Resistance Responses of Mycorrhizal Ri T-DNA–Transformed Carrot Roots to Infection by *Fusarium oxysporum* f. sp. *chrysanthemi*

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ABSTRACT

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The influence exerted by arbuscular mycorrhizal fungi in stimulating plant defense reactions was investigated using an in vitro system in which Ri T-DNA–transformed carrot roots were infected with *Fusarium oxysporum* f. sp. *chrysanthemi*. This experimental model, based on the use of root-organ culture to obtain typical mycorrhizal infections in axenic conditions, was selected as a reliable means of studying the events associated with subsequent pathogen attack. Cytological investigations of root samples revealed striking differences in the extent of plant defense reactions between mycorrhizal and nonmycorrhizal carrot roots after inoculation with the fungal pathogen. In nonmycorrhizal roots, the pathogen multiplied abundantly through much of the tissue, including the vascular stele, whereas in mycorrhizal roots, pathogen growth was restricted to the epidermis and the outer cortex. The accumulation of an unusual material in both colonized and noncolonized host cells and the coating of most intercellular spaces with similar substances were among the most typical features of host reactions. The newly formed deposits were free of cellulosic or pectic substances, as judged by gold labeling. According to their texture and electron density, the possibility that such deposits may be infused with phenolics has been suggested. Support for this hypothesis came from the observation that the deposited material often interacted physically with the walls of invading hyphae exhibiting morphological changes and cytological alterations. These observations lead us to suggest that this material, in addition to acting as a barrier to fungal spread, also may display a fungitoxic activity. A few electron-opaque structures resembling the deposits found in mycorrhizal-infected carrot roots were seen in some cells and intercellular spaces of noninfected, mycorrhizal carrot roots. By contrast, they were absent in cells of infected, nonmycorrhizal carrot roots. Cytochemical labeling of chitin using wheat germ agglutinin, a lectin with *N*-acetylglucosamine–binding specificity, in conjunction with gold-complexed ovomucoid, showed that disorganization of *Fusarium* hyphae colonizing mycorrhizal roots correlated with chitin degradation. These observations provided indirect evidence for the production of plant chitinases. However, the finding that some chitin molecules still occurred over cell walls of empty hyphae was taken as an indication that chitinases were probably not among the first determinants in the sequence of events leading to the establishment of plant resistance. Thus, restriction of pathogen growth together with an increase in hyphal alterations and accumulation of new plant products appears to be a typical feature observed in mycorrhizal carrot roots. Because these reactions were not seen in nonmycorrhizal roots, it is likely that mycorrhizal infection is responsible, at least in part, for the activation of the plant defense system that, in turn, provides enhanced protection against pathogen attack.

Additional keywords: gold cytochemistry, hydrolytic enzymes, induced resistance, phenolic compounds, ultrastructure.

In recent years, the management of crop diseases caused by root pathogens has become one of the most challenging research areas in plant pathology (17). Increasing knowledge and concern about environmental consequences of fungicide applications have prompted scientists to explore the potential for alternative strategies of disease and pest management (10,27,28). Among the suggested strategies that are promising for minimizing damage from plant pathogens, biological control of pathogenic populations by microorganisms such as *Trichoderma* spp. (16) and induced systemic resistance in plants (35) are two of the most talked about yet controversial issues in agriculture. Another exciting alternative

strategy that has not yet received much attention concerns the potential value of arbuscular mycorrhizal (AM) fungi in promoting plant disease resistance (19,25). Although most efforts have been concentrated on the beneficial effects of AM fungi in increasing phosphorus and other nutrient uptake and stimulating plant growth (35), some lines of evidence have shown convincingly that inoculation of a host plant with an AM mycorrhizal fungal symbiont could reduce incidence and severity of root diseases in a large number of crops (13,18,19,34).

The exact mechanisms by which AM fungi contribute to restrict fungal colonization in root tissues are not fully understood, although a number of hypotheses, including microbial competition for nutrients and increased host phosphorus levels (24), have been raised. However, these explanations are still a matter of speculation, and the role of phosphorus in disease resistance remains controversial (34). Although water status of the surrounding environment as well as translocation features of AM fungi may be responsible, in part, for the enhanced protection of plant roots against attack by pathogens, the possibility that mycorrhizal infections sensitize the plant to defend itself through the activation of defense genes is of particular interest. A growing body of evidence from several investigations indicates that increased resistance of AM roots could be associated, at least in part, with marked host metabolic changes, including enhanced production of phenolic compounds (19,26), accumulation of hydrolases such as chitinases with antimicrobial potential (37), and deposition of structural polymers such as lignin (20) and hydroxyproline-rich glycoproteins (11). If one considers that increased production of phenolic compounds may be of key importance in the resistance process due to their multifaceted role (2) and that accumulation of structural substances may increase the mechanical strength of host cell walls, induction of such defense mechanisms by AM fungi would likely inhibit or at least restrict pathogen invasion. At present, the situation is not clearly defined, and contradictory results about the accumulation of hydrolases (29) and deposition of structural substances (20) have been reported. Additional research is obviously needed to confirm that effective stimulation of the plant defense system occurs after mycorrhizal infection.

As a preface to further investigations of the effect of mycorrhizal infection on the induced resistance of whole plants to pathogen attack, the present study was undertaken to gain better insight into the influence exerted by AM fungi in stimulating plant defense mechanisms. An in vitro system, previously designed by Bécard and Fortin (4) to study the sequence of colonization steps taking place during the first days of contact between plants and AM fungi, was used to investigate the cytology of infection of mycorrhizal and nonmycorrhizal Ri T-DNA–transformed carrot roots by *Fusarium oxysporum* f. sp. *chrysanthemi*, a root pathogen of *Chrysanthemum* sp. against which nontransformed carrot is normally resistant. The advantages of such an experimental system include the absence of confounding effects caused by some undesirable contaminants in nature and the ability to precisely monitor the events associated with pathogen attack in mycorrhizal roots.

MATERIALS AND METHODS

Ri T-DNA–transformed carrot roots. Transformed carrot (*Daucus carota* L.) roots were obtained from Y. Piché, Faculté de Foresterie, Université Laval, Québec. They were prepared according to the procedure described by Bécard and Fortin (4). Maintenance of transformed carrot roots was made on modified White's medium solidified with 0.4% (w/v) gellan gum (ICN Biochemical Inc., Cleveland).

Fungal cultures and growth conditions. Spores of *Glomus intraradix* Schenck & Smith were provided by V. Furlan (Agriculture Canada, Sainte-Foy, Québec). They were recovered from the rhizosphere of mycorrhizal leeks (*Allium porrum* L.) grown in a calcined montmorillonite clay (IMC Imcore, Mundelein, IL). The spores were extracted by density-gradient centrifugation in diatrizoate meglumine (Winthrop Laboratories, Aurora, Ontario, Canada) after wet-sieving of soil. Spores were then washed by centrifugation for 1–2 min in sterile distilled water containing one drop of Tween 80, surface-sterilized twice by soaking for 10 min in 2% (w/v) chloramin T in a vacutainer-tube, and rinsed five times for 1–2 min in 1% (w/v) streptomycin sulfate with 0.5% (w/v) gentamycin sulfate solution. Spores were kept overnight at 4 C in the antibiotic solution, and the surface-sterilization procedure was repeated. Finally, the spores were spread on a 1.5% (w/v) water-agar plate.

The root pathogen *F. o. chrysanthemi* (ATCC 66279) was routinely grown on potato-dextrose agar (Difco Laboratories, Detroit) in a dark incubator at 26 C.

