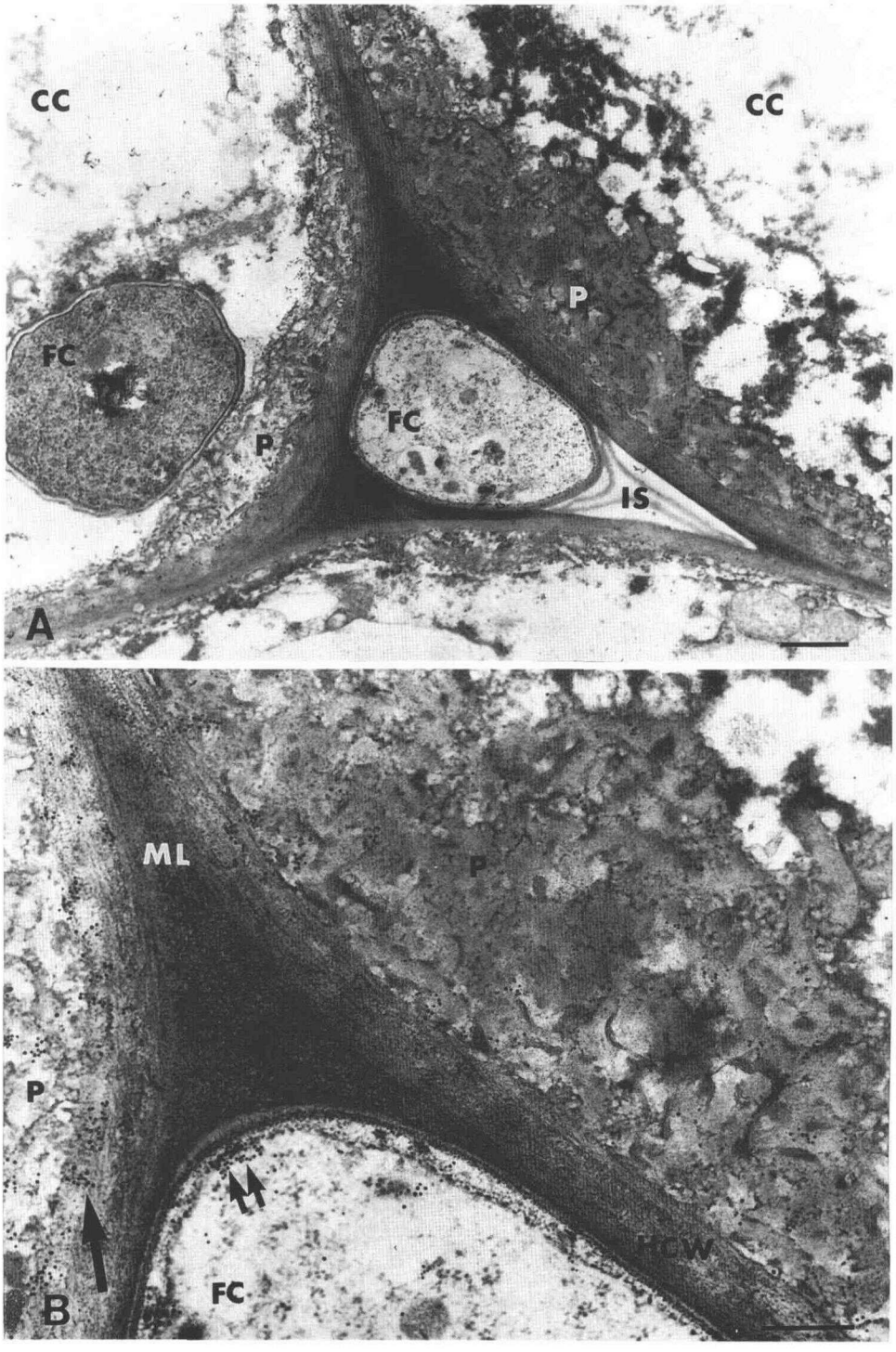

Figure 2. *Transmission Electron Micrographs of Tomato (cv Bonny Best, Susceptible Cultivar) Root Tissues Infected by FORL, 72 hr after Inoculation.*

Treatment with the antiserum raised against one tobacco β-1,3-glucanase (PR-N) and with GAR-gold antibodies.
(A) Fungal cells are growing inter- and intracellularly in the cortical area. Wall appositions, referred to as papillae, are formed on both sides of an invaded intercellular space (IS). Magnification ×18,000; bar = 0.5 μm.

Immunogold Localization of β-1,3-Glucanase in Eggplant Stem Cells Infected by *Verticillium alboatrum*

Figure 6A illustrates that fungal colonization was restricted to xylem vessels. Pronounced host cell reactions were observed. In xylem vessels, these reactions were characterized by the deposition of a network of intertwined fibrils in the lumen (Figure 6A). In addition, most fungal cells were surrounded by an electron-opaque material likely formed in response to infection (Figure 6B). Partial to complete retraction of the plasmalemma was observed in parenchyma cells adjacent to colonized vessels. Numerous vesicles accumulated usually in the newly formed paramural spaces (Figure 6B, arrow).

Following incubation with the β-1,3-glucanase antiserum and GAR-gold antibodies, an intense gold deposition occurred over secondary thickenings of infected vessels (Figure 6). Few gold particles were associated with the fibrillar material filling the vessel lumen (Figure 6B). Interestingly, there was an association of gold particles with the fungal cell surface (Figure 6B, arrow). The structural integrity of fungal cell walls was generally altered. This could explain the diffusion of gold particles toward the inside of fungal cells.

