

Induction of Differential Host Responses by *Pseudomonas fluorescens* in Ri T-DNA–Transformed Pea Roots After Challenge with *Fusarium oxysporum* f. sp. *pisii* and *Pythium ultimum*

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ABSTRACT

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In the current study, the influence exerted by the plant growth-promoting bacterium *Pseudomonas fluorescens* strain 63-28 in stimulating plant defense reactions was investigated at the ultrastructural level by an *in vitro* system in which Ri T-DNA pea roots were either infected with the pea root rot fungus *Fusarium oxysporum* f. sp. *pisii* or with the soil-borne pathogen *Pythium ultimum*. Scanning electron microscopy (SEM) observations showed that bacteria abundantly colonized the root surface and established close contact with the outermost host cell layers through a thin mucilage. When bacterized pea roots were challenged with either *P. ultimum* or *F. oxysporum* f. sp. *pisii*, strong differences in the extent of fungal damage were observed. Hyphae of *P. ultimum* were markedly collapsed, as illustrated by their wrinkled appearance, whereas *Fusarium* cells were apparently undamaged. In line with these SEM observations, cytological investigations of the root surface confirmed that the interaction between *Pseudomonas fluorescens* and *F. oxysporum* f. sp. *pisii* did not result in hyphal disturbances similar to those seen with *Pythium* hyphae. Restriction of *Fusarium* growth and development to the epidermis and outer root cortex and a marked decrease in pathogen viability were features observed only in bacterized pea roots. In pretreated roots,

striking modifications of the epidermal and cortical cell walls as well as deposition of newly formed barriers were seen in response to *Fusarium* infection. Deposition onto the inner surface of the cell walls of callose-enriched wall appositions was associated with a lack of fungal ingress toward the vascular stele. Fungal cells in the vicinity of wall appositions frequently were surrounded by an aggregated material containing phenolic compounds, as shown by laccase-gold labeling. The labeling pattern obtained with this probe showed that phenolic compounds were widely distributed in *Fusarium*-challenged, bacterized roots. In addition to their infiltration at strategic sites of potential penetration, phenolic compounds were detected in the host cell walls. Use of the wheat germ agglutinin/ovomucoid-gold complex provided evidence that the wall-bound chitin component in *Fusarium* hyphae colonizing bacterized roots was disrupted in places. However, chitin molecules still occurred over cell walls at a time when hyphae had undergone substantial degradation. The results reported in this paper show that pea root bacterization with *Pseudomonas fluorescens* strain 63-28 induces a set of plant defense reactions that culminates in the elaboration of physical barriers and the creation of a fungitoxic environment that adversely affects *Fusarium* growth and development.

Additional keywords: biological control, gold cytochemistry, induced resistance.

There are increasing expectations in the area of plant disease management concerning new strategies that have the potential to be efficient, reliable, and safe for the environment (30). Among the avenues being explored, induced resistance triggered by avirulent microorganisms (27), elicitor-active molecules (7), or chemicals (14) is one of the biocontrol approaches that offers excellent prospects for protecting plants against microbial infections and reducing environmental pollution. One facet of induced resistance that is attracting much attention concerns the potential of beneficial bacteria to protect plants from disease (29). Since the first demonstration that some free-living soil bacteria can stimulate plant growth (25), specific rhizobacteria, termed plant growth-

promoting rhizobacteria (PGPR), have been widely investigated for practical use in agriculture (22). The rationale for this interest is that these bacteria can not only enhance plant growth by increasing phosphorus and nitrogen uptake and providing roots with compounds such as phytohormones and solubilized iron (21), but they also can promote plant protection by controlling pathogen populations through an array of mechanisms, including nutrient competition (28), antibiosis, and production of antifungal factors (41). In addition, several lines of evidence from a number of studies have convincingly shown that PGPR can reduce incidence and severity of foliar and root diseases by increasing plant resistance to pathogen attack (26,34). It has become more and more apparent that PGPR-mediated induced resistance is associated with marked host metabolic changes, culminating in a number of physical (1) and biochemical responses (36,42) designed to limit, either indirectly (reinforcement of plant cell walls) or directly (antimicrobial activity), pathogen penetration and development in host tissues. However, in spite of these encouraging findings, the exact

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mechanisms by which PGPR trigger the plant defense system are not fully understood.

With the refinement of cytological and molecular techniques and the application of these approaches to biological systems, several unanswered questions regarding the relationship between the plant cell, the bacteria, and the challenging pathogen can now be addressed. In a recent report (8), we provided evidence that transformed pea roots are useful tools for elucidating the cytological aspects of the interaction between *Pseudomonas fluorescens* and root tissues, in addition to bringing new insights into the mechanisms underlying the enhanced resistance of bacterized roots to *Pythium ultimum* Trow attack. Transformed roots, obtained by inoculating plant tissues with virulent strains of the soil bacterium *Agrobacterium rhizogenes* and isolating the adventitious roots arising from the wound sites (32), offer the advantages of being genetically and biochemically stable and exhibiting fast growth compared to untransformed root systems. Such transformed roots have been used extensively to study the biosynthetic pathways of phenolic compounds (17) and also have proven useful for investigating the influence of endomycorrhizal infection on pathogen-induced resistance (10).