Establishment of mycorrhizal infection. A minimal medium previously described by Bécard and Fortin (4), but solidified with 0.4% (w/v) gellan gum instead of 1% (w/v) Bacto-agar, was used to initiate mycorrhizal colonization. Mycorrhizal colonization was achieved by placing 10–15 spores of *G. intraradix* on petri dishes containing a 2-cm-long vigorous transformed carrot root apex on minimal medium. Plates were incubated in the dark at 27 C. Plates in which the mycorrhizal fungus had contacted the root and had started growing and forming spores around root segments were selected for dual cultures with the pathogen.

Inoculation of mycorrhizal carrot roots with *Fusarium*. The mycelium of a 6-day-old colony of *F. o. chrysanthemi* was scraped from the surface of a petri plate and homogenized for 5 s in 15 ml of sterile distilled water in a Waring blender. Twenty 9-day-old cultures of mycorrhizal- and nonmycorrhizal-transformed carrot roots growing at the surface of the minimal medium were inoculated with 0.5 ml of the *F. o. chrysanthemi* hyphal and spore suspension or with 0.5 ml of sterile distilled water. The pathogen then was allowed to grow for 7 days in contact with the roots before tissue processing for electron microscopy.

Tissue processing for transmission electron microscopy. Samples (2 mm^3) were carefully excised from mycorrhizal and nonmycorrhizal carrot roots at sites of pathogen penetration detected by the formation of local lesions. They were immediately immersed in 3% (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2, for 2 h at room temperature and postfixed with 1% (w/v) osmium tetroxide in the same buffer for 1 h at 4 C. Samples were dehydrated in a graded ethanol series and finally embedded in Epon 812 (JBEM Chemical Co., Pointe-Claire, Québec, Canada). Ultrathin sections were collected on Formvar-coated nickel grids and were either contrasted with uranyl acetate and lead citrate for direct examination with a JEOL 1200 EX electron microscope (Tokyo) at 80 kV or processed for cytochemical labeling. For each treatment (mycorrhizal uninoculated; mycorrhizal inoculated; nonmycorrhizal uninoculated; nonmycorrhizal inoculated), an average of five samples from three roots was examined with five sections per sample.

Preparation of the colloidal gold probes. Colloidal gold with particles averaging 15 nm in diameter were prepared according to Frens (22). Wheat germ agglutinin (WGA), a lectin with *N*-acetylglucosamine–binding specificity, was used for localizing *N*-acetylglucosamine residues (chitin) according to a previously described procedure (5). Because of its low molecular weight, this lectin could not be directly complexed to colloidal gold. It was used in a two-step procedure, using ovomucoid as a second-step reagent. Ovomucoid was conjugated to gold at pH 5.4 (5).

For the localization of cellulosic β-1,4-glucans, an exoglucanase (β-1,4-D-glucan cellobiohydrolase, EC 3.2.1.21) purified from a cellulase produced by the fungus *Trichoderma harzianum*, was complexed to colloidal gold at pH 9.0 and used in a one-step procedure (7).

The *Aplysia* gonad lectin (AGL), a lectin isolated from the gonads of the sea mollusk *Aplysia depilans*, was used to localize polygalacturonic acid-containing molecules (pectin) (8). This lectin was complexed to colloidal gold at pH 9.5.

Cytochemical labeling. For the indirect labeling of *N*-acetylglucosamine residues, sections were first floated on a drop of phosphate buffered saline (PBS), pH 7.4, for 5 min, then transferred to a drop of WGA (25 μg/ml in PBS, pH 7.4) for 60 min at room temperature in a moist chamber. After washing with PBS, pH 7.4, sections were incubated on a drop of the ovomucoid-gold complex for 30 min at room temperature.

Sections were washed with PBS, rinsed with distilled water and contrasted with uranyl acetate and lead citrate.

For the direct labeling of cellulosic β-1,4-glucans and polygalacturonic acids, sections were first incubated on a drop of PBS containing 0.02% (w/v) of polyethylene glycol (PEG) for 5 min at room temperature. The pH of the PBS-PEG was adjusted according to the pH of optimal activity of each protein (pH 6.0 for the exoglucanase and pH 8.0 for the AGL). Thereafter, sections were transferred to a drop of the gold-complexed probe for 30 min at room temperature in a moist chamber. After washing with PBS and rinsing with distilled water, grids were contrasted as described above.

Cytochemical controls. Specificity of the labelings was assessed by the following control tests: 1) incubation with WGA, to which an excess of *N-N'-N"*-triacetylchitotriose (1 mg/ml in PBS) was previously added; 2) incubation with WGA, followed by unlabeled ovomucoid and finally by ovomucoid-gold complex; 3) incubation with the gold-complexed exoglucanase or AGL to which the corresponding substrate (β-1,4-glucans from barley for the exoglucanase and polygalacturonic acids from citrus for the AGL, 1 mg/ml in PBS) was previously added; and 4) incubation with stabilized or unstabilized gold suspension.

Reagents. The exoglucanase was provided by C. Breuil, Forintek, Canada, and the AGL was obtained from N. Gilboa-Garber, Bar Ilan University, Israël. Tetrachloroauric acid was purchased from BDH Chemicals, Montréal. All other reagents for electron microscopy were obtained from JBEM Chemical Co., Pointe-Claire, Québec.

RESULTS

Cytology of infection of Ri T-DNA-transformed carrot roots by *F. o. chrysanthemi*. Examination of samples from Ri T-DNA-transformed roots collected 7 days after inoculation with *F. o. chrysanthemi* revealed that fungal colonization was intense and paralleled marked host tissue damage (Fig. 1A). Although this pattern of fungal growth may be surprising, at first sight, because this fungus is not known to be pathogenic on carrot, it is likely that root transformation through the introduction of the A_4 *Agrobacterium rhizogenes* strain (4) is responsible for the loss of genetic resistance. Observation of a large number of sections showed that hyphae had ramified abundantly through much of the cortex, endodermis, and paratracheal parenchyma cells and reached the xylem vessels by centripetal growth (Fig. 1A). Fungal growth was mainly intracellular but also occurred intercellularly (Fig. 1A, small arrow) and sometimes intramurally. Cell invasion through host-wall penetration was frequently observed (Fig. 1B). Channels of penetration were usually narrower than the hyphal diameter and were associated with wall displacement in the growth direction (Fig. 1B, arrow).

Pathogen ingress toward the vascular stele coincided with extensive cell alterations, such as plasmolysis and aggregation of the cytoplasm, organelle disintegration, and host cell-wall breakdown (Fig. 1A). In massively invaded root cells, host cell walls were markedly altered, as judged by their decrease in electron density and loss of structural integrity (Fig. 1A, arrowheads). Dissolution of middle lamella matrices and disruption of primary walls also were observed frequently. In some colonized areas, host cell walls were no longer discernible (Fig. 1A, large arrow). Cell breakdown due to host cell-wall disruption was associated with tissue maceration and coincided with the presence of macroscopically visible lesions in the main and lateral roots. Fungal hyphae growing in these host tissues displayed a typical ultrastructure characterized by a regular cytoplasm that appeared closely appressed against the thin cell wall (Fig. 1B). Characteristic features of host reactions such as wall appositions, intracellular deposits, intercellular plugging, and xylem vessel coating were not detected (Fig. 1A).

