Cytological Analysis of Defense-Related Mechanisms Induced in Pea Root Tissues in Response to Colonization by Nonpathogenic *Fusarium oxysporum* Fo47

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Accepted for publication 30 April 2001.

ABSTRACT

Benhamou, N., and Garand, C. 2001. Cytological analysis of defense-related mechanisms induced in pea root tissues in response to colonization by nonpathogenic *Fusarium oxysporum* Fo47. Phytopathology 91:730-740.

The ability of nonpathogenic *Fusarium oxysporum*, strain Fo47, to trigger plant defense reactions was investigated using Ri T-DNA-transformed pea roots. Cytological investigations of strain Fo47-inoculated roots showed that the fungus grew actively at the root surface and colonized a number of epidermal and cortical cells, inducing marked host cell metabolic changes. In roots inoculated with pathogenic *F. oxysporum* f. sp. *pisi*, the pathogen multiplied abundantly through much of the tissues, whereas in Fo47-inoculated roots, fungal growth was restricted to the epidermis and the outer cortex. Invading cells of strain Fo47 suffered from serious alterations, a phenomenon that was not observed in control roots in which *F. oxysporum* f. sp. *pisi* grew so actively that the vascular stele was invaded within a few days. Strain Fo47 establishment in the root tissues resulted in a massive elaboration of hemispherical wall appositions and in the deposition of an electron-opaque material frequently encircling pathogen hyphae and accumulating in the noninfected xylem vessels. This suggests that the host roots were signaled to defend themselves through the rapid stimulation of a general cascade of nonspecific defense responses. The specific relationship established between strain Fo47 and the root tissues is discussed in relation to other types of plant–fungus interactions, including pathogenic and symbiotic associations.

Additional keywords: biological control, fungal endophytes, fungal-mediated induced resistance, gold cytochemistry, phenolic compounds.

Development of high-yielding, better quality greenhouse crop varieties with reduced pest resistance has led to increased use of agro-chemicals, which eventually disrupted the fragile balance in nature. In many cases, such disruptions have reduced or even eliminated the competitiveness of some of the naturally occurring beneficial agents, which greatly helped plants to remain healthy in their environment. Because the rhizosphere provides the first line of defense for roots against attack by pathogens and because plants have sophisticated defense mechanisms that can be naturally activated by environmental factors and microorganisms, the possibility of enriching the rhizosphere with adapted microorganisms has become a challenging priority for plant pathologists (1,36). Therefore, a number of biological approaches have been proposed to control soilborne pathogens but, so far, none of them has reached the performance of chemical control. This renewed interest in the development of biorational strategies reflects (i) a response to public concern about the harmful impact of repeated fungicide applications, and (ii) a possibility to circumvent chemical-mediated resistance of pathogenic strains.

Among the strategies that have been explored for minimizing damage from plant pathogens, biological control of pathogenic populations by fungal antagonists, such as *Trichoderma* spp. (14), *Pythium oligandrum* (17), and *Verticillium lecanii* (5,13), and elicitor-mediated plant-induced resistance (9) are currently two of the most investigated options. Another alternative strategy that is receiving increasing attention concerns the potential value of nonpathogenic *Fusarium oxysporum* strains in promoting plant disease resistance (24). Although most efforts have been concentrated on the beneficial effects of these fungi in both interacting with the pathogen population in the rhizosphere (23) and competing for nutrients and infections sites in the rhizosphere (1,29, 34), some lines of evidence indicate that induced resistance mediated by nonpathogenic *F. oxysporum* strains, such as strain Fo47, may be involved in the observed reduction of Fusarium wilt incidence in tomato (23,24).

How strain Fo47 contributes to enhance tomato plant protection against root pathogens is largely unknown, although a number of hypotheses including microbial competition for nutrients (e.g., carbon and iron) (1), microbial antagonism (23), and physicochemical factors (e.g., pH, moisture and temperature, nitrogen content, and soluble salt content) (4) have been raised. However, these explanations are still a matter of speculation, and the role of biotic and abiotic parameters in the promotion of disease resistance remains controversial (1). Although there is no doubt that direct antagonistic effects of strain Fo47 on the pathogenic *Fusarium* populations are responsible, at least partly, for the enhanced plant protection observed by several authors in suppressive soils (25), the possibility that this fungus may also trigger indirect effects by sensitizing the plant to defend itself through the activation of defense genes has been raised (22,24) but not yet fully investigated at the cellular level. Recent studies suggested that increased resistance of tomato plants grown in the presence of strain Fo47 correlated with accumulation of some pathogenesis-related (PR) proteins including chitinases and β-1,3-glucanases (22,24). In spite of such indicative results, the situation regarding Fo47-mediated induced resistance is not clearly defined and further work is needed to elucidate the complex relationships established between this fungus and the root tissues (30).

The recent demonstration that nonpathogenic, mycoparasitic fungi such as *T. harzianum* (38) and *Pythium oligandrum* (16) could act as elicitors of plant defense reactions, thereby promoting the overall plant protection, raises the questions as to what extent

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Publication no. P-2001-0607-01R

strain Fo47 could be capable of stimulating the plant to defend itself through the formation of structural and biochemical barriers. As a preface to further investigations of the effect of Fo47 on whole plants, an in vitro root system, developed by Bécard and Fortin (6) and recently used to probe the sequence of events taking place during bacterial-mediated induced resistance (11), was selected. This experimental system offers multiple advantages including the absence of confounding effects caused by some undesirable contaminants in nature, the exclusion of a direct effect of competition for nutrients as a mechanism of disease suppression, and the possibility to precisely monitor the ultrastructural features associated with strain Fo47 colonization in pea roots. Data indicate that pea root cells undergo marked ultrastructural modifications upon inoculation with strain Fo47, which correlates with the creation of a fungitoxic environment that protects the roots by restricting fungal growth and development to the outermost tissues.

MATERIALS AND METHODS

Fungal culture and growth conditions. Nonpathogenic *F. oxysporum* Fo47, initially isolated by Alabouvette et al. (3) from a suppressive soil located at Châteaurenard in France, was supplied by Natural Plant Production (Noguères, France) as a liquid formulation termed "Fusaclean" and contained spores at a concentration of 10^{11} CFU/liter. The root pathogen, *F. oxysporum* f. sp. *pisi* (Van Hall) Snyd and Hans, was provided by C. Richard (Agriculture Canada, Ste-Foy, Quebec). Both fungi were grown on potato dextrose agar (Difco Laboratories, Detroit) at 24°C in the dark and subcultured weekly.

Preparation of Ri T-DNA-transformed cucumber roots. Transformed pea (*Pisum sativum* L.) roots were prepared according to the procedure described by Bécard and Fortin (6) by infecting 1-month-old pea shoots with *Agrobacterium rhizogenes* (ATCC 15834). Adventitious roots were transferred onto modified White's medium, pH 5.5, and solidified with 0.4% (wt/vol) gellan gum (ICN Biochemical Inc., Cleveland). Clonal lines were established as axenic cultures after several transfers of root tips onto fresh media. Stock cultures were maintained in large petri plates (150 × 15 mm) at 24°C in the dark and subcultured 1 week prior to fungal inoculation.