In the current study, *Pseudomonas fluorescens* strain 63-28 was used to determine whether this rhizobacterium, shown to protect transformed pea roots against *P. ultimum* attack through the synergistic action of strong antimicrobial activity and induction of plant defense mechanisms (8), also was effective in controlling infection by a major pea root rot pathogen, *Fusarium oxysporum* Schlechtend.:Fr. f. sp. *pisi* (J.C. Hall) W.C. Snyder & Hanna. Our results demonstrate that pea root bacterization with *Pseudomonas fluorescens* strain 63-28 induces a set of plant defense reactions that culminate in the elaboration of physical barriers and in the creation of a fungitoxic environment that adversely affects pathogen growth and development.

MATERIAL AND METHODS

Preparation of Ri T-DNA-transformed pea roots. Transformed pea (*Pisum sativum* L.) roots were obtained from Y. Piché, Faculté de Foresterie, Université Laval, Québec, Canada. They were prepared according to the procedure described by Bécard and Fortin (4), by infecting 1-month-old pea shoots with *A. rhizogenes* (American Type Culture Collection [ATCC] 15834). Adventitious roots were transferred onto modified White's medium 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 to fresh media. Stock cultures were maintained in petri dishes at 24°C in the dark.

Bacterial culture and growth conditions. The isolate of *Pseudomonas fluorescens* (strain 63-28R) obtained from Agrium Inc. (formerly ESSO Agricultural Biologicals), Saskatoon, SK, Canada, was stored in nutrient broth containing 10% glycerol at -80°C. To produce bacterial cells for root inoculation, the bacterial strain was retrieved from storage and streaked on King's medium B agar (24). Two days later, bacterial cells were suspended in sterile distilled water and pelleted by low-speed centrifugation. The pellet was resuspended in sterile distilled water, and the density of the bacterial suspension was adjusted to 10⁶ cells per ml by measuring the absorbance at 640 nm and comparing with a standard curve.

Fungal culture and growth conditions. The root pathogens *P. ultimum*, isolate BARR 447 (Center for the Land and Resources Research, Ottawa, ON, Canada), and *F. oxysporum* f. sp. *pisi* (provided by C. Richard, Agriculture and Agri-Food Canada, Ste-Foy, QB), known to be virulent on pea, were routinely grown on Difco (Detroit) potato dextrose agar at 26°C in the dark.

Root inoculation with *Pseudomonas fluorescens*. Inoculation of Ri T-DNA-transformed pea roots growing on White's modified medium was performed by inoculating the main root with 2 ml of the bacterial suspension at 10⁶ cells per ml with a sterile

micropipette. Bacteria-inoculated roots were kept at 24°C for 2 to 3 days prior to being either directly processed for electron microscopy or challenged with one of the two fungal pathogens. Samples from bacterized roots were collected from five petri dishes in three replicate experiments.

Root inoculation with fungal pathogens. Two to three days after inoculation with *Pseudomonas fluorescens*, transformed pea roots were inoculated with the mycelium of 3-day-old colonies of *P. ultimum* or *F. oxysporum* f. sp. *pisi*. For both fungi, inoculation was performed either by placing 3-mm mycelial disks at 2-cm intervals along the root surface or using a hyphal and spore suspension (10⁸ CFU/ml) prepared by scraping the surface of a petri plate and homogenizing for 5 s in 15 ml of sterile distilled water in a blender. Transformed pea roots growing at the surface of the minimal medium were inoculated with 0.5 ml of the fungal suspension or with 0.5 ml of sterile distilled water. Controls included pea roots that were not preinoculated with *Pseudomonas fluorescens*. The pathogen was allowed to grow for 1 to 3 days in contact with the roots before sampling for electron microscopy. Root samples were collected from five petri dishes per time period in three replicate experiments.

Tissue processing for scanning electron microscopy (SEM). Samples (1 cm²) from control and challenged pea roots were vaporized with 2% (wt/vol) osmium tetroxide in distilled water for 20 h at room temperature, air-dried, and sputter-coated with gold palladium in a Nanotech (Cambridge, England) sputter coater. Samples were kept in a dessicator until examination with a JEOL (Tokyo) JSM-35CF scanning electron microscope operating at 15 kV. Micrographs were taken on Polapan 400 positive films (Polaroid Corporation, Cambridge, MA). Two samples per petri dish per time period were examined.

Tissue processing for transmission electron microscopy (TEM). Samples (2 mm³) were carefully excised from control and pathogen-inoculated pea roots at sites of potential pathogen penetration. They were preembedded in 2% (wt/vol) aqueous Bacto agar to preserve the rhizosphere microbial populations and immersed in 3% (vol/vol) glutaraldehyde in 0.1 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 prior to embedding in Epon 812. 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 operating at 80 kV or processed for cytochemical labeling. For each treatment (bacteria alone, pathogen alone, or combination of bacteria and pathogen), an average of five samples from three roots were examined, using five sections per sample.