Immunogold Localization of β-1,3-Glucanase in Noninoculated Control Plants

Following incubation with the β-1,3-glucanase antiserum and GAR-gold antibodies, a slight and uneven labeling was found over primary walls and secondary thickenings of tomato root cells, as shown in Figure 7A. Few randomly distributed gold particles were present in intracellular vacuoles (Figure 7B). Similar results were observed with eggplant tissues (not illustrated).

Immunocytochemical Control Tests

Several control tests were performed to assess the validity of labeling patterns obtained with the β-1,3-glucanase antiserum and GAR-gold antibodies. Thus, pre-incubation of the β-1,3-glucanase antiserum with an excess of purified β-1,3-glucanase (PR-N from tobacco) prior to section treatment resulted in a considerable reduction of labeling in infected plant tissues (Figure 7C). Incubation of sections with rabbit pre-immune serum instead of β-1,3-glucanase antiserum yielded negative results (Figure 7D). Similarly, sections incubated with GAR-gold antibodies only or with the gold suspension alone did not show any labeling (not illustrated).

DISCUSSION

The role of lytic enzymes in reducing the intensity of pathogen invasion in plant tissue has often been emphasized (Netzer and Kritzman, 1979; Young and Pegg, 1981; Ferraris et al., 1987). However, the exact role of plant hydrolases in defense reactions against pathogen attack is still a matter of speculation, although recent studies have convincingly demonstrated that chitinases and β-1,3-glucanases from pea pods acted synergistically in the degradation of isolated fungal cell walls and caused swelling as well as disruption of hyphal tips in vitro (Mauch et al., 1988b). In light of such results, it was of interest to investigate whether or not plant hydrolases played a role in the outcome of host-pathogen relationships.

Results of the present immunocytochemical study performed on tomato and eggplant tissues infected by pathogenic fungi provide support to the hypothesis that β-1,3-glucanases induced upon infection are implicated in plant defense against fungal attack. Support for this is drawn by the observation that the β-1,3-glucanase increase in resistant tomato plants is an early event associated with the limited spread of the fungus, whereas in susceptible plants, it appears to occur as a result of successful tissue colonization. These findings confirm and extend previous biochemical data reported by Netzer and Kritzman (1979) on muskmelon infected by *Fusarium oxysporum* f. sp. *melonis* and by Ferraris et al. (1987) on tomato infected by *F. oxysporum* f. sp. *lycopersici*. In both cases, it was found that the increase in β-1,3-glucanase activity occurred earlier and to a higher extent in resistant than in susceptible plant cultivars.

In the compatible tomato-FORL interaction, the increase in β-1,3-glucanase was found to follow closely the progressive tissue colonization. Our observations at various times after inoculation revealed that enzyme increase started after close contact between both partners and occurred as a result of damage of the plant tissue by the pathogen. By contrast, in the incompatible tomato-FORL interaction, the accumulation of β-1,3-glucanases was not restricted to colonized tissues only but also occurred in uninvaded inner cells. This indicated that the enzyme

Figure 2. (continued).

(B) Portion of **(A)** at higher magnification. Gold labeling accumulates preferentially over host wall areas in the vicinity of fungal cells (arrow). Few irregularly distributed gold particles are present over papillae. Host middle lamella is nearly free of labeling. Although unevenly distributed, few gold particles occur in some peripheral areas of the fungal cell (double arrows). Magnification ×45,000; bar = 0.25 μm. The abbreviations used are: CC, cortical cell; FC, fungal cell; HCW, host cell wall; IS, intercellular space; ML, middle lamella; P, papilla.