Root inoculation with either Fo47 or *F. oxysporum* f. sp. *pisi*. Inoculation of Ri T-DNA-transformed pea roots was carried out by depositing, along the main root in the apex region, mycelial plugs (3-mm diameter) taken from an actively growing colony of strain Fo47 or *F. oxysporum* f. sp. *pisi*. The fungi were allowed to grow for 1 to 5 days in contact with the roots before tissue processing for electron microscopy. Samples were collected from five petri dishes per time period in three replicate experiments.

Tissue processing for light and transmission electron microscopy. Root samples (2 mm^3) were carefully excised from colonized cucumber roots at sites of potential fungal penetration. They were preembedded in 2% aqueous bacto-agar in order to preserve the rhizosphere microbial populations and immersed in a mixture of 3% (vol/vol) glutaraldehyde and 3% (wt/vol) paraformaldehyde in 0.2 M sodium cacodylate buffer, pH 7.2, for 2 h at room temperature. Samples were subsequently postfixed with 1% (wt/vol) osmium tetroxide in the same buffer for 1 h at 4°C and dehydrated in a graded ethanol series before embedded in Epon 812 (JBEM Chemical Co. Pointe-Claire, Québec). Thin sections (0.7 μm), cut from the Epon-embedded material with glass knives, were mounted on glass slides and stained with 1% aqueous toluidine blue prior to examination with a microscope (Axioscope; Carl Zeiss, Don Mills, Ontario,). Ultrathin sections (0.1 μm) were collected on Formvar-coated nickel grids and were either contrasted with uranyl acetate and lead citrate for direct examination with an electron microscope (1200 EX; JEOL, Tokyo) at 80 kV or processed for cytochemical labeling. An average of five samples from three different plants per sampling time was investigated. For each sample, 10 to 15 ultrathin sections were examined under the electron microscope.

Cytochemical labeling. The colloidal gold suspension, with particles averaging 15 nm in diameter (BDH Chemicals, Montréal), was prepared as described by Benhamou (7). 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 *T. harzianum* was provided by C. Breuil (Forintek, Canada). It was complexed to colloidal gold at pH 9.0 and used in a one-step procedure (12). Ultrathin sections were first incubated on a drop of phosphate-buffered saline (PBS), pH 6.0, containing 0.02% (wt/vol) polyethylene glycol 20,000 (PEG 20,000) for 5 min at room temperature. They were, thereafter, transferred to a drop of the gold-complexed exoglucanase for 30 min at room temperature in a moist chamber. After washing with PBS and rinsing with distilled water, grids were contrasted as described previously. Specificity of the labeling was assessed by incubating the sections either with the gold-complexed exoglucanase to which were previously added β-1,4-glucans from barley (1 mg/ml in PBS) or with stabilized or an unstabilized gold suspension.

For the localization of callose, a β-1,3-glucan polymer, i.e., a β-1,3-glucanase, purified from tobacco reacting hypersensitively to *Tobacco mosaic virus*, was used according to a previously described method (8). The enzyme was conjugated to colloidal gold at pH 5.5, and incubation of sections was carried out as described for the gold-complexed exoglucanase.

Wheat germ agglutinin (WGA), a lectin with *N*-acetylglucosamine-binding specificity (Sigma Chemical Co., St. Louis) was used for localizing *N*-acetylglucosamine residues (chitin) (7). Because of its low molecular weight, the lectin could not be directly complexed to colloidal gold. It was used in a two-step procedure, using ovomucoid, and conjugated to gold at pH 5.4 as a second step reagent. For the labeling of *N*-acetylglucosamine residues, sections were first floated on a drop of PBS at pH 7.4 for 5 min and transferred to a drop of WGA (12 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 (1:30 in PBS-PEG, pH 6.0) for 30 min at room temperature. Sections were washed with PBS, rinsed with distilled water, and contrasted as described previously. Controls included incubation with the WGA, to which was previously added an excess of *N*-*N*′-*N*″-triacetylchitotriose (1 mg/ml in PBS), and incubation with the WGA, followed by unlabeled ovomucoid and ovomucoid-gold complex.

RESULTS

Histological observations. Observations of transversally sectioned pea root samples that were inoculated with *F. oxysporum* f. sp. *pisi* showed that all tissues were massively invaded by hyphae of the pathogen (Fig. 1A). At 3 days after fungal inoculation, root tissues were intensely colonized, evidenced by the occurrence of *Fusarium* hyphae through much of the epidermis, the cortex, the endodermis, and the paratracheal parenchyma cells (Fig. 1A). Fungal growth occurred inter- and intracellularly, and direct host cell wall penetration was frequently recorded (Fig. 1B, arrow). Pathogen ingress toward the vascular stele did not coincide with extensive host cell wall damage, except in the endodermis where a decrease in staining intensity of the cell wall was noticed (Fig. 1A, double arrows). Host reactions such as wall appositions, intercellular plugging, and xylem vessel coating were not detected. In all cases, the observed pattern of fungal colonization coincided with a marked loss of root firmness.

Examination of sections from Fo47-inoculated pea roots revealed that a large number of fungal cells had grown on the root surface and established an intimate contact with the host exodermis (Fig. 1C). Observation of about 50 sections from five dif-

ferent roots provided evidence that penetration and colonization occurred, but to a much lesser extent than in pathogen-inoculated plants (Fig. 1C). Fungal growth was mainly restricted to the outermost root cell layers (Fig. 1C), and a number of the invading hyphae appeared to be severely damaged as evidenced by the frequent occurrence of empty hyphal shells (Fig. 1D, arrow). Fungal ingress toward the inner root tissues was apparently halted, because Fo47 hyphae were very seldom seen in the endodermis and the vascular stele. Fungal invasion of the outermost root cortex always coincided with host cell changes mainly characterized by the elaboration of structural barriers in the regions proximal to potential fungal penetration (Fig. 1D). A typical feature of the host response concerned the formation of hemispherical protuberances, the so-called papillae (35), at the sites of potential fungal penetration (Fig. 1D). Another reaction was the deposition of an amorphous material that stained densely with toluidine blue in some infected cells (data not shown). Hyphae, trapped in this material, were apparently immobilized. Such host reactions were not seen in the uncolonized tissues beneath the invaded cell layers.

Cytological observations. As expected from the light microscope observations, an intense fungal development was seen in *F. oxysporum* f. sp. *pisi*-inoculated-transformed pea roots (Fig. 2). Pathogen growth occurred intracellularly through direct host wall penetration, which was achieved by means of constricted hyphae (Fig. 2A, C, and D, arrows). Surprisingly, such a massive fungal invasion did not result in marked host wall alterations as estimated by the electron density of the host cell walls in areas adjacent to the points of fungal penetration (Fig. 2A). Cells of the pathogen were also frequently seen in intercellular spaces (Fig. 2B). All invading hyphae showed a typical ultrastructure mainly characterized by a thin cell wall surrounding a dense polyribosome-enriched cytoplasm in which a large number of organelles, including mitochondria, endoplasmic reticulum, nuclei, and vacuoles were found (Fig. 2B and D). In these control pea roots, pathogen invasion failed to stimulate host reactions such as wall appositions, intercellular plugging, and xylem vessel occlusions (Fig. 2D).