Preparation of colloidal gold probes. Colloidal gold with particles averaging 15 nm in diameter was prepared according to Frens (18). 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 (9).

Wheat germ agglutinin (WGA), a lectin with *N*-acetylglucosamine binding specificity, was used for localizing fungal 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. Ovomuroid was conjugated to gold at pH 5.4 (5).

Callose, a polymer of β -1,3-glucans, was detected by purified tobacco β -1,3-glucanase (pathogenesis related [PR] protein, PR-N) complexed to colloidal gold at pH 5.5 according to a recently described method (6). Localization of lignin-associated phenolic compounds was performed with a laccase (EC 1.10.3.2) purified from the white rot fungus *Rigidoporus lignosus* (20). The enzyme

was complexed to colloidal gold at pH 4.0, a pH value close to its reported isoelectric point of 3.83 (12). All gold complexes were stored at 4°C until use.

Cytochemical labeling. For direct labeling with the enzyme-gold complexes, ultrathin sections mounted on nickel grids were floated for 5 min on a drop of 0.01 M sodium phosphate-buffered saline (PBS) containing 0.02% of polyethylene glycol 20,000 at the pH corresponding to the optimal activity of the protein tested (6.0 for the three tested enzymes). Sections were transferred to a drop of each gold-complexed enzyme for 30 to 60 min at room temperature in a moist chamber. They were washed thoroughly with PBS, pH 7.4, rinsed with distilled water, and allowed to dry before staining with uranyl acetate and lead citrate.

For indirect labeling of *N*-acetylglucosamine residues, sections were floated on a drop of PBS, pH 7.4, for 5 min and 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 as described above.

Specificity of the different labelings was assessed by the following control tests: (i) addition of the corresponding substrate to each protein-gold complex for a competition experiment: β-1,4-glucans from barley (1 mg ml⁻¹) for the β-1,4-exoglucanase-gold complex; laminarin or laminaribiose (1 mg ml⁻¹) for the β-1,3-glucanase; and *p*-coumaric acid, ferulic acid, or sinapinic acid for the laccase (1 mg ml⁻¹); (ii) substitution of the protein-gold complex under study by bovine serum albumin-gold complex to assess the nonspecific adsorption of the protein-gold complex to the tissue sections; (iii) incubation of the tissue sections with the protein-gold complexes under nonoptimal conditions for biological activity; (iv) incubation of the tissue sections with colloidal gold alone to assess the nonspecific adsorption of the gold particles to the tissue sections; and (v) for indirect labeling of chitin, controls included both incubation with WGA to which was previously added an excess of *N*-*N'*-*N''*-triacetylchitotriose (1 mg ml⁻¹ in PBS) and incubation with WGA followed by unlabeled ovomucoid and ovomucoid-gold complex.

Reagents. The exoglucanase was provided by C. Breuil, Forintek, Canada, and the laccase was obtained from M. Nicole, ORSTOM, Montpellier, France. Tetrachloroauric acid was purchased from BDH Chemicals, Montreal. All other reagents for electron microscopy were obtained from JBEM Chemical Company, Pointe-Claire, QB, Canada.

RESULTS

SEM observations. Examination of samples from transformed pea roots collected as early as 2 days after inoculation with *Pseudomonas fluorescens* revealed that the bacterium abundantly colonized the root surface (Fig. 1A). Observation of several samples from five roots provided evidence of the bacterium's ability to establish close contact with the root surface through a fine mucilaginous matrix that could be readily detected in some areas (Fig. 1B, arrows). In spite of marked bacterial colonization of the root surface, features of root alteration, such as morphological changes, loss of turgor pressure, or collapse, were never seen, even 3 days after inoculation.

Observations of pathogen-inoculated pea root samples showed that both *P. ultimum* (Fig. 1C) and *F. oxysporum* f. sp. *pisi* formed densely branched mycelium on the root surface. Although features of potential host penetration were suspected in many places (Fig. 1C, arrow), it was nearly impossible to obtain clear evidence of this process through SEM, mainly because sites of possible entry in the roots were masked by a large number of intermingled hyphae.

Examination of challenged, bacterized root samples revealed marked differences between *P. ultimum* (Fig. 1D) and *F. oxy-*

sporum f. sp. *pisi* (Fig. 1E and F) in the extent of colonization. Although cells of *Pseudomonas fluorescens* closely interacted with hyphae of the two pathogens, growth and structural integrity of the hyphal cells differed strongly. As early as 2 days after contact between both partners, nearly all *Pythium* hyphae were characterized by pronounced loss of turgor and general collapse (Fig. 1D). Cell walls were not visually altered, whereas most hyphae of the pathogen appeared devoid of cytoplasm (Fig. 1D). Such hyphal alterations were not seen when *Pythium* developed at the root surface in the absence of *Pseudomonas fluorescens* (Fig. 1C). In contrast, interaction of *Pseudomonas fluorescens* with *F. oxysporum* f. sp. *pisi* did not result in such drastic fungal alterations (Fig. 1E and F). Indeed, *Fusarium* hyphae were seemingly turgid with no apparent signs of cellular disorganization (Fig. 1F). Whether this apparent structural preservation correlated with lack of damage or was due simply to the physical properties of the *Fusarium* cell walls could not be determined through SEM.