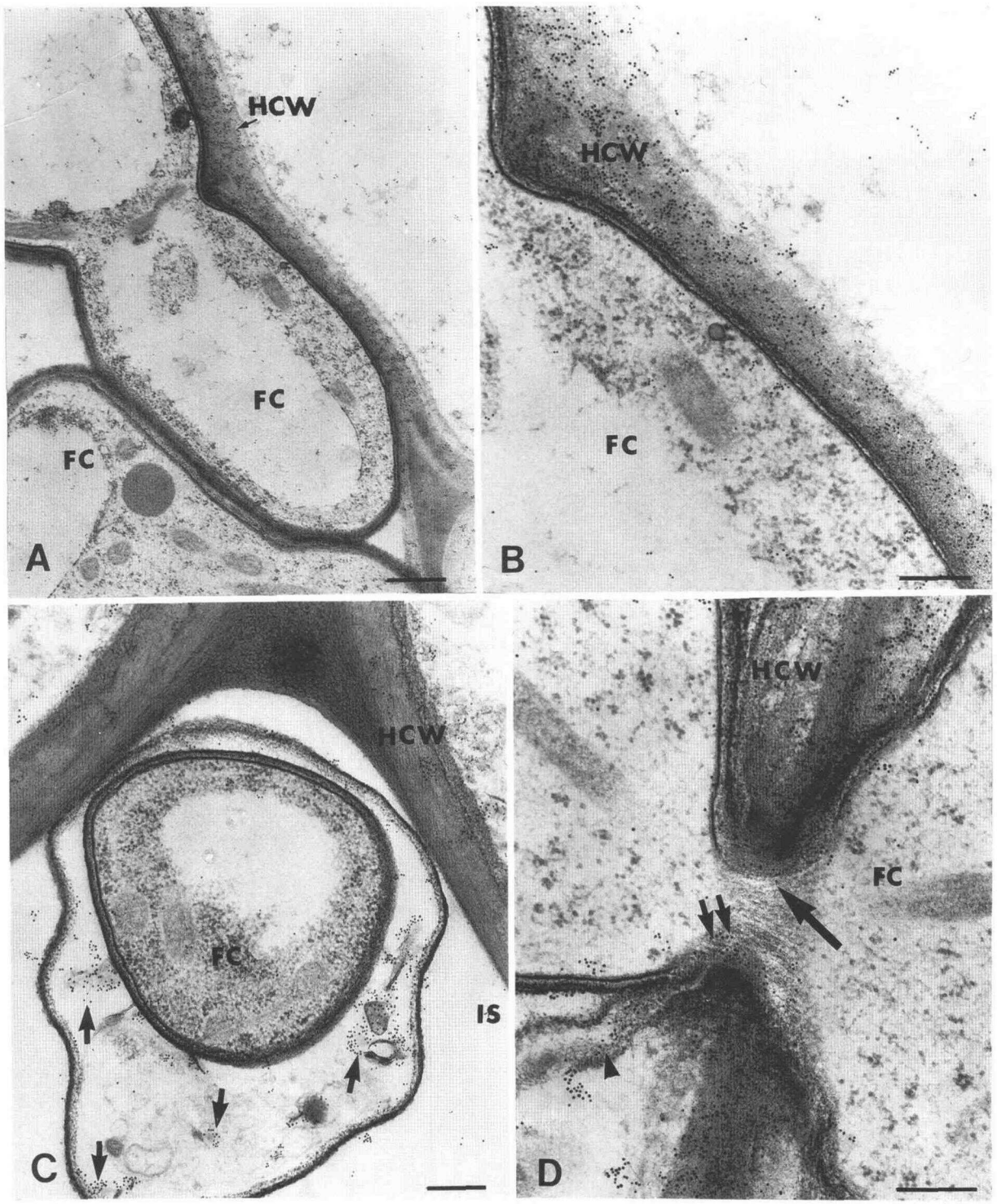

Figure 3. Transmission Electron Micrographs of Tomato (Susceptible Cultivar) Root Tissues Infected by FORL, 96 hr to 120 hr after Inoculation.

Treatment with the antiserum raised against one tobacco β-1,3-glucanase (PR-N) and with GAR-gold antibodies.
(A) In the intensely colonized pericycle, fungal cells are growing both inter- and intracellularly. Some fungal cells are closely appressed against the host cell wall. Magnification ×18,000; bar = 0.5 μm.

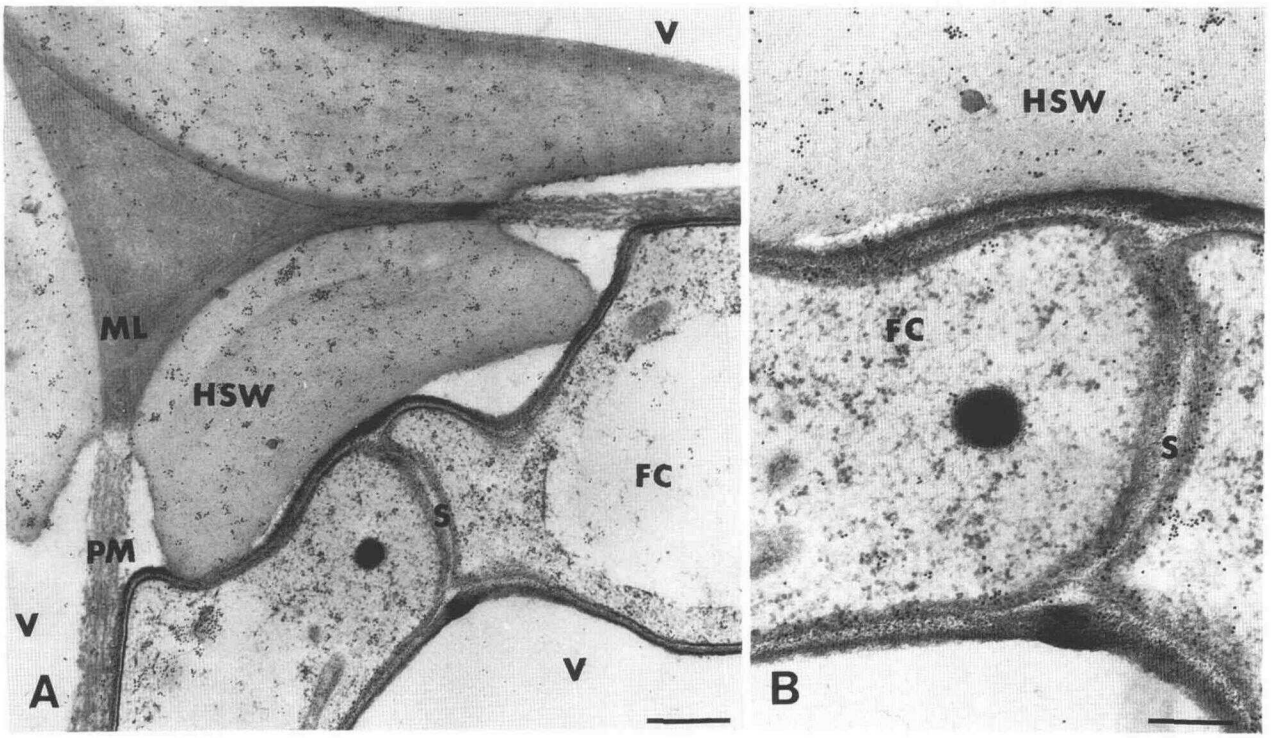

Figure 4. Transmission Electron Micrographs of Tomato (Susceptible Cultivar) Root Tissues Infected by FORL, 120 hr after Inoculation.