In Fo47-inoculated pea roots, the pattern of fungal colonization differed markedly from that observed in roots colonized by *F. oxysporum* f. sp. *pisi* (Fig. 3A). Although extensive fungal multiplication was seen at the root surface (Fig. 3A), fungal growth in planta was mainly restricted to the outermost cell layers, including the epidermis and the outer cortex. Hyphae of the nonpathogenic *Fusarium* strain were seldom seen in the inner tissues and were never detected in the vascular stele. One of the most striking changes observed in Fo47-inoculated roots compared with roots inoculated with the pathogenic *Fusarium* strain, was the damaged aspect of most fungal cells. Indeed, a large number of invading hyphae (approximately 80%) showed various degrees of alteration, including distortion, retraction, and even breakdown of the plasma membrane (Fig. 3C, arrowheads), as well as pronounced disorganization of the cytoplasm in which typical organelles such as nuclei and mitochondria were no longer discernible (Fig. 3C). In some cases, aggregation of the cytoplasm and formation of electron-dense, polymorphic inclusions between the cell wall and the retracted cytoplasm were typical features of fungal reaction (Fig. 3D, arrow). Attempts of host cell wall penetration by morphologically and structurally altered hyphae of strain Fo47 were frequently seen (Fig. 3B). A marked loss of electron density was usually observed over host wall areas bordering the channels of fungal penetration (Fig. 3B, arrow).

Beside fungal cell alterations, root inoculation with strain Fo47 triggered the elaboration of several host reactions. One of the most prominent host responses was the formation of multitextured wall appositions at sites of potential fungal penetration (Fig. 3B to D). These hemispherical appositions or papillae were apparently made of an amorphous matrix that was impregnated by osmiophilic substances and delimited by a loosely arranged layer of cytoplasm (Fig. 3B and D). Except for the areas neighboring channels of fungal penetration, the host cell wall displayed a higher electron density than normal, thus, indicating the probable infiltration of structural molecules. Both the impregnated host cell wall and the wall appositions were efficient in preventing fungal ingress because successful hyphal penetration of the appositions was seldom observed. Hyphae penetrating papillae showed marked signs of degradation characterized by necrosis of the penetration peg (Fig. 3B, double arrows) and cytoplasmic disorganization of the external portion.

As expected, application of the β-1,4-exoglucanase gold complex to sections of Fo47-inoculated root tissues for the localization of cellulose resulted in a heavy deposition of gold particles over the host cell walls (Fig. 4A). In contrast, labeling was absent over the host cytoplasm and organelles as well as over cells of strain Fo47. Labeling occurred over the wall appositions, but it was irregularly distributed and reduced to a few randomly distributed gold particles (Fig. 4A). Control tests, including preincubation of the enzyme-gold complex with β-1,4-glucans prior to section labeling, resulted in the absence of labeling over both the cell walls and the wall appositions (data not shown).

Upon incubation of sections with the gold-complexed tobacco β-1,3-glucanase for the localization of callose, a substantial number of gold particles was detected over all wall appositions, regardless of their size and texture, whereas only a few scattered gold particles were found over the host cell walls (Fig. 4B to D). A close examination of the wall appositions that were in the process of being penetrated by strain Fo47 hyphae revealed that lysis areas occurred along the penetration pegs (Fig. 4C) and extended at some distance from it (Fig. 4D, arrows). Complete penetration of the wall appositions was never recorded. Control tests, including incubation of the enzyme-gold complex with β-1,3-glucans from barley prior to section labeling, resulted in a near absence of gold particles (data not shown).

Another striking feature of host reactions was the deposition of either a compact material (Fig. 5A, double arrows) or a cloudy, electron-opaque network (Fig. 5B and C) in a large number of invaded cells and intercellular spaces. The osmiophilic material lining the primary walls usually extended toward the inside to form polymorphic deposits that frequently interacted with the wall of invading hyphae (Fig. 5B, arrow) and occasionally formed a coating band at the fungal cell surface (Fig. 5B, arrow). Invading hyphae trapped in these materials exhibited extensive disorganization of their cell content (Fig. 5C). Interestingly, host reactions were also detected in noninvaded xylem vessels (Fig. 5D). These were mainly characterized by the coating of secondary walls and pit membranes with a band of electron-opaque material that could extend in the vessel lumen to form osmiophilic droplets (Fig. 5D).

When the WGA-ovomucoid-gold complex was applied to sections of Fo47-inoculated pea roots for localization of chitin, the pattern of labeling over fungal cell walls varied from one cell to another (Fig. 6). In all cases, the cell wall of fungal hyphae showing discrete signs of alteration was heavily labeled (Fig. 6A, arrow), whereas gold particles were generally less abundant over the walls of substantially altered fungal cells (Fig. 6C and D). A close examination of the penetrating hyphae revealed that labeling disappeared over the walls of the constricted fungal portion that was encased in the host cell wall (Fig. 6B, arrow), whereas gold particles were quite regularly distributed over the external hyphal cell walls. Most interesting was the considerable decrease in labeling intensity of the fungal cell wall areas that were neighboring the host cell wall (Fig. 6D, arrow). The wall appositions, as well as the amorphous material interacting with fungal cells, were unlabeled (Fig. 6C and D). All control tests, including previous adsorption of the WGA with *N-N′-N″*-triacetylchitotriose, resulted in an absence of labeling over the fungal cell walls (data not shown).