Cytology of infection of Ri T-DNA-transformed carrot roots by *G. intraradix*. Examination of root samples inoculated with the AM fungus *G. intraradix* showed a high preservation of the host cell integrity in all tissues (Fig. 1C). Cells of the symbiont were usually detected in the epidermis (not shown) but were seldom seen in most inner tissues at this early stage of symbiosis (Fig. 1C). Signs of host cell-wall alterations similar to those observed in *Fusarium*-infected carrot roots were never observed. Similarly, cytoplasm breakdown did not occur. Host cells in the parenchyma were characterized by a large, centrally located vacuole and by a layer of dense cytoplasm appressed against the cell wall (Fig. 1C). Although at first sight these host cells resembled those observed in uninoculated, healthy tissues (not shown), a close examination revealed the accumulation of unusual structures that were either embedded in the cytoplasm (Fig. 1C') or free in the vacuole (Fig. 1C, arrow). These electron-opaque structures displayed an amorphous texture and were usually spherical in shape although, in some cases, they appeared more elongated and branched (Fig. 1C'). They were not restricted to colonized, epidermal host cells because they also occurred in vascular parenchyma cells. Another reaction feature, which was detected in only a few (10%) intercellular spaces, was the deposition of an osmiophilic matrix in which were embedded amorphous globules (Fig. 1D). Such globules were polymorphic and appeared to be made of a material that was structurally different from that of the intracellular spherical structures in terms of texture and electron density. Except for these two types of reactions, other host responses such as formation of wall appositions and vessel coating were not seen.

Cytology of infection of mycorrhized Ri T-DNA-transformed carrot roots by *F. o. chrysanthemi*. Preinfection of carrot roots with *G. intraradix* prior to inoculation with *F. o. chrysanthemi* did not halt pathogen spread in the host tissues. However, marked changes in the pattern of fungal colonization were observed. Pathogen growth was usually restricted to the epidermis and the cortical area although, in some cases, a few fungal cells were seen in parenchyma cells neighboring xylem vessels. Cells of the AM symbiont also were restricted to the epidermis, and physical contact between the AM fungus and the pathogen was never observed (Fig. 2A). However, examination of about 50 sections revealed that most hyphae of the pathogen (80%) exhibited considerable changes in both morphology (Fig. 2C) and ultrastructure (Fig. 2B). These changes included a pronounced disorganization of the cytoplasm that was most often accompanied by the involution of vacuole membranes and the formation of polymorphic vesicles (Fig. 2B). In addition, the plasma membrane was no longer discernible, and the innermost wall layers appeared to suffer from some damage, as judged by their irregular aspect (Fig. 2B, arrows). Lipid bodies often were the only preserved organelles.

Cell alterations of the pathogen were closely associated with an increased expression of host reactions. One of the most typical host reactions was the deposition of an electron-dense material lining the primary walls in all infected intercellular spaces (Fig. 2C). This material usually extended toward the inside to form polymorphic deposits that frequently physically interacted with the wall of invading hyphae of the pathogen (Fig. 2C, arrows). Interestingly, host cell walls appeared well preserved and of higher electron density than normal. Another typical reaction that was observed in about 50% of the infected host cells was the formation of hemispherical protuberances at the point of fungal penetration (Fig. 2D). These protuberances, which resembled papillae, were irregular in size and shape and appeared heterogeneous in nature.

Besides the plugging of intercellular spaces and the formation of papilla-like structures at sites of attempted fungal penetration, striking host-cell reactions were frequently observed in the inner cortex (Fig. 3). These reactions were mainly characterized by the deposition of an amorphous, electron-opaque material coating the host cell walls and surrounding the invading hyphae by means of elongated strands of aggregated material (Fig. 3A). Hyphal cells trapped by this material were always abnormally shaped and showed various degrees of alteration, including distortion and retraction of the plasma membrane (Fig. 3B and C) as well as cell-wall disruption in places (Fig. 3C, arrows). In some host cells (about 20% of the examined cells), the pathogen was reduced to empty hyphal shells neighboring osmiophilic deposits (Fig. 3D). Such host reactions were associated with a considerable restriction of fungal growth toward the vascular stele.

Cytochemical localization of *N*-acetylglucosamine residues. Application of the WGA-ovomucoid-gold complex to sections of *Fusarium*-infected carrot roots that were nonmycorrhizal resulted in a regular deposition of gold particles over hyphal cell walls (Fig. 4A). Cytoplasm, organelles, and vacuoles were nearly unlabeled. Over the thin cell wall, gold particles appeared to be preferentially associated with the innermost layers (Fig. 4A).

When WGA-ovomucoid-gold complex was applied to sections of mycorrhizal carrot roots inoculated with *F. o. chrysanthemi*, a marked decrease in labeling intensity was observed over the