Ultrastructural features of *Pseudomonas fluorescens*-infected pea root tissue challenged with *P. ultimum*. In agreement with earlier observations (8), the current investigation of samples from *Pseudomonas fluorescens*-infected pea roots challenged with *P. ultimum* confirmed that bacterial interactions with the pathogen at the root surface resulted in limited host colonization. Although some hyphae of *P. ultimum* could penetrate the root tissues, their growth and development were restricted to the epidermis and the first outer cortical cell layers (Fig. 2A). In line with the SEM observations, our investigation revealed that bacterial cells developed abundantly at the root surface and established close contact with the pathogen, causing severe damage, as judged by the appreciable morphological and structural changes observed (Fig. 2A and B). Osmiophilic droplets often interacted with the cell surface of markedly altered *Pythium* hyphae (Fig. 2B, arrowheads).

Pathogen ingress toward the root cortical area was associated with a number of visible host defense reactions, such as the formation of polymorphic wall appositions along the host epidermal cells (Fig. 2C) and the occlusion of some intercellular spaces with fibrillo-granular material (Fig. 2D). Bacterial cells also were found in these outer tissues, and frequent associations between both microorganisms were detected in intercellular spaces (Fig. 2E). Most hyphae of the pathogen that penetrated the epidermis exhibited considerable morphological and structural changes, resulting in wall distortion, cytoplasm disorganization, and, often, loss of the protoplast (Fig. 2E).

Ultrastructural features of pea root tissues infected by *F. oxysporum* f. sp. *pisi*. Examination of *Fusarium*-infected samples from nonbacterized pea roots at the TEM level showed that hyphae of the pathogen developed abundantly through much of the cortex (Fig. 3A), endodermis, and paratracheal parenchyma cells and rapidly reached the xylem vessels. Fungal growth was mainly intracellular but also occurred intercellularly (Fig. 3A). Most fungal cells showed a typical ultrastructure with a dense cytoplasm closely appressed against the thin cell wall (Fig. 3A). Pathogen ingress toward the vascular stele coincided with extensive host cell alterations, such as aggregation of the cytoplasm, organelle disintegration, and cell wall dissolution (data not shown). In these nonbacterized roots, pathogen invasion failed to stimulate host reactions such as wall appositions, intracellular deposits, intercellular plugging, and xylem vessel occlusions.

Ultrastructural and cytochemical features of *Pseudomonas fluorescens*-infected pea root tissues challenged with *F. oxysporum* f. sp. *pisi*. In line with the SEM observations, our ultrastructural investigations provided evidence that close interaction of the pathogen with the bacteria at the root surface did not prevent fungal development nor did it cause structural hyphal damage (Fig. 3B and C). Indeed, extensive fungal growth was seen at the surface of bacterized pea roots (Fig. 3B). Although bacteria frequently closely interacted with *Fusarium* hyphae, the metabolic activity of the fungal cells appeared to be preserved, as judged by

the presence of a regular cytoplasm in which was embedded numerous organelles (Fig. 3D). Osmiophilic droplets, most likely produced by the bacterial cells, were often seen at the cell surface of *Fusarium* hyphae (Fig. 3C, arrows). However, this interaction did not correlate with fungal alterations in a way similar to that observed with *Pythium* hyphae (Figs. 3C and 2D). Incubation of ultrathin sections with the WGA/ovomucoid-gold complex for the localization of chitin resulted in regular and specific deposition of gold particles over the cell walls of *F. oxysporum* f. sp. *pisi* hyphae (Fig. 3D). The active growth of *Fusarium* hyphae at the surface of bacterized roots together with the preservation of their structural integrity suggested there was no direct antimicrobial action exerted by *Pseudomonas fluorescens* on *F. oxysporum* f. sp. *pisi*.

Pathogen ingress in the root epidermis occurred often through direct wall penetration (Fig. 4A) but also through localized cell wall disruptions. These alterations were usually characterized by

an apparent disruption of the fibrillar network at the junction between epidermal cells and the accumulation of an electron-dense granular material that interacted with the fungal cells without causing obvious physical damage. Pathogen growth toward the cortical area was apparently prevented by the formation at the epidermis level of heterogeneous wall appositions that varied in size, shape, and texture (Fig. 4B). *Fusarium* hyphae were frequently encased in this newly formed material (Fig. 4B). In spite of this structural defensive line in the epidermis, a few fungal cells could penetrate and colonize some intercellular spaces in the outer cortex (Fig. 4C). *Fusarium* hyphae adjacent to bacteria in the colonized intercellular spaces were not altered. In contrast, some host wall alteration could be detected in invaded areas, as exemplified by the apparent decrease in the electron density of the primary walls (Fig. 4C, arrows) and the punctual disruption of middle lamellae in places (Fig. 4C, arrowhead). Despite this apparent wall degradation, labeling