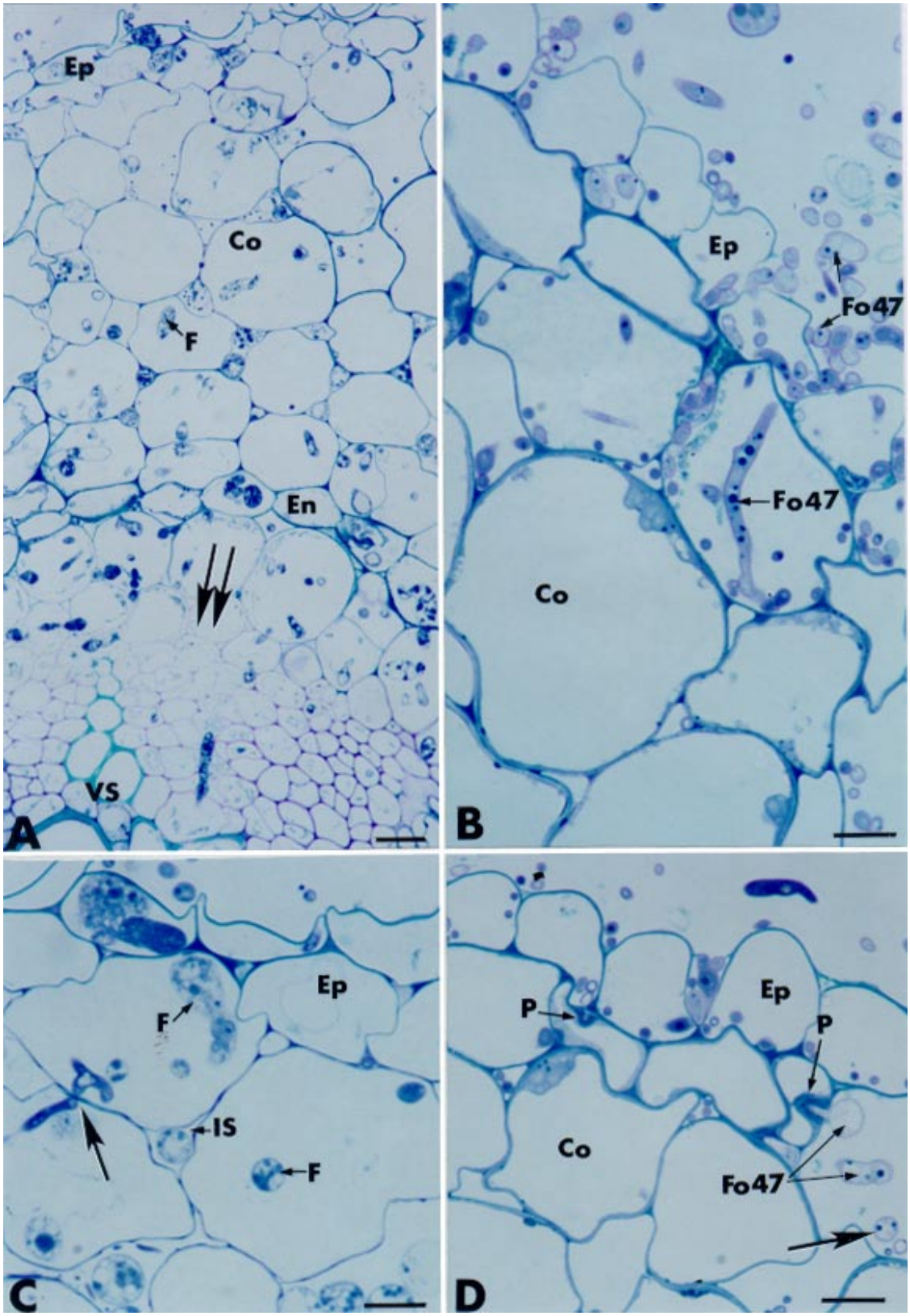

Fig. 1. Cross-sections of pea roots and staining with toluidine blue. **A and B,** Roots inoculated with *Fusarium oxysporum* f. sp. *pisi*. Hyphae of *F. oxysporum* f. sp. *pisi* (F) develop abundantly through much of the root tissues including the epidermis (Ep), cortex (Co), endodermis (En), and vascular stele (VS). Fungal growth occurs in intercellular spaces (IS) as well as intracellularly. **B,** Direct host cell wall penetration indicated by arrow. **A,** In the endodermis, a decrease in staining intensity of the host cell walls indicated by double arrows. **A,** Bar = 20 µm. **B,** Bar = 10 µm. **C and D,** Roots inoculated with *F. oxysporum* Fo47. The fungus develops actively at the root surface and penetrates the epidermis (Ep). Colonization is restricted to the epidermis and the outer cortex (Co) and correlates with the formation of hemispherical protuberances, the papillae (P). **D,** Most invading hyphae appear severely damaged (arrow). Bar = 10 µm.