Treatment with the antiserum raised against one tobacco β-1,3-glucanase (PR-N) and with GAR-gold antibodies.

(A) Three adjacent xylem vessels are intensely colonized. Vessel penetration occurs through the fragile pit membrane. Host secondary walls are heavily labeled, whereas middle lamella and pit membranes are only labeled by a few scattered gold particles. Magnification ×21,500; bar = 0.5 μm.

(B) Portion of **(A)** at higher magnification. Gold particles are present over the fungus wall but are unevenly distributed. The fungal septum appears more regularly labeled. Magnification ×45,000; bar = 0.25 μm.

The abbreviations used are: FC, fungal cell; HSW, host secondary wall; ML, middle lamella; PM, pit membrane; S, septum; V, vessel.

increase in resistant cultivars did not proceed via tissue colonization as it appeared to be the case in susceptible plants. In light of these observations, it can be suggested that the accumulation of β-1,3-glucanases is a protective mechanism that may account, at least partially, for the effective resistance of some tomato cultivars to invasion by FORL. It is likely that other stress-induced molecules including chitinases, pathogenesis-related-1 (PR-1) proteins (Benhamou et al., 1989a), hydroxyproline-rich glycoproteins (Benhamou et al., 1989b), and phytoalexins (Darvill and Albersheim, 1984) are also important determinants of resistance expression. Preliminary results dealing with the subcellular localization of chitinases in tomato root cells infected by FORL (Benhamou et al., 1989c) indicate that this enzyme activity is preceded by the hydrolytic action of β-1,3-glucanases since chitin appears inaccessi-

Figure 3. (continued).

(B) Portion of **(A)** at higher magnification. An intense deposition of gold particles occurs over the host wall area that is in close contact with the fungal cell. Magnification × 45,000; bar = 0.25 μm.

(C) In an invaded intercellular space, a fungal cell is surrounded by a wavy electron-opaque envelope. Gold particles, mainly as clusters, are associated with the amorphous material surrounding the fungal cell and delimited by the envelope (arrows). Magnification ×36,000; bar = 0.25 μm.

(D) A hypha is penetrating the wall of two adjacent paratracheal parenchyma cells. The channel of penetration is narrower than the average hyphal diameter. Fungal growth causes little wall displacement (arrowhead). A heavy deposition of gold particles occurs over host wall areas adjacent to the channel of penetration. Fungal wall alteration is apparent during host penetration (arrow). Gold particles accumulate over these altered fungal wall areas (double arrows). Magnification ×54,000; bar = 0.25 μm.

Abbreviations are as in Figure 2.