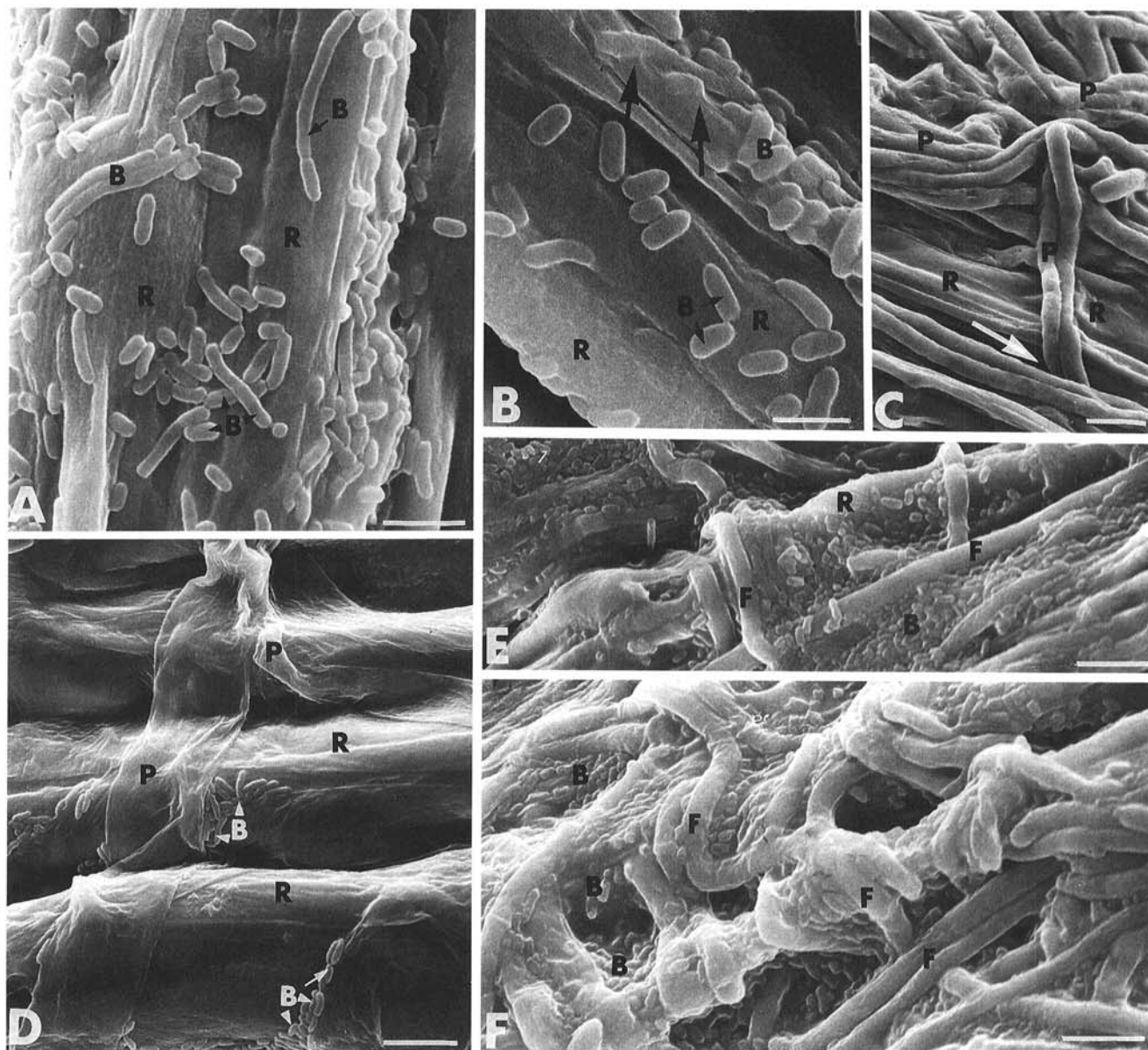


Fig. 1. Scanning electron micrographs of samples from Ri T-DNA-transformed pea roots. **A and B**, Samples from transformed pea roots collected 2 days after inoculation with *Pseudomonas fluorescens* strain 63-28. Bacteria (B) abundantly colonize the host roots (R). Close contact is apparently established with the root surface through a fine mucilaginous matrix (B, arrows). **A**, 4,000 \times ; bar = 3 μ m. **B**, 6,000 \times ; bar = 2 μ m. **C**, Sample from transformed pea roots collected 2 days after inoculation with *Pythium ultimum* (P). Densely branched mycelium occurs on the root surface. Features of potential root (R) penetration are suspected in places (arrow). 800 \times ; bar = 10 μ m. **D**, Sample from *Pseudomonas fluorescens*-inoculated pea roots collected 2 days after challenge with *P. ultimum*. Hyphae of the pathogen (P) show pronounced collapse and loss of turgor. R = root; B = bacteria. 3,000 \times ; bar = 4 μ m. **E and F**, Sample from *Pseudomonas fluorescens*-inoculated pea roots collected 2 days after challenge with *Fusarium oxysporum* f. sp. *pisi*. The fungus (F) does not apparently suffer from the presence of bacteria, because no apparent signs of cell disorganization are observed. R = root. **E**, 2,000 \times ; bar = 5 μ m. **F**, 3,000 \times ; bar = 4 μ m.