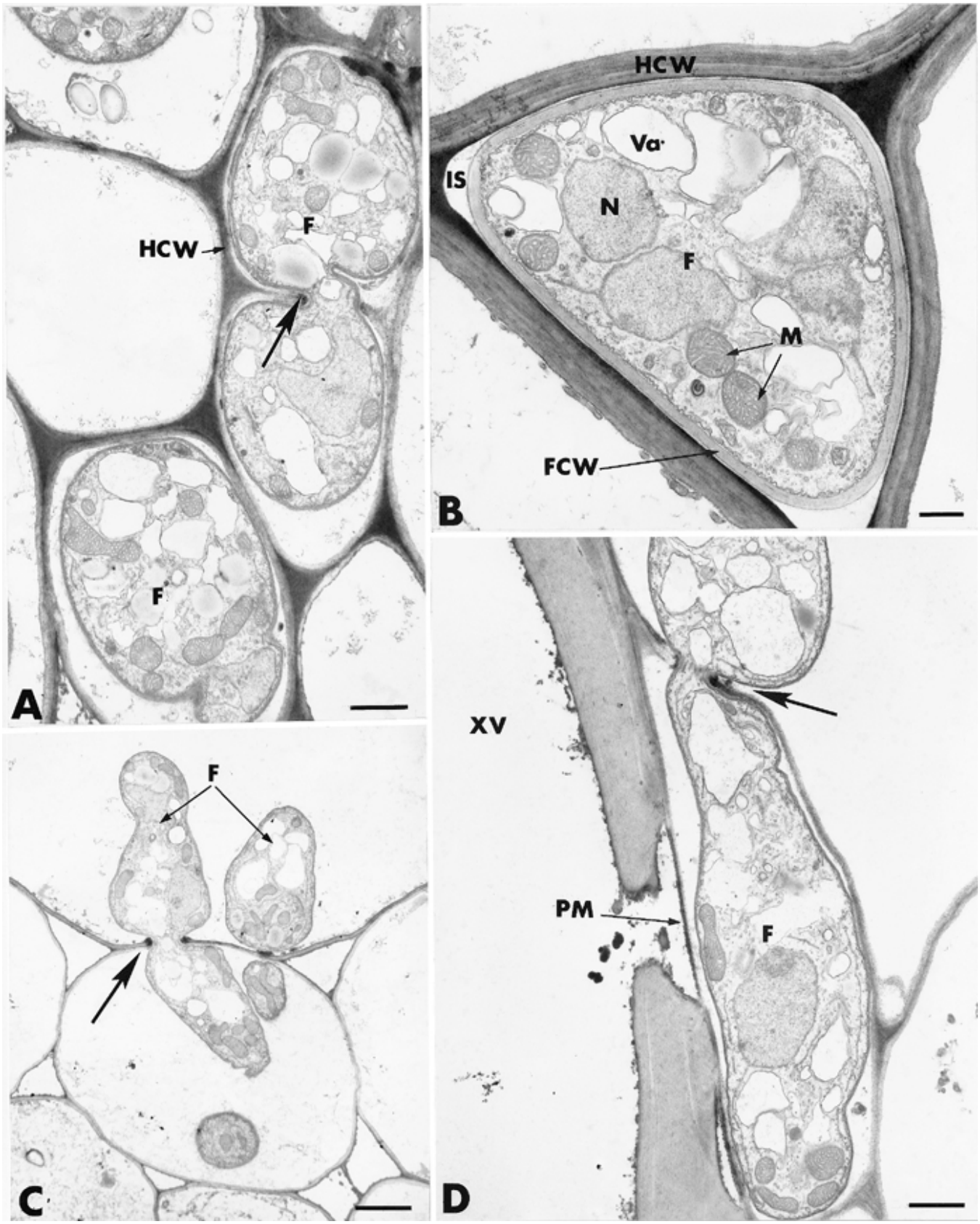

Fig. 2. Transmission electron micrographs of cross-sections from pea roots inoculated with *Fusarium oxysporum* f. sp. *pisi*. Fungal cells (F) develop abundantly in the root tissues without causing extensive host cell wall alterations. **A, C, and D,** Cell invasion by host wall penetration is frequently observed (arrows). **B,** Cells of the pathogen showing a dense polyribosome-enriched cytoplasm containing mitochondria (M), nuclei (N), and vacuoles (Va) are seen in intercellular spaces (IS). **D,** The pathogen reaches the xylem vessels (XV) by centripetal growth. FCW = fungal cell wall; HCW = host cell wall; and PM = pit membrane. **A,** Bar = 1 μm; **B,** bar = 0.5 μm; **C,** bar = 2 μm; and **D,** bar = 1 μm.

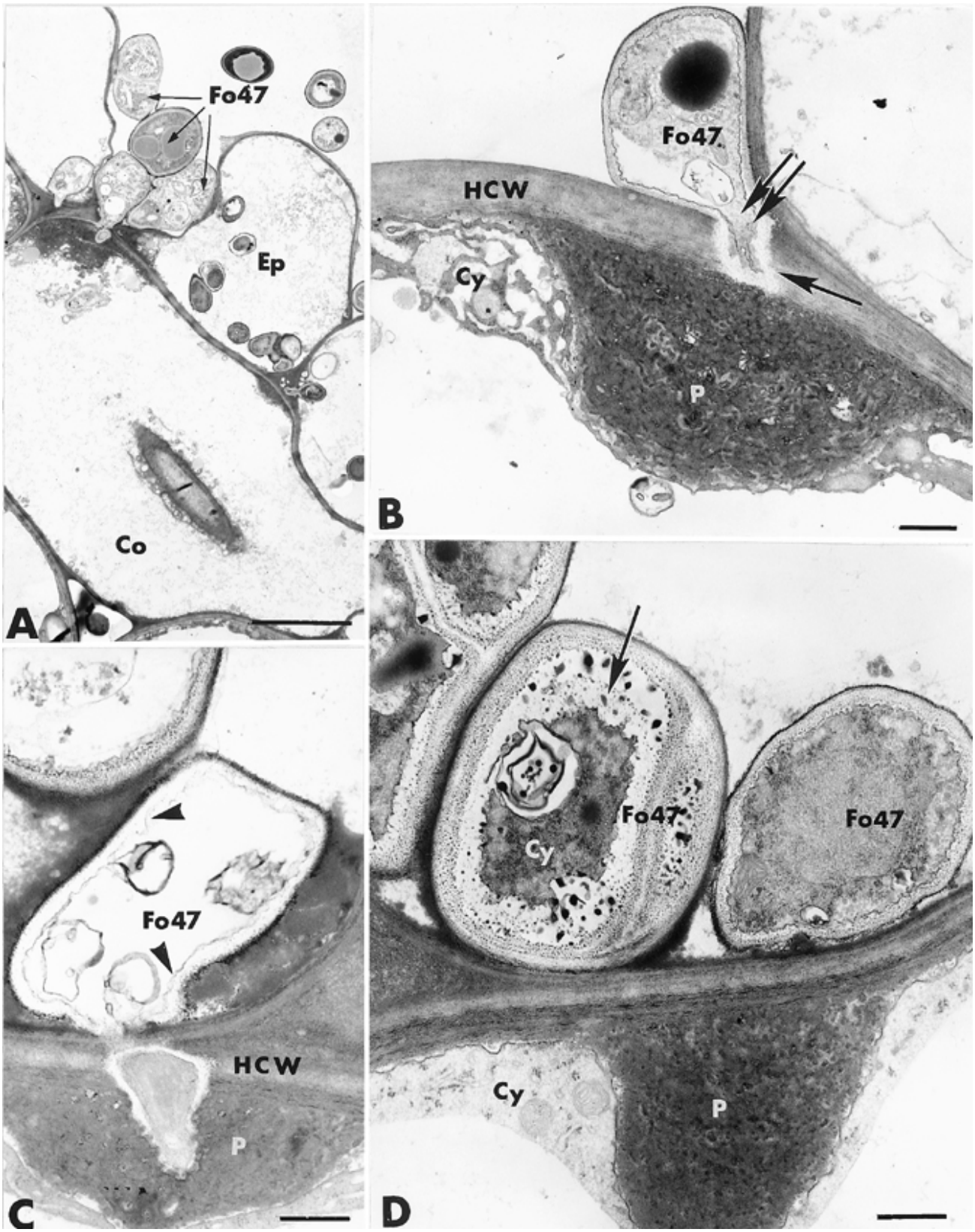

Fig. 3. Transmission electron micrographs of cross-sections from pea roots inoculated with nonpathogenic *Fusarium* strain Fo47. **A,** Although extensive fungal multiplication is seen at the root surface, fungal growth in planta is restricted to the outermost root cell layers. **C,** Most fungal cells are markedly altered as shown by the pronounced distortion of the plasma membrane (arrowheads). **D,** In some fungal cells, aggregation of the cytoplasm (Cy) is accompanied by the formation of electron-dense, polymorphic inclusions between the cell wall and the retracted cytoplasm (arrow). **B,** Attempts of host cell wall penetration by altered hyphae of strain Fo47 are seen. Lysis areas surround the channels of fungal penetration indicated by arrow. **B to D,** Strain Fo47 ingress is associated with the formation of multitextured papillae (P) at sites of potential fungal penetration. These structures are delimited by a loosely arranged layer of cytoplasm (Cy). **B,** Hyphae penetrating papillae showed marked signs of degradation characterized by necrosis of the penetration peg (double arrows). Ep = epidermis; and HCW = host cell wall. **A,** Bar = 2 μm; and **B to D,** bars = 0.5 μm.