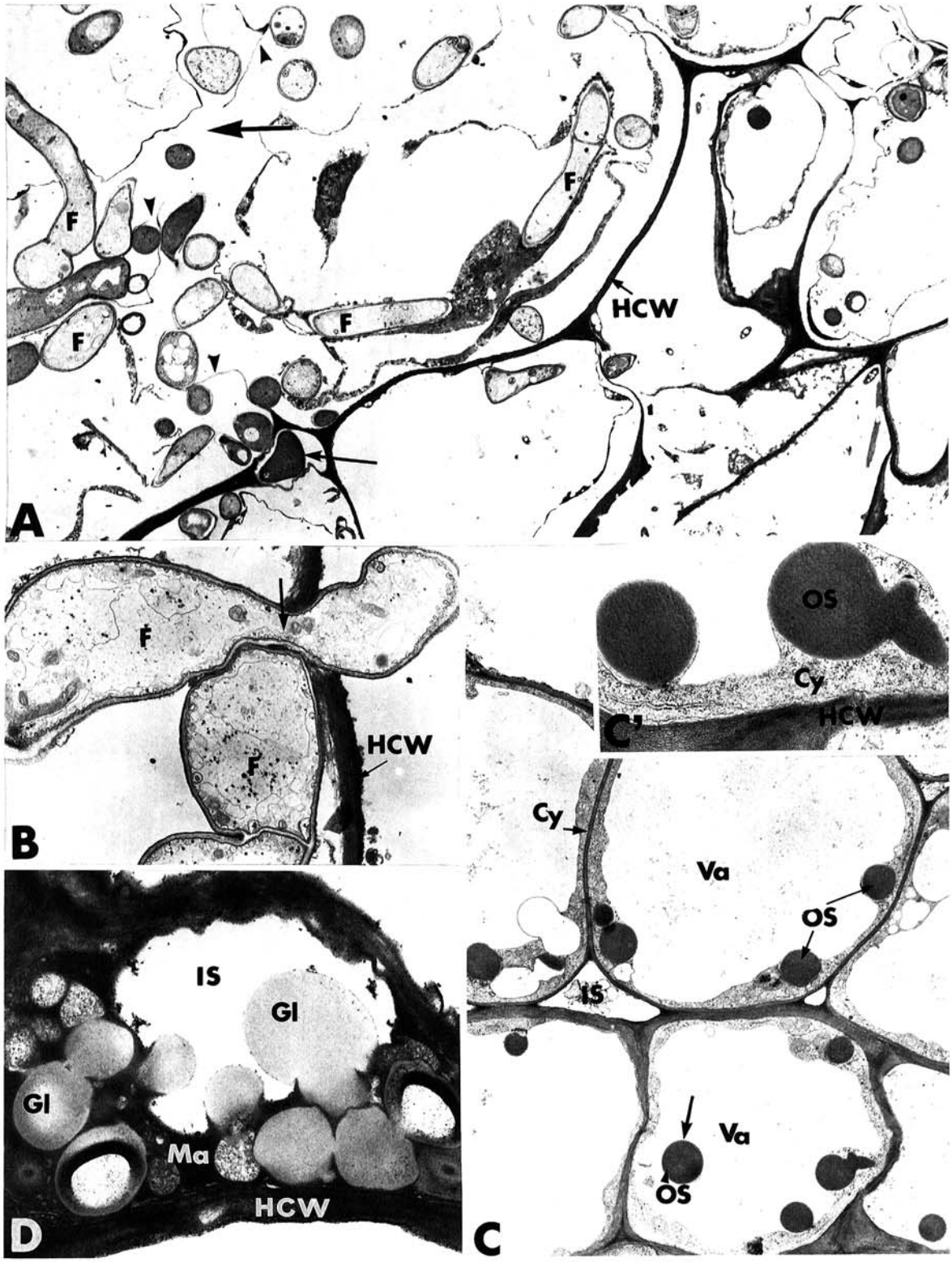

Fig. 1. Transmission electron micrographs of Ri T-DNA–transformed carrot roots infected either with *Fusarium oxysporum* f. sp. *chrysanthemi* **(A and B)** or with *Glomus intraradix* **(C and D)**. **A,** The pathogen (F) multiplies abundantly in the root tissues. Fungal growth is mainly intracellular but also occurs intercellularly (small arrow). Fungal colonization is associated with marked host cell wall (HCW) alterations, ranging from loss of electron density (arrowheads) to complete disruption (large arrow). ×2,700. **B,** A host cell wall (HCW) is penetrated by a fungal cell (F). The channel of penetration is narrower than the average hyphal diameter (arrow). ×9,000. **C and C',** Root cells infected by *G. intraradix* show good ultrastructural preservation. The only sign of reaction is the formation of electron-opaque structures (OS) that are either free in the vacuole (Va) **(C,** arrow) or embedded in the cytoplasm (Cy) **(C')**. IS = intercellular space. **C,** ×4,500; **C',** ×9,000. **D,** An osmiophilic matrix (Ma) is accumulating in an intercellular space (IS). Amorphous globules (Gl) are formed over this matrix. ×14,500.

pathogen cell walls (Fig. 4B–D). Gold particles appeared to be more randomly distributed over the walls of empty fungal cells (Fig. 4B). In some cases, the release of labeled fungal wall fragments could be seen (Fig. 4B, arrowheads). In contrast, the electron-opaque material lining the host primary walls (Fig. 4D) and surrounding the invading hyphae was free of labeling (Fig. 4B). Labeling also was distributed unevenly over hyphae that did not show extensive damage (Fig. 4C). Most interesting was the complete absence of labeling over the fungal cell wall areas neighboring the host cell wall (Fig. 4C, arrow).

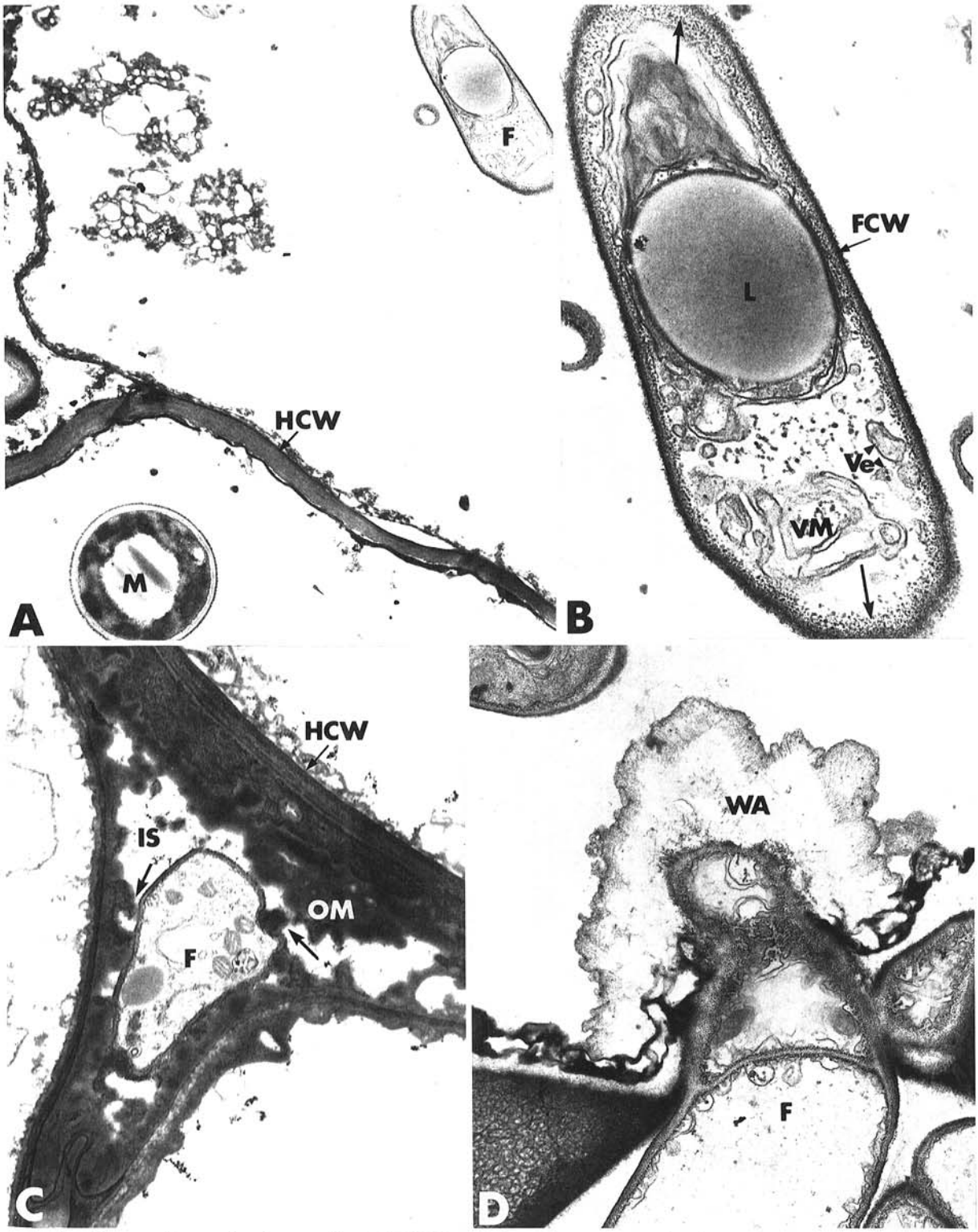

Fig. 2. Transmission electron micrographs of mycorrhizal Ri T-DNA–transformed carrot roots after inoculation with *Fusarium oxysporum* f. sp. *chrysanthemi*. **A,** The symbiont (M) is seen only in the epidermis. The pathogen (F) is apparently damaged. HCW = host cell wall. ×9,000. **B,** Portion of **A** at a higher magnification showing the marked alteration of a pathogen cell. The cytoplasm is disorganized, and the only discernible organelle is a lipid body (L). The plasma membrane is no longer visible, and the innermost wall layers are apparently altered (arrow). Vesicles (Ve) as well as vacuolar membranes (VM) are seen in the space previously occupied by the cytoplasm. FCW = *Fusarium* cell wall. ×27,000. **C,** A *Fusarium* cell (F) with an abnormal shape is colonizing an intercellular space (IS) coated by a band of osmiophilic material (OM) that interacts with the pathogen cell wall (FCW) (arrows). ×14,500. **D,** A wall apposition (WA) is formed at the site of attempted penetration by a *Fusarium* cell (F). This wall apposition is irregular in shape and appears heterogeneous in nature. ×14,500.