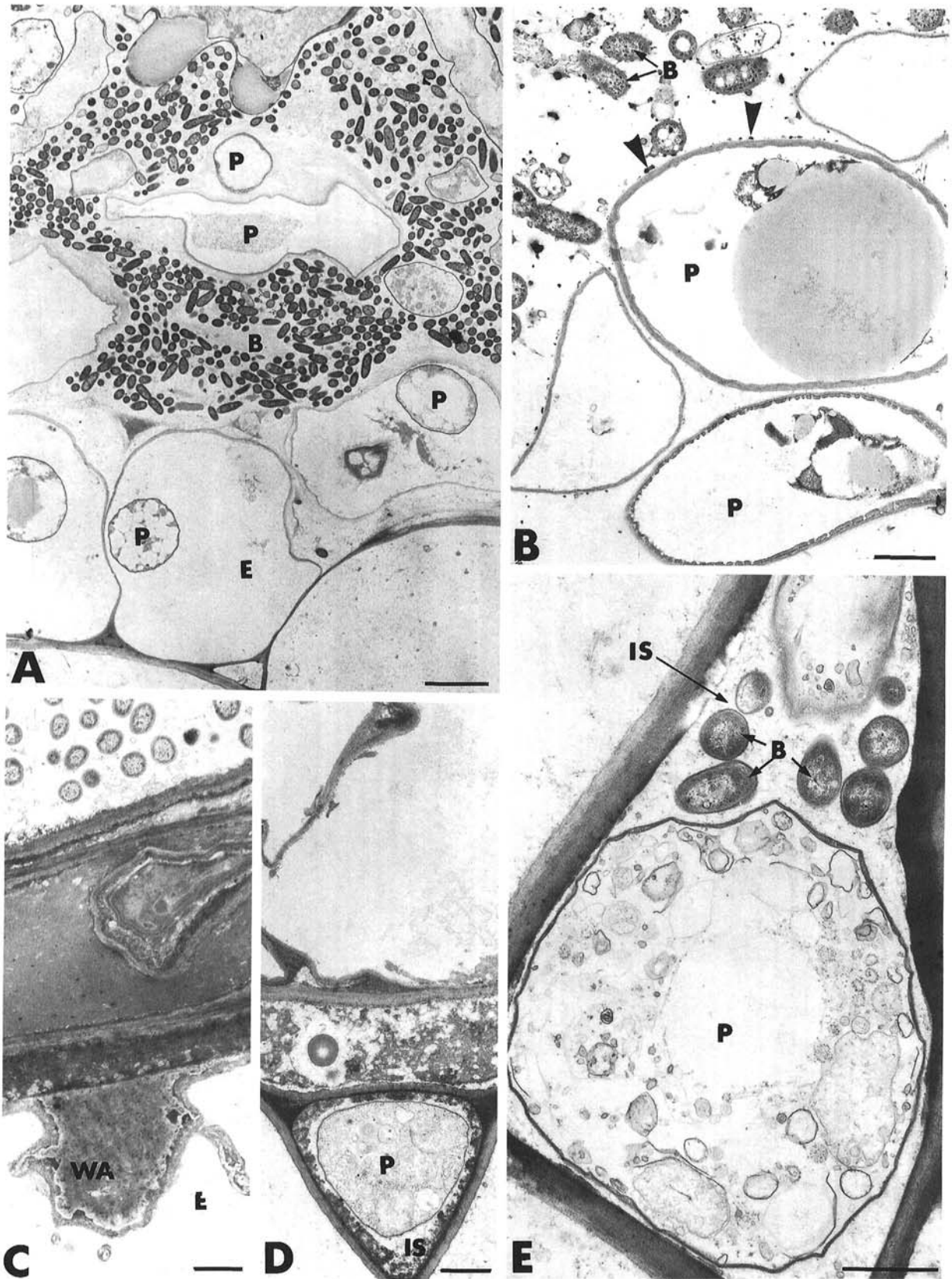


Fig. 2. Transmission electron micrographs of *Pseudomonas fluorescens*-inoculated pea root tissues collected 2 days after challenge with *Pythium ultimum*. **A and B,** Interaction between *Pseudomonas fluorescens* and *P. ultimum* at the root surface. Hyphae of the pathogen (P) show marked alterations, as judged by their abnormal shape accompanied by strong plasmalemma retraction and organelle breakdown. Droplets of osmiophilic material produced by the bacteria (B) are seen at the fungal cell surface (B, arrowheads). Some *Pythium* (P) hyphae are seen in the root epidermis (E). **A,** 3,000X; bar = 3 μ m. **B,** 10,000X; bar = 1 μ m. **C,** A wall apposition (WA) in the epidermis (E). 8,000X; bar = 2 μ m. **D,** Fibrillo-granular material in an intercellular space (IS) colonized by a cell of *P. ultimum* (P). 8,000X; bar = 2 μ m. **E,** A dead cell of *P. ultimum* (P) neighboring bacterial cells (B) occurs in an intercellular space (IS). 16,000X; bar = 1 μ m.