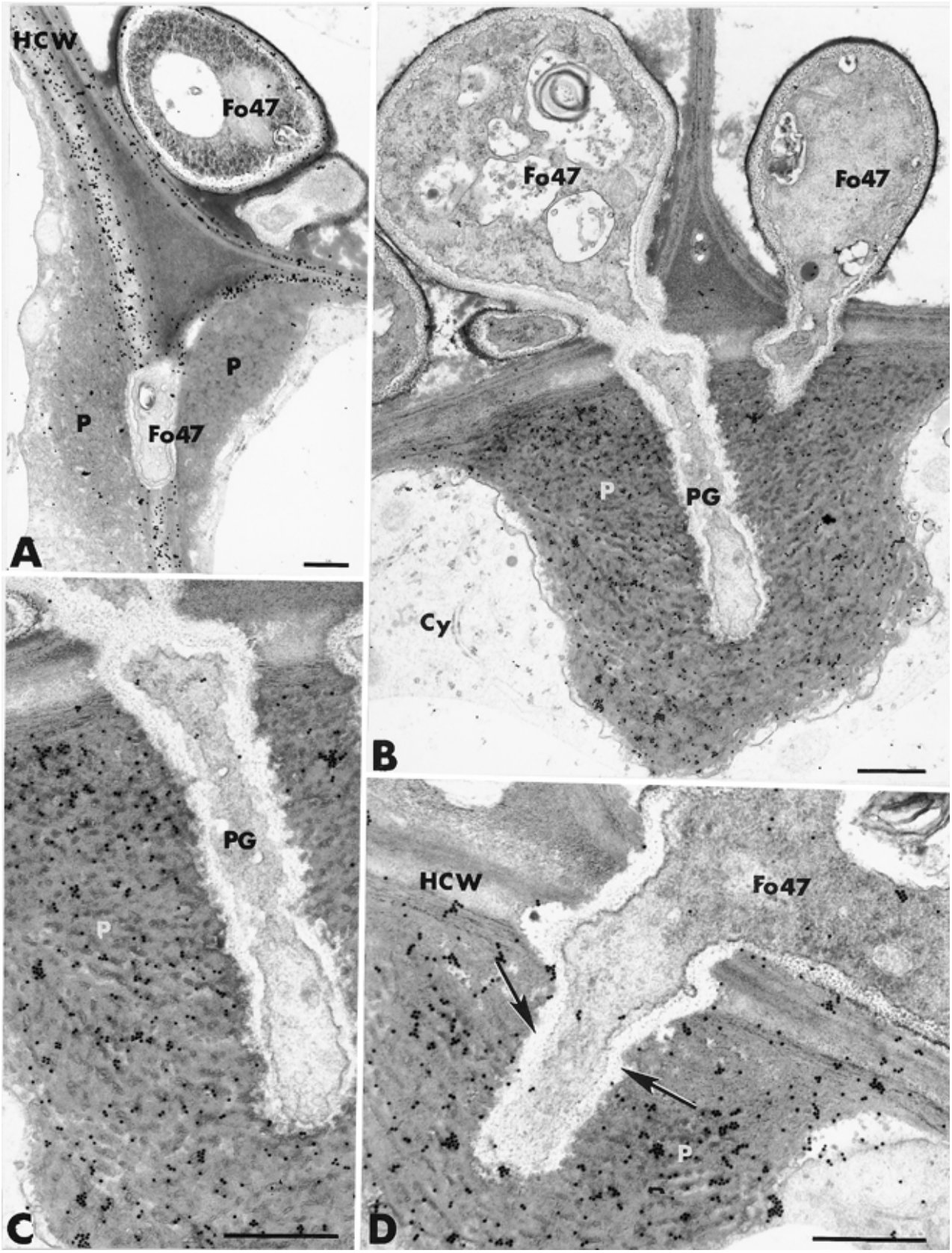

Fig. 4. Transmission electron micrographs of cross-sections from pea roots inoculated with nonpathogenic *Fusarium* strain Fo47. **A,** Labeling of cellulose with the gold-complexed exoglucanase. A heavy deposition of gold particles occurs over the host cell walls (HCW), whereas only a few scarce gold particles are seen over the papillae (P). Bar = 0.5 μm. **B to D,** Labeling of callose with the gold-complexed tobacco β-1,3-glucanase. Papillae (P) are intensely labeled. By contrast, the host cell walls (HCW) are weakly labeled. **D,** Lysis areas are present along the penetration pegs (PG) and extend at some distance from it (arrows). Bars = 0.5 μm.

DISCUSSION

In recent years, interest in the ability of nonpathogenic microorganisms to induce plant disease resistance has increased with respect to their potential use for enhancing the level of suppressiveness that exists in any soil (1,16,21,24,36). In spite of the increasing amount of research devoted to plant disease suppression by nonpathogenic *Fusarium* strains (2), our knowledge of the exact mechanisms underlying the bioprotective effect of these fungi is still incomplete. Indeed, most efforts have focused on