All control tests, including previous adsorption of WGA with *N-N'-N"*-triacetylchitotriose yielded negative results (not shown).

Cytochemical localization of cellulosic β-1,4-glucans. In nonmycorrhizal carrot roots, the massive host-tissue colonization by *F. o. chrysanthemi* was responsible for extensive host cell-wall damage. After incubation with the gold-complexed exoglucanase, a significant number of gold particles was associated with these altered host cell walls (Fig. 5A). In most cases, the occurrence of gold particles was indicative of the presence of a host cell wall that was no longer discernible ultrastructurally (Fig. 5A).

In mycorrhizal carrot roots, host cell walls of *Fusarium*-infected tissues showed a higher preservation than those in nonmycorrhizal

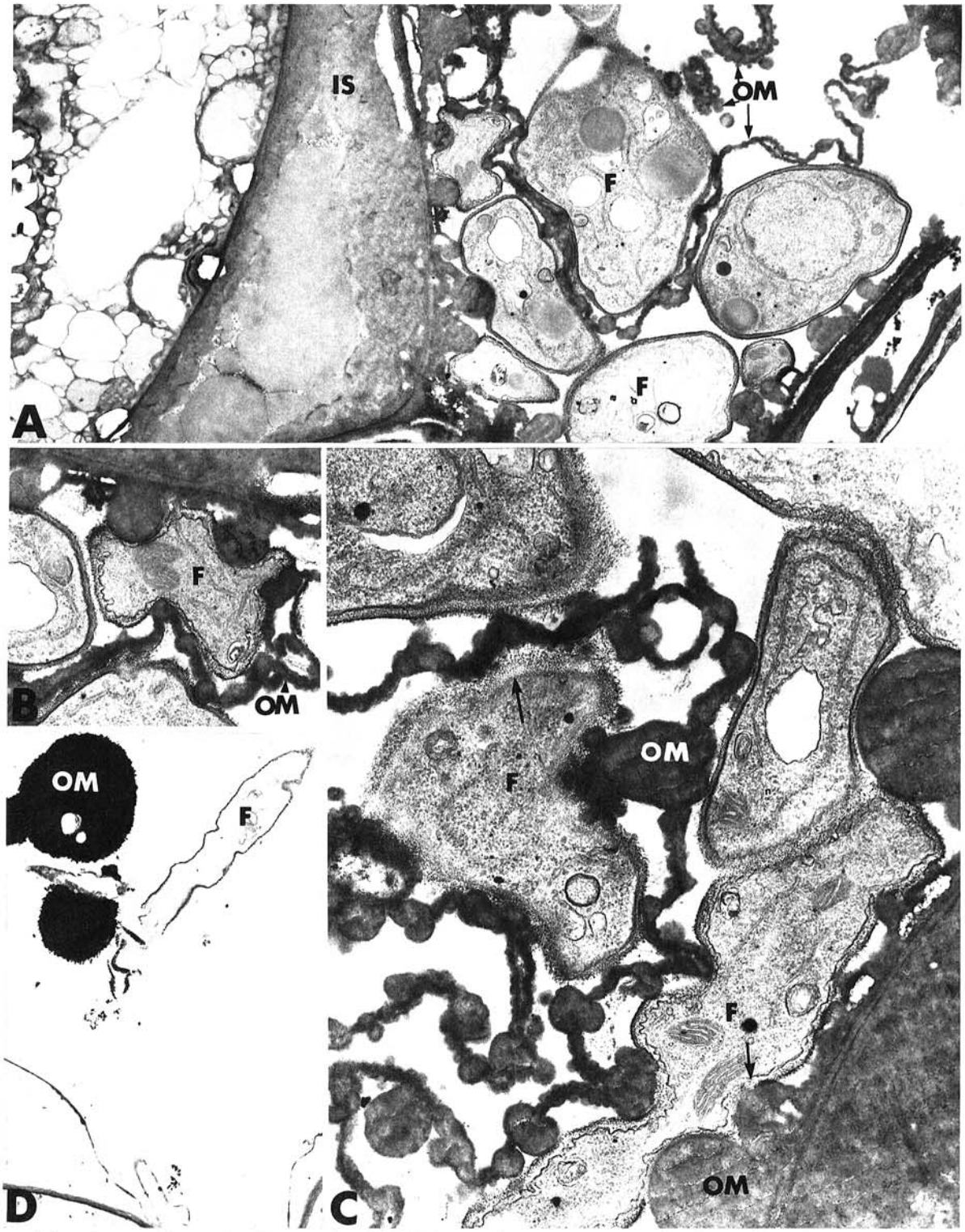

Fig. 3. Transmission electron micrographs of mycorrhizal Ri T-DNA–transformed carrot roots after inoculation with *Fusarium oxysporum* f. sp. *chrysanthemi*. **A,** A strong host reaction characterized by extensive production of electron-opaque material (OM) is seen in a colonized host tissue area. The intercellular space (IS) is completely plugged by the dense material that extends into the adjacent host cell and surrounds the invading hyphae of the pathogen. F = *Fusarium* cell. ×9,000. **B,** Portion of **A** at a higher magnification showing the physical interaction of the electron-opaque (OM) material with a cell of the pathogen (F). Considerable changes in fungal morphology are observed. ×18,000. **C,** Cells of *F. o. chrysanthemi* (F) in close contact with the electron-opaque material (OM) are abnormally shaped and suffer from marked damage, including cell wall disruption in places (arrows). ×36,000. **D,** In some cells, the pathogen is reduced to an empty hyphal shell (F). ×5,500.

roots (Fig. 5B–D). Treatment with the gold-complexed exoglucanase resulted in a specific deposition of gold particles over the host cell walls, whereas the amorphous material coating some cells (Fig. 5B) and plugging some intercellular spaces (Fig. 5D) was unlabeled. Similarly, wall appositions formed between the host cell wall and the retracted plasma membrane were free of labeling (Fig. 5C). Control tests performed to assess the specificity of labeling were negative (not shown).

Cytochemical localization of pectic substances. Application of AGL to sections of *Fusarium*-infected carrot roots resulted in