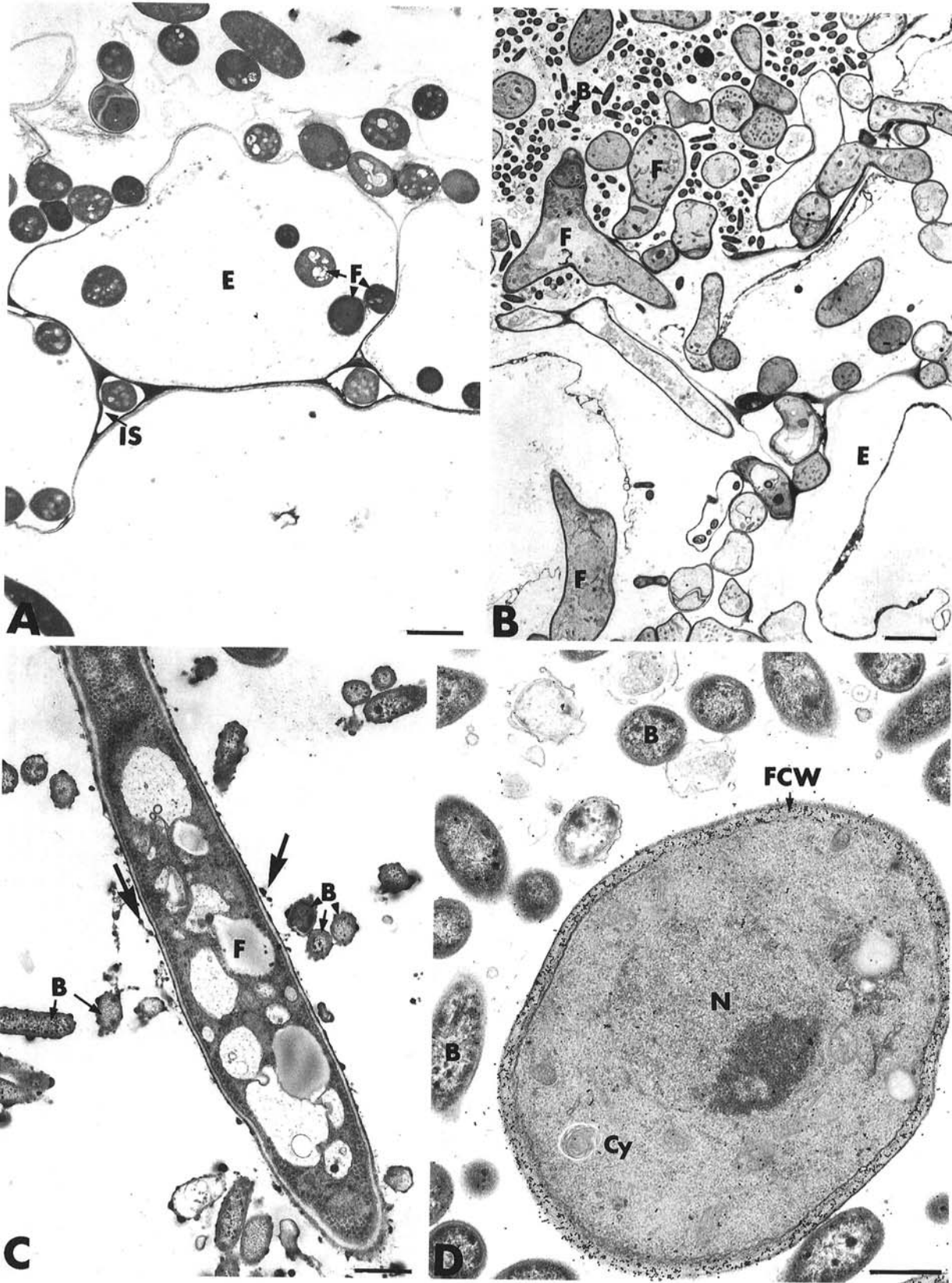


Fig. 3. Transmission electron micrographs of *Pseudomonas fluorescens*-inoculated pea root tissues collected 2 days after challenge with *Fusarium oxysporum* f. sp. *pisi*. **A**, In control roots grown in the absence of *Pseudomonas fluorescens*, *Fusarium* hyphae (F) grow abundantly at the root surface and rapidly colonize the root tissues by centripetal growth. Fungal cells are seen in intra- and intercellular compartments. E = epidermis; IS = intercellular space. 3,000X; bar = 3 μ m. **B and C**, Interaction of *Fusarium* hyphae (F) with the bacteria (B) at the root surface does not prevent fungal penetration of the root epidermis (E). Osmiophilic droplets (C, arrows), likely produced by the bacterial cells, are seen at the cell surface of *Fusarium* hyphae (F), which apparently do not suffer from any damage. **B**, 3,000X; bar = 3 μ m. **C**, 10,000X; bar = 1 μ m. **D**, Incubation of ultrathin sections with wheat germ agglutinin/ovomuroid-gold complex for localization of chitin results in regular and specific deposition of gold particles over the cell walls (FCW) of a *Fusarium* cell. The metabolic activity of the fungal cell appears to be preserved, as judged by the presence of a regular cytoplasm (Cy) in which are embedded numerous organelles, including the nucleus (N). 25,000X; bar = 0.5 μ m.