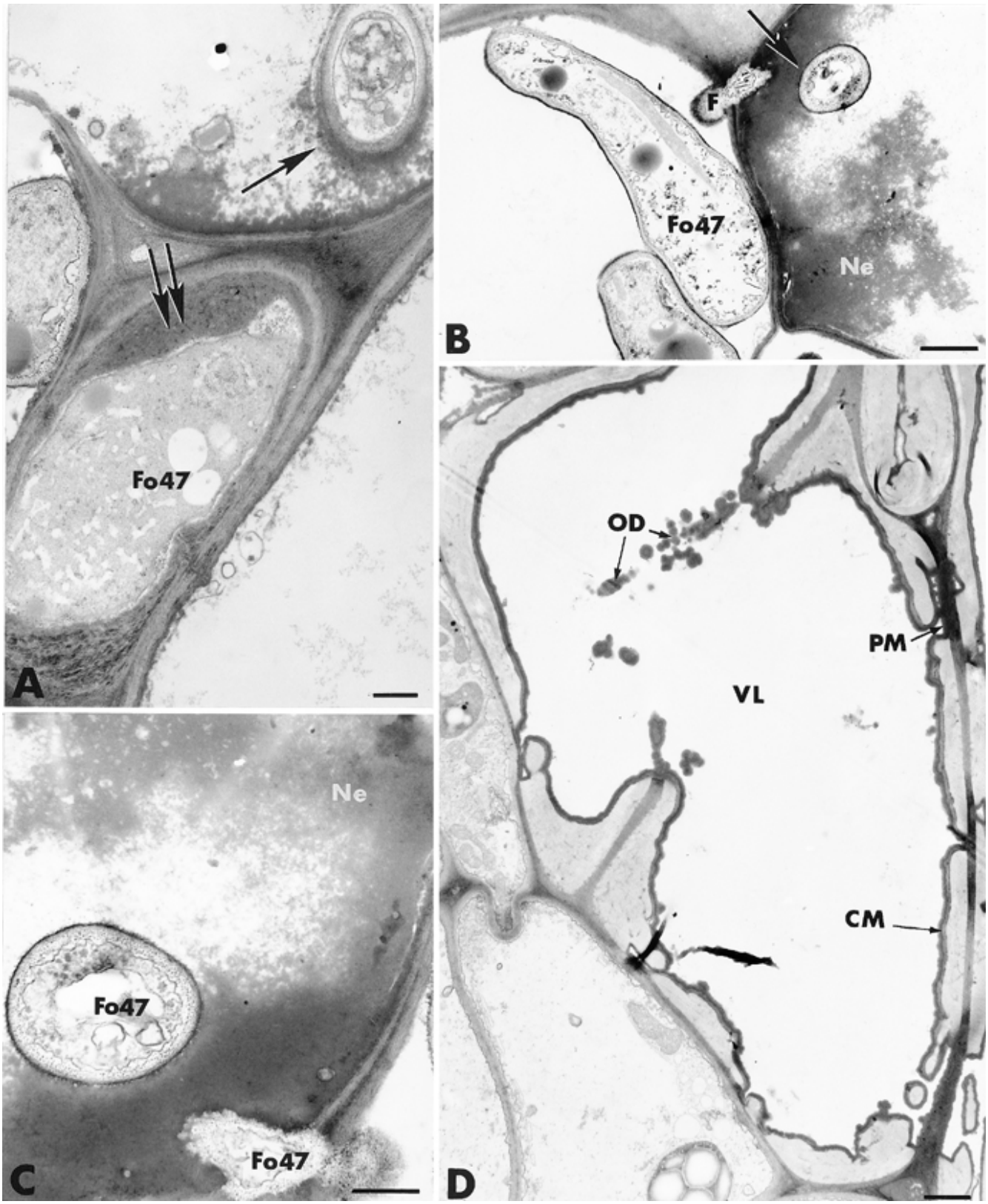

Fig. 5. Transmission electron micrographs of cross-sections from pea roots inoculated with nonpathogenic *Fusarium* strain Fo47. A typical host reaction concerns the deposition of either **A,** a compact material (double arrows) or **B and C,** a cloudy network (Ne) in invaded cells and intercellular spaces. **A and B,** This material surrounds the wall of invading hyphae (arrows). **D,** In noninvaded xylem vessels, a coating material (CM) accumulates along the secondary walls and pit membranes (PM) and extends in the vessel lumen (VL) to form osmiophilic droplets (OD). **A,** Bar = 1μm; **B,** bar = 1 μm; **C,** bar = 0.5 μm; and **D,** bar = 1 μm.

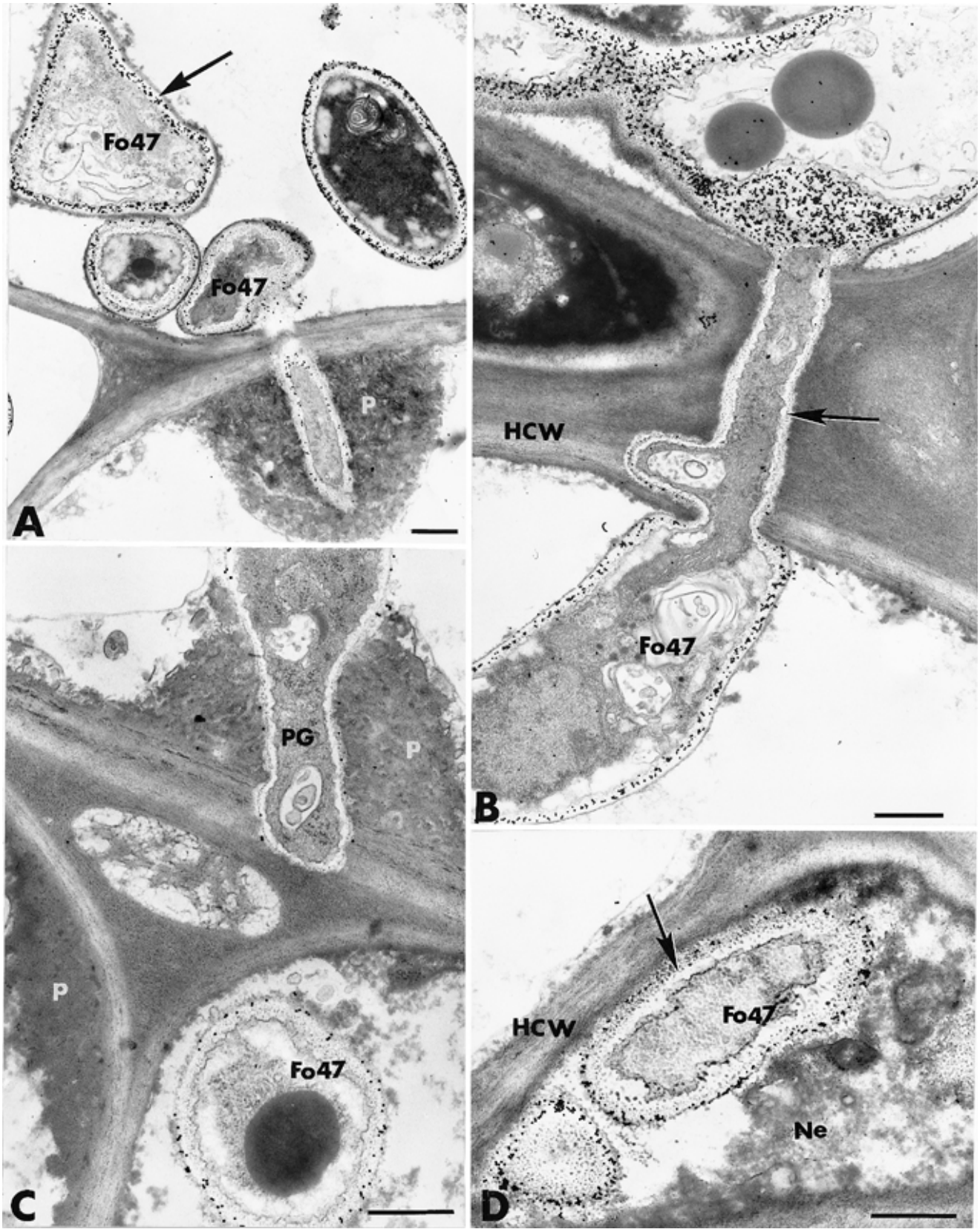

Fig. 6. Transmission electron micrographs of cross-sections from pea roots inoculated with nonpathogenic *Fusarium* strain Fo47. Labeling of chitin with the wheat germ agglutinin/ovomucoid-gold complex. **A,** The cell wall of fungal hyphae showing discrete signs of alteration is heavily labeled (arrow), whereas **C and D,** gold particles are nearly absent over the walls of substantially altered fungal cells. **B,** Labeling nearly disappears over the walls of the constricted fungal portion that is encased in the host cell wall (HCW) (arrow). **D,** Similarly, labeling is absent over the fungal cell wall areas neighboring the HCW (arrow). **C and D,** Papillae as well as the amorphous network (Ne) formed in invaded host cells are unlabeled. Bars = 0.5 µm.

microbial interactions and not on the possible involvement of the host plant, although circumstantial evidence correlating fungal penetration into the root tissues and induced resistance has been reported (25,28). Results of the current study demonstrate that Fo47, a nonpathogenic strain of *F. oxysporum*, is capable of evoking biochemical events characteristic of the natural plant disease resistance process. These results are of particular relevance because they highlight, to our knowledge for the first time, the properties of Fo47, which, in addition to being a strong competitor for carbon compounds in the rhizosphere (29), is also an elicitor of plant defense reactions. A similar host response that normally correlates with genetically determined resistance has been frequently obtained upon exogenous applications of chitin and chitosan oligosaccharides (15), and of certain chemicals including 2,6-dichloroisonicotinic acid, β-aminobutyric acid (20), and benzo (1,2,3) thiadiazole-7-carbothioic acid *S*-methyl ester (10).