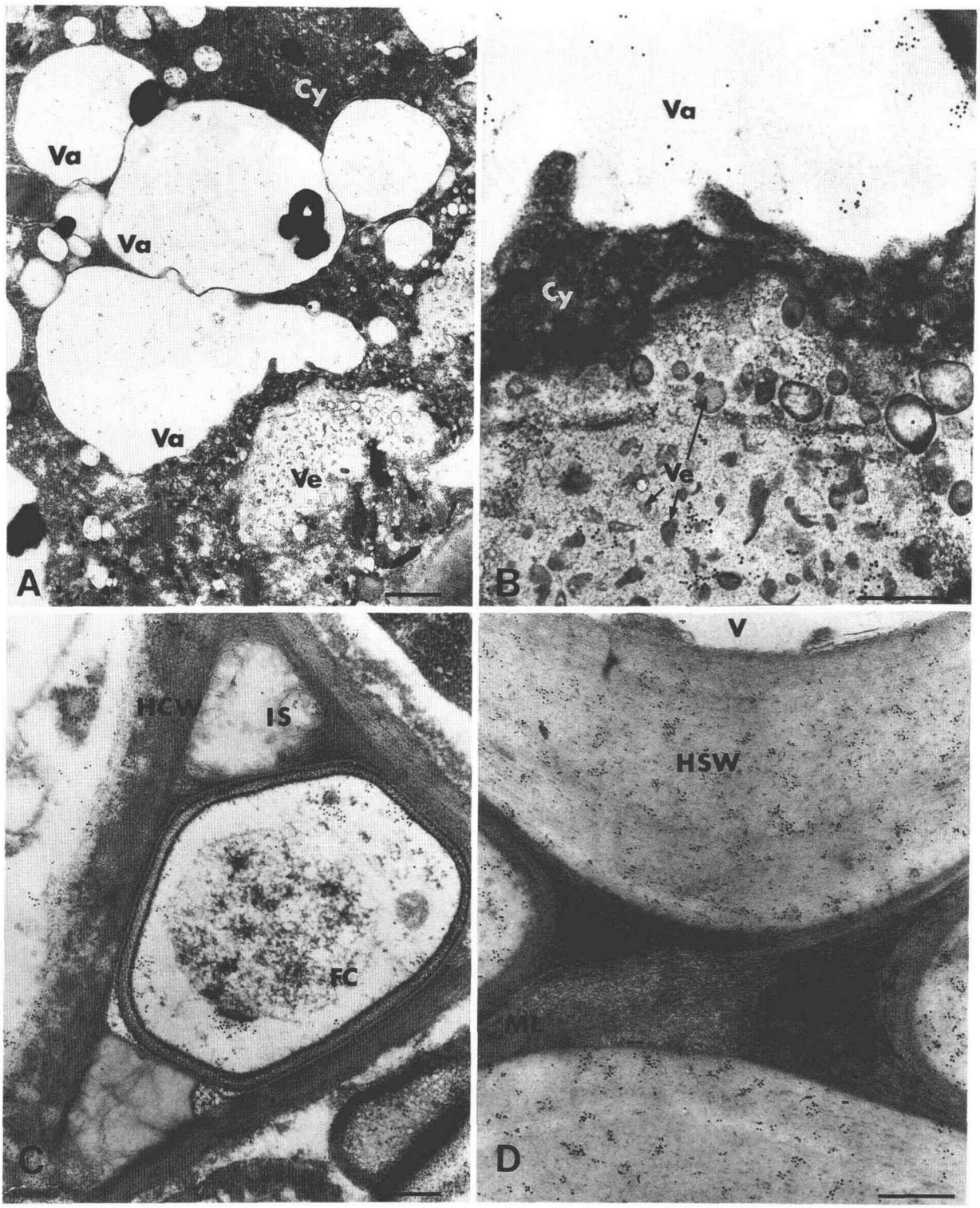

Figure 5. Transmission Electron Micrographs of Tomato [Susceptible Cultivar **(A)** and **(B)** and Resistant Cultivar **(C)** and **(D)**] Root Tissues Infected by FORL, 120 hr after Inoculation.

Treatment with the antiserum raised against one tobacco β-1,3-glucanase (PR-N) and with GAR-gold antibodies.

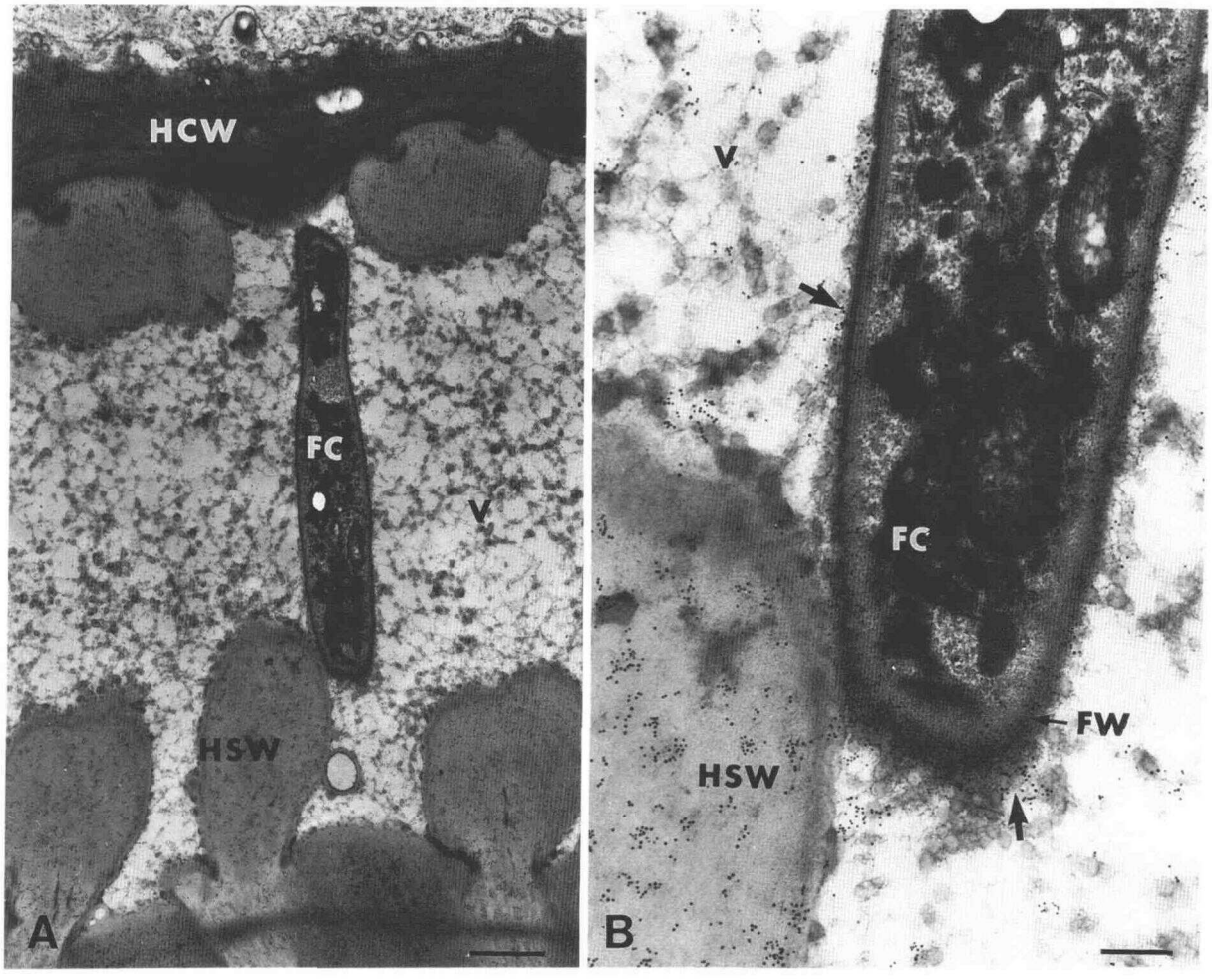

Figure 6. Transmission Electron Micrographs of Eggplant (cv Imperial Black Beauty, Susceptible Cultivar) Stem Tissues Infected by *V. albo-atrum*.