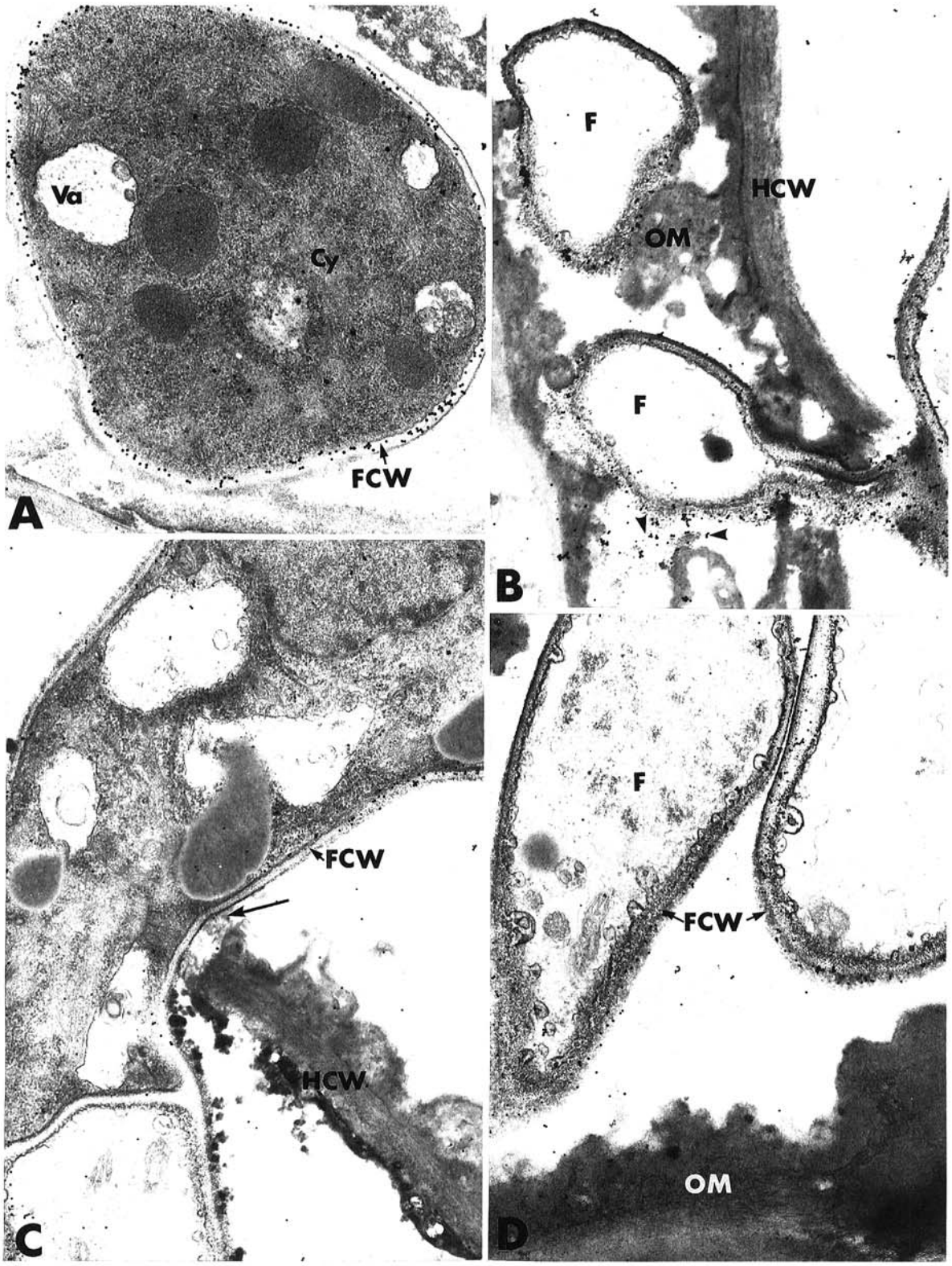

Fig. 4. Transmission electron micrographs of mycorrhizal Ri T-DNA–transformed carrot roots after inoculation with *Fusarium oxysporum* f. sp. *chrysanthemi*. Labeling with the wheat germ agglutinin-ovomucoid-gold complex for localization of *N*-acetylglucosamine residues (chitin). **A,** Control, absence of mycorrhization. The cell wall of the pathogen (FCW) is regularly labeled. Gold particles appear preferentially located over the innermost wall layers. Cy = cytoplasm; Va = vacuole. ×45,000. **B,** In mycorrhizal carrot roots, the pathogen cell walls (F) are more irregularly labeled. Detached fragments, apparently released from the pathogen cell walls, are labeled (arrowheads). The osmiophilic material (OM) surrounding the invading hyphae is unlabeled. HCW = host cell wall. ×21,500. **C,** Labeling disappears over fungal wall (FCW) areas adjacent to the host cell wall (HCW) (arrow). ×21,500. **D,** A noticeable decrease in fungal cell wall (FCW) labeling is observed. OM = osmiophilic material. ×21,500.

a near absence of gold labeling over host primary walls and middle lamellae (Fig. 6A).

In contrast, a more intense labeling was observed over host cell walls and middle lamella matrices of mycorrhizal root tissues (Fig. 6B). The electron-opaque, amorphous material found in reactive host cells was unlabeled (Fig. 6B). However, in many instances, this material was decorated at its periphery by gold particles (Fig. 6D, arrowheads). Another interesting feature was the occurrence of aggregated fragments that were specifically labeled (Fig. 6C, arrows). These fragments accumulated in the lumen of some host cells and apparently impregnated the cell wall of invading hyphae (Fig. 6C, arrowheads).