In the present study, we compared the cytology of root infection by strain Fo47 with that associated to a pathogenic *Fusarium* strain. The rationale for the use of an experimental model as a prelude to further experiments on whole plants was that not only could transformed pea roots provide a means to avoid the influence of other parameters such as competition for nutrients in the substrate, but they also could allow an accurate investigation of the cellular events related to strain Fo47-mediated induced resistance. Under the conditions of this experimental system, strain Fo47 proliferated at the root surface and penetrated the root epidermis, causing marked changes of the host metabolism. Examination of tissues from *F. oxysporum* f. sp. *pisi* and Fo47-inoculated pea roots showed substantial differences in both the amount of fungal colonization and the number of viable hyphal cells. Although the extent of tissue invasion could vary from one root to another, growth and development of Fo47 hyphae were markedly restricted to the outer tissues in all examined samples. In addition, fungal cells suffered from pronounced damage, a phenomenon that was not observed in control roots in which *F. oxysporum* f. sp. *pisi* grew so actively that the vascular stele was invaded within a few days. Such differences in fungal growth and activity correlated with the spatial expression of plant defense components, likely laid down to barricade Fo47 cells from invading the tissues through physical exclusion. This observed restriction of Fo47 growth to the outermost root tissues contrasts with the histological observations of Salerno et al. (32) who reported that *F. oxysporum* Fo47 colonized all root tissues of Eucalypt seedlings without, however, causing extensive cell alteration. In the absence of ultrastructural investigation, Salerno et al. (32) could not correlate the protective effect of strain Fo47 with the possible expression of plant defense mechanisms.

The current results confirmed that strain Fo47 displayed the ability to colonize the outer root tissues without inducing cell damage, which is known to occur in several host–*Fusarium* spp. interactions (18). This property implies that at least small amounts of cell wall hydrolytic enzymes, such as pectinases and cellulases, are produced by strain Fo47 to locally breach the host cell walls, thus, facilitating spread into the root tissues. Support of this concept is drawn by the observation that well-delineated, digested host wall areas were consistently seen along the channels of fungal penetration. However, the regular pattern of cellulose distribution over host cell walls adjacent to invaded areas was taken as an indication that cell wall-degrading enzymes were slightly produced inside the plant. Synthesis of extracellular lytic enzymes by nonpathogenic *Fusarium* strains has not been reported, although the possibility that these fungi may produce pectinases and cellulases as part of their enzymatic arsenal appears realistic. In line with this concept, our results suggest that pectin hydrolysis is one of the main mechanisms involved in root colonization by strain Fo47, evidenced by the presence of the fungus in a large number of intercellular spaces.

Based on the production of cell wall-degrading enzymes, one may assume that pathogenic and nonpathogenic fungal associations share some similarities in terms of parasitism. However, despite obvious similarities in the infection processes, the remarkable alteration of most invading fungal cells and the rapid elaboration of physical barriers by the host plant in the nonpathogenic fungal association clearly distinguish both types of fungal interactions. Considering the specific relationship established between strain Fo47 and the root tissues, one may be tempted to establish a parallel with endomycorrhizal associations in which hyphae penetrate the root epidermis and proliferate in the cortical parenchyma to form highly specialized structures, the arbuscules (26). However, at least two quite different reasons may explain why such a correlation between these two types of associations should be excluded. First, the induction of host metabolic changes, leading to the elaboration of structural barriers at sites of potential fungal penetration, has never been reported to occur in the cortical parenchyma of endomycorrhizal roots (27). Second, endomycorrhizal fungi undergo a degenerative process during root colonization as exemplified with cells of strain Fo47 (19). Thus, the relationship established between the host plant and nonpathogenic *F. oxysporum* Fo47 appears to follow a well-defined scheme of events including proliferation along the elongating root and local penetration of the epidermis, probably resulting in the release of pectic fragments that, in turn, may act as elicitors of the plant defense strategy (9). Because strain Fo47 establishes itself in the cortical tissues, it may be considered a fungal endophyte, which, according to Wilson (37), are microorganisms that, for all or part of their life cycle, invade the tissues of living plants without causing disease symptoms. Recent studies have shown that nonpathogenic, endophytic mutualists, obtained through genetic conversion of fungal plant pathogens, could become potential biocontrol agents via their ability to trigger the plant defense system (31).

The main facet of the altered metabolism in Fo47-infected roots concerns the massive elaboration of hemispherical wall appositions in the invaded epidermis and outer cortex. Such cellular changes were associated with marked fungal disorganization. Whether the observed fungal alterations correlate with the creation of a fungitoxic environment due to the synthesis and accumulation of antimicrobial compounds by the reacting host cells or simply relate to a specific behavior of the fungus in planta deserves to be investigated. However, the finding that a material of high electron density frequently encasing fungal hyphae accumulated in a large number of host cells and intercellular spaces can be taken as an indication that the host cells were signaled to protect themselves by the production of antifungal substances such as phenolic compounds, which appear opaque under the electron microscope (33). According to their wide pattern of distribution, phenolic-like compounds may play a key role in Fo47-mediated induced resistance by directly inhibiting fungal growth due to their antimicrobial potential and indirectly protecting the tissues underlying or neighboring the invaded areas by acting as impervious, mechanical barriers capable of restricting fungal ingress.

Cytochemical analysis of the pattern of chitin distribution revealed a marked decrease in the amount of chitin over Fo47 cell walls, especially at sites where the fungus was closely appressed against the host cell wall. Collectively, these results indicate that the root cells were signaled to produce chitinases that likely accumulated extracellularly, as previously shown in the tomato–*F. oxysporum* f. sp. *radicis-lycopersici* interaction (18). Our observations indicate that the root cells exert a control over Fo47 development by restricting its propagation to the outermost tissues through rapid cell wall modifications, secondary metabolite production, and PR proteins (i.e., chitinase) accumulation. Because the plant did not react similarly in response to infection by the pathogenic *Fusarium* strain, it may be suggested that the stress signaling systems are either distinct or transduced by different pathways. Recent advances in molecular biology have clearly shown that,

within the syndrome of biological stress, different signal transduction pathways were involved in defense gene activation (9). Understanding the molecular mechanisms underlying the regulatory circuit involved in plant gene activation following root infection by nonpathogenic fungal strains such as Fo47 represents a major challenge for future research.

In any event, evidence is provided in this study that nonpathogenic *F. oxysporum* Fo47 stimulates the plant to defend itself. Because this type of induced resistance is very difficult to overcome by a virulent pathogen, strain Fo47 may be considered a powerful biocontrol agent for long-term root disease management.

ACKNOWLEDGMENTS

This study was supported by grants from the Natural Sciences and Engineering Research Council of Canada (NSERC), and the Fonds Québécois d'Aide à la Recherche (FCAR). We thank A. Goulet for excellent technical assistance.

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