Treatment with the antiserum raised against one tobacco β-1,3-glucanase (PR-N).
(A) Fungal colonization is restricted to xylem vessels. Host reactions are characterized by the accumulation of a network of intertwined fibrils in the vessel lumen. In an adjacent parenchyma cell, a partial retraction of the plasmalemma is visible (arrow). Few vesicles are formed in the paramural space. Magnification ×9,000; bar = 1 μm.
(B) Portion of **(A)** at higher magnification. Numerous gold particles are associated with the host secondary wall. An intense labeling is also associated with the fungal wall surface (arrows). Because of the loss of structural integrity, some compounds may have diffused from the fungus wall toward the inside of the fungal cell, thus explaining the presence of internal gold particles. Magnification ×36,000; bar = 0.25 μm.
The abbreviations used are: FC, fungal cell; FW, fungal wall; HCW, host cell wall; HSW, host secondary wall; V, vessel.

Figure 5. (continued).

(A) In an uninvaded parenchyma cell adjacent to a colonized one, gold labeling is mainly associated with vacuoles and intracytoplasmic vesicles. Large number of particles are present over the intravacuolar inclusions, but they are not discernible because of the high electron density of these inclusions. Magnification ×18,000; bar = 0.5 μm.
(B) Portion of **(A)** at higher magnification. Gold particles, mainly found as clusters, occur over vacuoles and intracytoplasmic vesicles. Magnification ×54,000; bar = 0.25 μm.
(C) In the epidermis, a fungal cell is growing intercellularly. Few, unevenly distributed gold particles are associated with the host cell wall. The amorphous material filling the intercellular space is nearly free of labeling. Magnification ×35,000; bar = 0.25 μm.
(D) In uninvaded xylem vessels, an intense labeling occurs over host secondary walls, whereas the middle lamella is nearly unlabeled. Magnification ×30,000; bar = 0.5 μm.
The abbreviations used are: Cy, cytoplasm; FC, fungal cell; HCW, host cell wall; HSW, host secondary wall; IS, intercellular space; ML, middle lamella; V, vessel; Va, vacuole; Ve, vesicle.