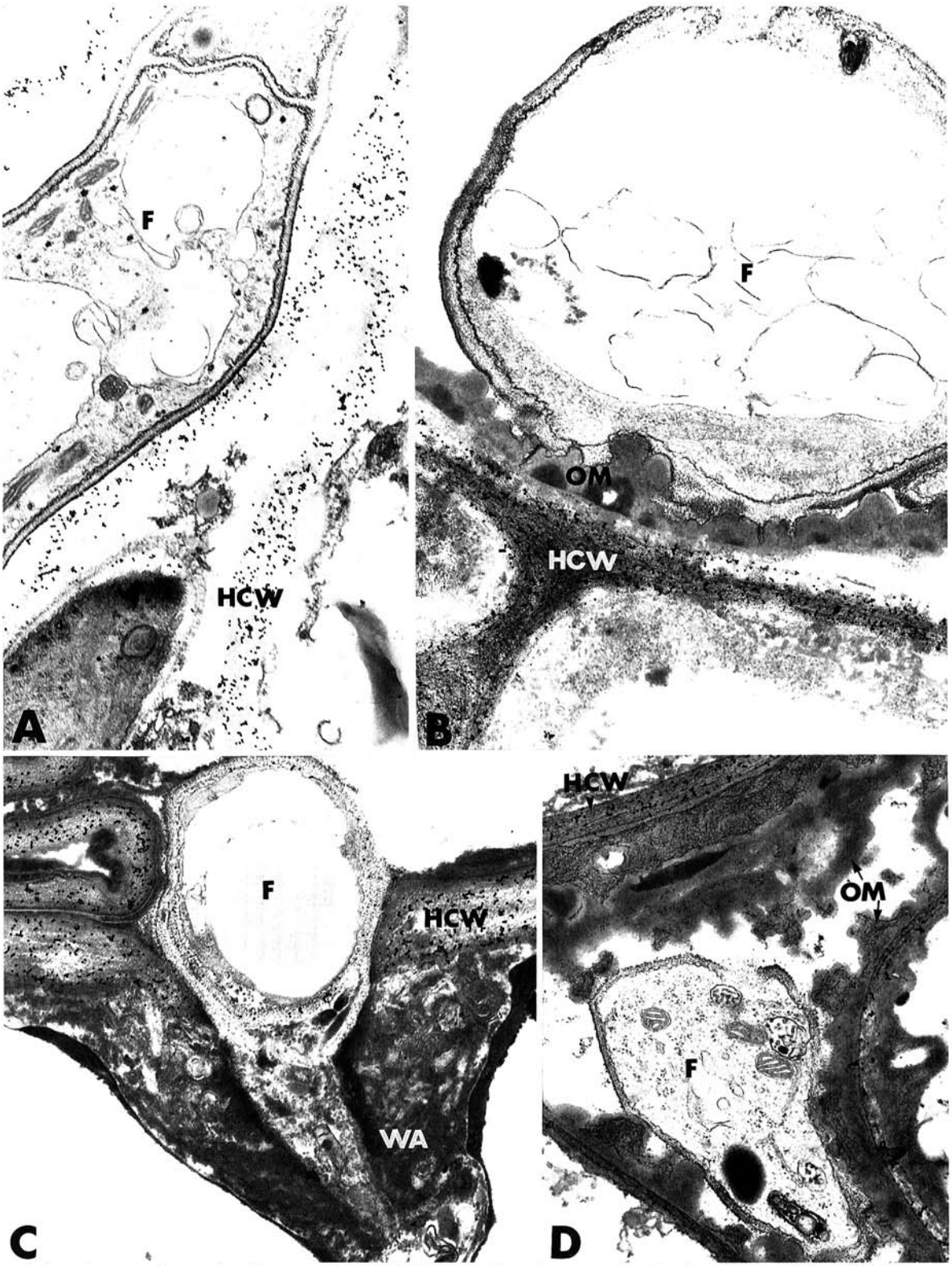

Fig. 5. Transmission electron micrographs of mycorrhizal Ri T-DNA–transformed carrot roots after inoculation with *Fusarium oxysporum* f. sp. *chrysanthemi*. Labeling with the exoglucanase-gold complex for the localization of cellulosic β-1,4-glucans. **A,** Control, nonmycorrhized carrot roots. Gold particles are regularly distributed over altered host cell walls (HCW). F = *Fusarium* cell. ×27,000. **B,** In mycorrhized carrot roots, an intense labeling occurs over the host cell walls (HCW), whereas the osmiophilic material (OM) formed in response to infection is unlabeled. ×45,000. **C,** A wall apposition (WA) formed between the host cell wall (HCW) and the retracted plasma membrane is free of labeling. ×36,000. **D,** The electron-opaque material (OM) plugging an intercellular space is unlabeled. ×36,000.

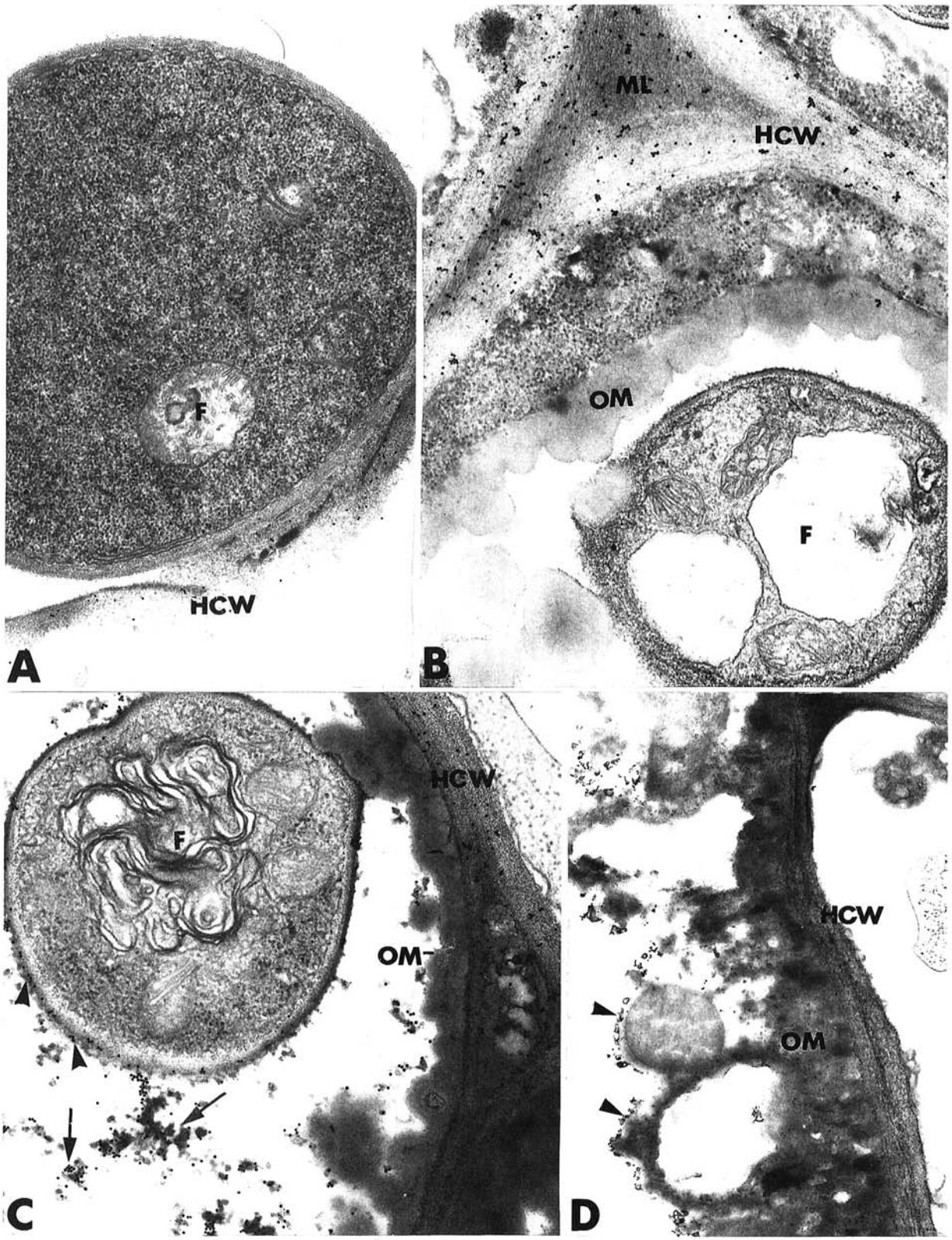

Fig. 6. Transmission electron micrographs of mycorrhizal Ri T-DNA–transformed carrot roots after inoculation with *Fusarium oxysporum* f. sp. *chrysanthemi*. Labeling with the *Aplysia* gonad lectin-gold complex for the localization of pectic substances. **A,** Control, nonmycorrhizal carrot roots. The altered host cell wall (HCW) is nearly unlabeled. F = *Fusarium* cell. ×27,000. **B,** In mycorrhizal carrot roots, gold particles occur over host primary walls (HCW) and middle lamellae (ML). By contrast, the electron-opaque material (OM) coating the host cell is unlabeled. ×54,000. **C,** Labeled fragments of aggregated material (arrows) are seen inside a host cell. These fragments apparently interact with the cell wall of invading hyphae (arrowheads). ×36,000. **D,** The electron-dense coating material (OM) is decorated at its periphery by gold particles (arrowheads). ×21,500.

Control tests, including previous adsorption of AGL with polygalacturonic acids, resulted in an absence of labeling over host cell walls and middle lamellae (not shown).

DISCUSSION

In the past 20 years, correlations between endomycorrhizal infections and enhanced resistance to soilborne pathogens have been established in a number of host-pathogen interactions, and this topic has been reviewed recently (12,19,23,33). Although much attention has been paid to such correlations, the exact mechanisms underlying root protection by AM fungi against pathogen infection remains poorly understood and, in many cases, controversial (25,30). Several hypotheses have been put forward to explain the role that AM fungi may play in plant protection against root pathogens, but very few of them have been convincingly assessed through biochemical and cytological investigations of plant tissues challenged by pathogens. Indeed, most studies have been concerned with the possible induction of defense mechanisms in mycorrhizal plants (11,20,38,39) and have suggested that such reactions could be involved in subsequent pathogen inhibition (20,25). Such speculations should be viewed with caution because these studies did not specifically investigate the role of pathogen attack in the overall expression of plant defense mechanisms in mycorrhizal roots.