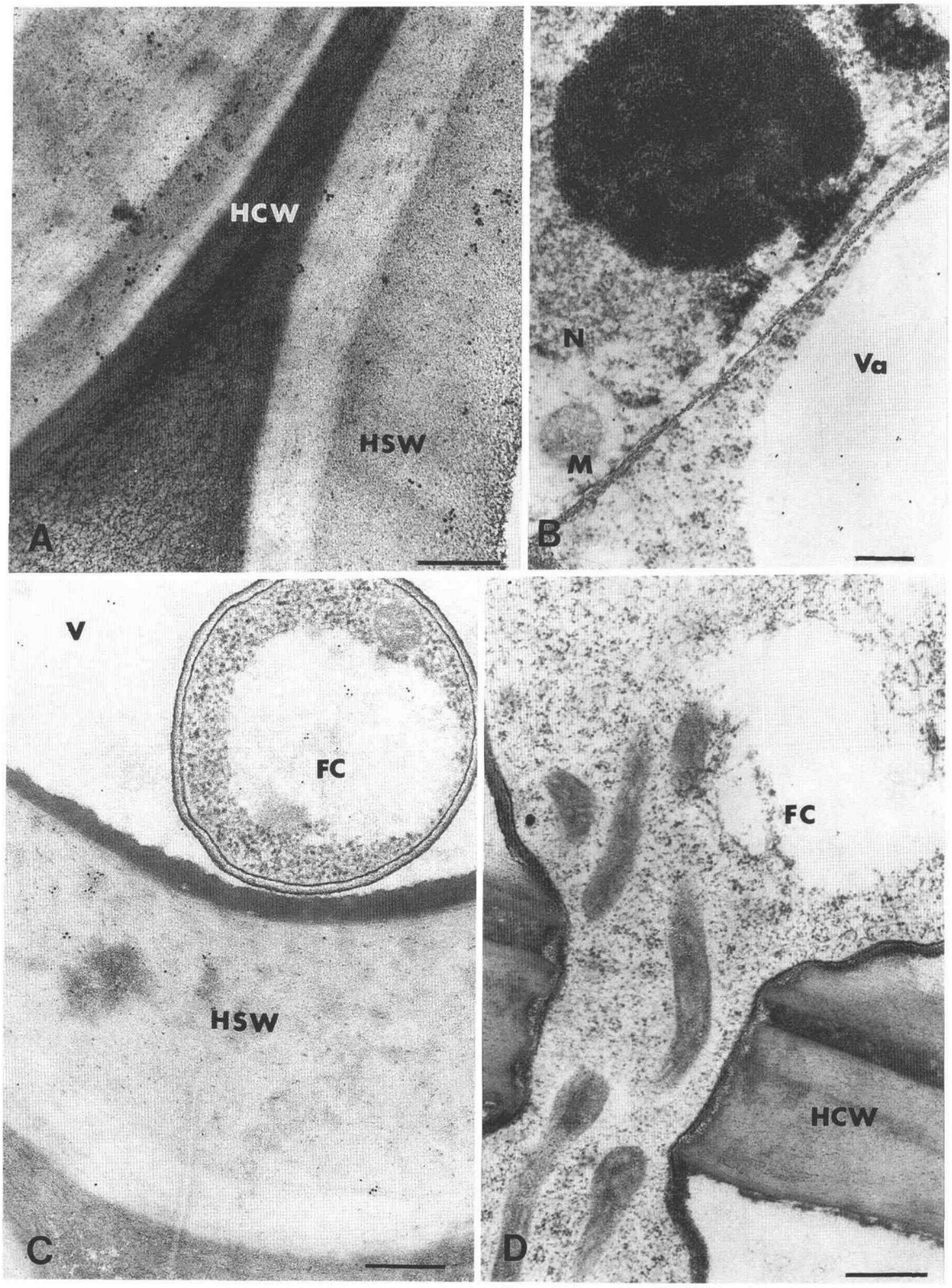

Figure 7. Control Tests.

ble to plant chitinases unless fungal cell walls are previously altered by enzymes such as β-1,3-glucanases.

Although a striking difference between resistant and susceptible tomato cultivars was observed in the rate of production of β-1,3-glucanase, the pattern of enzyme distribution in host tissues was found to be similar. In both interactions, a considerable amount of β-1,3-glucanases was associated with host cell walls, especially with secondary thickenings of xylem vessels, whereas only small quantities of enzyme were detected in vacuoles. In the susceptible tomato cultivar, cell wall accumulation of β-1,3-glucanases occurred mainly in areas adjacent to fungal cells and in secondary thickenings of intensely colonized vessels. By contrast, cell walls of the resistant tomato cultivar showed a generalized enrichment in β-1,3-glucanase soon after inoculation. The presence of β-1,3-glucanases in host cell walls was confirmed by the localization of these enzymes in secondary thickenings of invaded vessels in the compatible interaction between eggplant and *V. dahliae*. In this case, the limited association of β-1,3-glucanase with secondary walls corresponded to a fungal colonization restricted to xylem elements. In both interactions, the middle lamella region of host cell walls was nearly free of significant accumulation of β-1,3-glucanases.

Our immunocytochemical observations contrast with those of Mauch and Staehelin (1989), who found β-1,3-glucanases mainly associated with vacuoles and to a lesser extent with middle lamella regions in ethylene-stressed bean leaf cells. Whether this variation in the pattern of enzyme distribution reflects specific mobilization of β-1,3-glucanase according to the type of elicitor used for induction or is related to a different affinity of the immunological probe remains to be determined. Plant hydrolases are known to be similarly induced by chemical, hormonal, or pathogenic elicitors (Boller, 1985). However, it is possible that they accumulate at different strategic sites according to the nature of the inducing elicitor (Van den Bulcke et al., 1989). In the case of fungus-stressed plant tissues, these hydrolases may first accumulate in vacuoles and later be conveyed toward the cell surface. Such a pattern would explain the low amount of β-1,3-glucanase found in vacuoles of infected tomato and eggplant tissues. In addition to these considerations, the possibility that the antisera used as probes for β-1,3-glucanase detection may have a different affinity should not be ruled out. In the present study, we have used an antiserum raised against one purified acidic (PR-N) β-1,3-glucanase from tobacco (Kauffmann et al., 1987) that cross-reacts with the 33-kD tomato β-1,3-glucanase. However, this antiserum as well as others produced against acidic tobacco β-1,3-glucanases have little affinity for the 35-kD tomato β-1,3-glucanase (Joosten and De Wit, 1989), which is thought to be intravacuolar. It is, thus, possible that the use of a single antiserum may not give a real image of β-1,3-glucanase distribution in plant tissues. In this regard, it would be interesting to study the subcellular localization of these enzymes using also an antiserum raised against a basic β-1,3-glucanase.

In conclusion, the present study brings new insights into the in situ accumulation of β-1,3-glucanases during host-fungus interactions. Although our observations do not prove that these enzymes restrict fungal colonization, they provide additional support for their implication in disease resistance because they were found to accumulate earlier and to a higher extent in resistant than in susceptible cultivars. The accumulation of β-1,3-glucanases at the fungal cell surface does not bring conclusive evidence of an antifungal activity but represents an in situ indication that β-1,3-glucanases interact with fungal cell walls. Whether this accumulation is based on an enzyme-substrate relationship remains to be demonstrated.

METHODS

Fungal Culture

The isolate of *Fusarium oxysporum* f. sp. *radicis lycopersici* Jarvis and Shoemaker (FORL) was kindly supplied by Mr. P.O. Thibodeau (Complexe Scientifique, Sainte-Foy, Québec). It was grown on potato dextrose agar medium at 22°C and reinoculated and reisolated periodically from ripe tomato fruits.

Figure 7. (continued).

(A) and **(B)** Transmission electron micrographs of non-inoculated (control) plants. Treatment with the antiserum raised against a tobacco β-1,3-glucanase (PR-N) and with GAR-gold antibodies. A slight and uneven labeling occurs over secondary walls of two adjacent xylem vessels **(A)**, as well as over vacuoles **(B)**. Organelles such as nucleus and mitochondria are nearly unlabeled. **(A)** Magnification ×54,000; bar = 0.25 μm. **(B)** Magnification ×40,000; bar = 0.25 μm.

(C) and **(D)** Transmission electron micrographs of tomato (susceptible cultivar) infected by FORL, 120 hr after inoculation. Treatment with the β-1,3-glucanase antiserum to which was previously added an excess of its corresponding antigen results in a considerable reduction of labeling over host secondary walls of xylem vessels **(C)**. Similarly, negative results are obtained when preimmune serum is used instead of β-1,3-glucanase antiserum **(D)**. **(C)** Magnification ×45,000; bar = 0.25 μm. **(D)** Magnification ×54,000; bar = 0.25 μm.

The abbreviations used are: FC, fungal cell; HCW, host cell wall; HSW, host secondary wall; M, mitochondria; N, nucleus; V, vessel; Va, vacuole.

Plant Material

Seeds of tomato (*Lycopersicon esculentum* Mill, cv Bonny Best, highly susceptible to FORL, and cv Larma, resistant to FORL) were surface-sterilized in 0.5% (v/v) aqueous sodium hypochlorite for 30 min and sown in vermiculite at a density of five seeds per 6-cm pot. Plants were grown at 22 ± 2°C under normal greenhouse conditions and watered periodically with a nutrient solution.

At the three-leaf stage, tomato seedlings were inoculated with a suspension of microconidia (2×10^6 spores mL^{-1}) deposited on the roots with a sterile syringe. Control plants were treated with distilled water and incubated under the same conditions as the inoculated plants. Roots from inoculated and control plants were collected periodically from 48 hr to 120 hr after inoculation and processed either for electron microscope studies or for PAGE.

Eggplants (*Solanum melongena* cv Imperial black beauty) were grown from seeds and inoculated with conidia of *Verticillium albo-atrum* Reinke and Berth according to a previously described procedure (Bush and Edgington, 1967).

Preparation of Intercellular Fluids

IF from infected tomato and tobacco plants (*Nicotiana tabacum* cv Xanthi-nc) were extracted as previously described (Parent and Asselin, 1984) using 1 mM aqueous L-serine (tomato) or 1 mM aqueous thiamine-HCl (tobacco) as chemical inducers of PR proteins (Asselin, Grenier, and Côté, 1985). IF from plants injected with sterile distilled water were used as controls.

PAGE

IF extracts from infected and healthy plants were analyzed by PAGE under denaturing conditions in 15% (w/v) polyacrylamide gels as previously described (Parent and Asselin, 1984). Electrophoresis was run at 20 mA for 1.5 hr at room temperature. Molecular mass markers ranged from 14.4 kD to 92.5 kD (Bio-Rad) or from 14.3 kD to 200 kD (Bethesda Research Laboratories). Staining of gels was performed with Coomassie Blue G-250 as previously described (Asselin and Grenier, 1985).

Protein Gel Blotting

After electrophoresis, proteins were transferred electrophoretically to 0.45-μm nitrocellulose filters according to Towbin, Staehelin, and Gordon (1979). Nitrocellulose membranes were incubated for 1 hr in a blocking solution consisting of Tris-buffered saline (TBS), pH 7.4, to which were added 10% (v/v) fetal bovine serum, 1% (w/v) BSA, 1% (v/v) blocking reagent (Boehringer-Mannheim), and 0.2% (w/v) sodium azide. They were then incubated overnight in the primary antibody (1:2000 in TBS) raised against tobacco PR-N protein (Kauffmann et al., 1987). After rinsing in TBS containing 0.05% (v/v) Tween 20, nitrocellulose filters were incubated for 1 hr in peroxidase-conjugated goat anti-rabbit immunoglobulins. Antigens were visualized by the horseradish peroxidase conjugate as described in the Bio-Rad technical sheet.

Tissue Processing for Electron Microscopy

Root samples (1 mm^3) were fixed by immersion in 3% (v/v) glutaraldehyde buffered with 0.1 M sodium cacodylate buffer, pH 7.2, for 2 hr at 4°C. They were then dehydrated in a graded ethanol series and embedded in Epon 812. Ultrathin tissue sections were collected on Formvar-coated nickel grids.

Immunogold Labeling

Ultrathin sections of infected and non-inoculated tomato root samples were first incubated on a drop of PBS-ovalbumin, pH 7.4, for 5 min at room temperature. They were then transferred to a drop of normal goat serum diluted 1:20 in PBS-ovalbumin, pH 7.4, for 60 min at room temperature, and incubated with the primary antibody (rabbit anti-PR-N) diluted 1:200 in PBS-ovalbumin, pH 7.4, for 2 hr at 37°C. Grids were rinsed with Tris-HCl, pH 8.2, containing 0.5 M NaCl and 1% (w/v) BSA and incubated on a drop of colloidal gold (10 mn)-conjugated goat antiserum to rabbit immunoglobulins (GAR-gold antibodies), diluted 1:10 in the rinsing buffer, for 30 min at room temperature. After washing with PBS, pH 7.4, and rinsing with distilled water, grids were contrasted with uranyl acetate and lead citrate and examined with a JEOL 1200 EX electron microscope.

Immunocytochemical Controls

Specificity of labeling was assessed by the following control tests: (1) incubation with the antiserum against tobacco β-1,3-glucanase to which was previously added purified β-1,3-glucanase (PR-N) (approximately 1:1 molar ratio); (2) incubation with pre-immune serum diluted 1:100 in PBS-ovalbumin; (3) incubation with GAR-gold antibodies, the primary antibody step being omitted; and (4) successive incubations with primary antiserum, unlabeled goat antiserum to rabbit immunoglobulins, and, finally, GAR-gold antibodies.

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