The results of the present study demonstrate that mycorrhizal carrot roots afford increased protection against *F. o. chrysanthemi* and that this protection is associated, at least in part, with the accumulation of newly formed plant products at sites of fungal penetration. To our knowledge, this is the first report on the effect of mycorrhizal infection on the cytology of fungal colonization in a nonhost plant. The rationale for the use of an experimental model with transformed carrot roots is that not only this system could provide a means to avoid the influence of other parameters, such as competition for nutrients in the substrate, but it also could allow the precise and detailed investigation of cellular events related to mycorrhizal-mediated induced resistance. Because root receptivity to mycorrhizal colonization is greatly affected by the age and speed of tissue differenciation, the use of Ri T-DNA–transformed roots that display a remarkable consistency in the rate of root-tip growth as a consequence of clonal culture (4) was considered appropriate for accurately monitoring the plant reactions after mycorrhizal and pathogen establishment. In addition, the ease of manipulation of germ tubes from either the symbiont or the pathogen toward a selected region of the root allowed for standardization of the samples collected for cytological purposes. Under the conditions of this experimental system, *F. o. chrysanthemi* developed abundantly in transformed carrot roots, causing extensive tissue degradation. Although further studies are needed to clearly delineate the reasons why a plant, which is normally resistant to a pathogen, becomes susceptible, one may assume that root transformation is responsible for the observed changes. In the context of the present study, the susceptibility of transformed carrot roots to *F. o. chrysanthemi* attack was considered an interesting means of evaluating the effect of mycorrhizal infection on the induction of resistance against this pathogen.

The observation that defense reactions were expressed with a much higher magnitude in *Fusarium*-infected mycorrhizal roots than in uninoculated mycorrhizal roots supports the hypothesis that a signal produced by the pathogen is essential for triggering synthesis and accumulation of defense gene products. A similar conclusion was reached in the case of chitosan-treated tomato plants (9). Benhamou and Thériault (9) reported that defense reactions accumulating in *Fusarium*-infected, chitosan-coated tomato roots were seldom seen in noninfected, chitosan-treated tomato roots. Similarly, Chérif et al (15) studying the protective effect of silicon (Si) against fungal plant pathogens found that extensive defense reactions occurred in Si-treated cucumber plants only after pathogenic attack. These observations together with the present results suggest that biotic or abiotic agents sensitize the plant to respond more rapidly to microbial attack without causing accumulation of defense gene products that would require extensive loss of energy. Thus, it is reasonable to assume that gene activation at the posttranscriptional level occurs only after the perception of signals generated by the pathogen itself. This may explain why some authors failed to detect significant amounts of defense molecules in mycorrhizal roots (21,31,32,38). In a recent study, Spanu et al (37) observed a peak in chitinase activity in the early stage of mycorrhizal infection and a marked decrease of enzyme activity after establishment of the symbiosis. This finding supports the concept that AM fungi may be capable of evoking transcriptional activation of plant defense genes, the expression of which may be subsequently suppressed due to an unknown mechanism and restimulated after the perception of signals originating from contact with the pathogen. Analysis of the changes in mRNAs coding for defense molecules, such as pathogenesis-related (PR) proteins and enzymes of the phenyl propanoid biosynthetic pathway, would provide further insights into the molecular mechanisms by which AM fungi confer protection against microbial attack. It would be interesting also to investigate the expression of resistance in plants in which AM fungi have been inoculated after the pathogen. According to Caron et al (14), such plants afford increased resistance to *F. o. radicis-lycopersici* infection, indicating that microbial competition for nutrients may take place in addition to induction of resistance mechanisms in the observed protection against pathogen attack.

Striking differences in the extent of plant defense reactions were observed between mycorrhizal and nonmycorrhizal carrot roots after inoculation with *F. o. chrysanthemi*. One of the most common features observed was the extensive deposition of an unusual material at sites of fungal penetration as well as at strategic sites of fungal spread, such as intercellular spaces. The abnormal accumulation of electron-opaque deposits at these sites can be interpreted as indicating that these compounds are laid down to restrict the growth and the progression of the pathogen in the host tissues. The observation that colonization by *F. o. chrysanthemi* was mainly confined to the epidermis and the cortical area in mycorrhizal root tissues correlates well with the idea that the amorphous deposits contribute to prevent pathogen ingress toward the vascular stele. The absence of cellulose and pectic compounds in this coating material, as revealed by gold labeling, suggests that these products do not originate from the plant cell wall but instead likely correspond to the aggregation of newly formed compounds that, according to their electron density, may be phenolic in nature. In this context, it is interesting to note that this amorphous material, which often interacted with the fungal cell surface, was associated with morphological changes and cytological alterations of the invading hyphae.

In light of these observations, it appears that the coating material is more than a simple physical barrier to fungal invasion. The occurrence of secondary metabolites (i.e., phenolics and phytoalexins) in this material may explain the observed fungitoxic activity. It is well-known that such metabolites and the free radicals formed during the oxidative polymerization reactions display the ability to disturb the fungal metabolism and to cause a deregulation of enzyme production (36). The structural preservation of the host cell walls in mycorrhizal carrot roots may well be related to a marked decrease in host wall-degrading enzymes produced by the pathogen. However, the possibility that the coating material may protect the host cell walls by limiting the diffusion of deleterious substances cannot be ruled out.

Deposition of coating material was seldom seen in *Fusarium*-infected carrot roots that were not mycorrhizal. This observation suggests that expression of plant defense genes leading to the formation of these deposits cannot be triggered by the pathogen alone. In contrast, some electron-opaque structures were seen in mycorrhizal roots that were not inoculated with *F. o. chrysanthemi*. Although such deposits only occurred in a few cells and intercellular spaces, their unusual presence indicates that the plant was, at least in the early stages of the interaction, signaled to activate the genes coding for precursors of this electron-opaque material. Whether this discrete accumulation of unusual deposits results from a transient activation of the phenolic pathway that

is reactivated later on by a signal originating from the pathogen itself remains to be determined.

Evidence was provided through the use of the WGA-ovomucoid-gold complex that chitin in cell walls of *Fusarium* hyphae colonizing mycorrhizal roots was altered. The marked decrease in labeling intensity over *Fusarium* cell walls, especially at sites where the fungus was closely appressed against the host cell wall, suggests that the plant cells were signaled to produce chitinases that likely accumulated extracellularly, as previously shown in the tomato-*F. o. radicis-lycopersici* interaction (9). However, the finding that some chitin molecules were still present over cell walls of hyphae showing obvious signs of degradation leads us to hypothesize that production of chitinases does indeed occur but is not a primary determinant in the expression of plant resistance. It is more likely that toxic substances such as phenolics and phytoalexins are the first fungicidal factors followed by chitinases and other hydrolytic enzymes such as β-1,3-glucanases, which probably contribute to the complete disintegration of the fungal cells. A similar conclusion was reached by Benhamou and Thériault (9) who observed the same phenomenon in chitosan-treated tomato roots. Finally, the frequent observation that chitin molecules were released from *Fusarium* cell walls (Fig. 4B) and that pectic compounds were detached from the host cell wall (Fig. 6C and D) leads us to suggest that oligomers with eliciting potential (1,3) are produced during the course of infection. Whether these molecules participate in the continuous activation of the plant's defense system leading to the accumulation of defense molecules in a coordinated manner remains to be determined.

In conclusion, the present study, dealing with an experimental model, brings further insights into the mechanism by which AM fungi confer protection against pathogen infection. Further studies should be conducted on the changes in mRNA populations coding for biochemical markers of resistance, such as PR proteins and enzymes of the secondary metabolism, to better assess the exact mode of operation of AM fungi. It also will be necessary to investigate whether the reactions observed under experimental conditions also occur in whole mycorrhizal plants after infection with a pathogen.

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