with the gold-complexed exoglucanase resulted in regular distribution of gold particles over the host cell walls, indicating the cellulose framework was still in place (Fig. 4C).

Application of the WGA/ovomucoid-gold complex to sections of bacterized pea roots inoculated with *F. oxysporum* f. sp. *pisi* resulted in slight labeling of the pathogen cell walls (Fig. 4D). Gold particles were distributed more randomly than normal (Fig. 3D), and interestingly, they were nearly absent over large fungal cell wall areas (Fig. 4D, arrow). Electron-opaque flecks lining the host cell walls frequently extended toward the inside and interacted with the fungal cell surface (Fig. 4D, arrowheads). When such interactions occurred, they were associated with some disturbances of the fungal cells, such as abnormal circonvolution of the plasma membrane and loosening of the cytoplasm matrix.

A considerable number of structural plant defense reactions were seen in the regions proximal to fungal penetration (Fig. 5A through C). The wall appositions formed in the reacting cortical host cells varied enormously in their appearance, from elongated deposits along a large portion of the host cell wall (Fig. 5C) to hemispherical or domelike protuberances (Fig. 5A and B). Frequently, the wall appositions were delimited by a band of osmiophilic material (Fig. 5A). It was difficult to determine whether this densely stained layer originated from an aggregation of the remaining cytoplasm or was newly synthesized as a response to infection. Hemispherical protuberances, the so-called papillae, were highly heterogeneous in size, shape, and texture (Fig. 5A and B). They were usually made up of an amorphous matrix in which small electron-dense structures were embedded. After incubation of sections with the gold-complexed tobacco β -1,3-glucanase, a considerable number of gold particles were detected over all the wall appositions, regardless of their size, shape, texture, or architecture (Fig. 5B and C). A few scattered gold particles were detected over the host cell walls (Fig. 5B). Control tests, including incubation of the enzyme-gold complex with laminarin prior to section labeling, yielded negative results (data not shown).

In colonized areas, both cortical cells and intercellular spaces were frequently filled with aggregated deposits that often accumulated at the cell surface of markedly altered invading hyphae (Figs. 5C and 6A). The host cell wall itself displayed a higher electron density than normal, indicating the probable infiltration of structural molecules (Fig. 6B). Incubation of sections with the gold-complexed laccase for the localization of phenolic or lignin-like compounds resulted in specific deposition of gold particles over the host cell walls (Fig. 6C), wall appositions (Fig. 6C), and material filling some intercellular spaces (Fig. 6B and D). Although no labeling could be detected over the aggregates lining the host cell walls, probably because of the high compactness of this material, gold particles were specifically associated with the fine electron-opaque flecks likely released from the aggregated material and interacting with the fungal cell surface (Fig. 6B, arrows). Interestingly, gold particles were detected not only over the material formed in intercellular spaces (Fig. 6E, arrow) but also over the walls of fungal cells surrounded by this material (Fig. 6D and E). In all sections examined, these fungal cells showed signs of obvious alteration and were often depleted of protoplasm (Fig. 6C). Such a fungal cell alteration was never seen in nonbacterized, control roots. Incubation of the laccase-gold complex with either ferulic acid or *p*-coumaric acid prior to section treatment abolished the labeling over the cell walls, wall appositions, and dense material (data not shown).

DISCUSSION

The results of the current study demonstrate that pea roots gain increased protection against *F. oxysporum* f. sp. *pisi* as a result of preinoculation with *Pseudomonas fluorescens* strain 63-28. Evidence is provided that the beneficial effect of *Pseudomonas fluorescens* in reducing the extent of fungal colonization in the root

tissues is primarily associated with stimulation of the plant defense responses and not with a direct inhibitory effect on fungal growth, as occurs with *P. ultimum* (8). These observations support the concept that the mechanisms by which a single strain of *Pseudomonas fluorescens* provides biological control against soilborne pathogens are selective, probably because highly specific molecular events at the cell surface determine the outcome of a given interaction.

In line with our previous observations (8), the current results confirmed that *Pseudomonas fluorescens* strain 63-28 successfully penetrated the epidermis and established itself in the outer root tissues through colonization of intercellular spaces. SEM observations of samples from the interaction region showed that bacteria abundantly colonized the root surface and established close contact with the outermost host cell layers through a thin mucilage. Little is known about the mechanism of initial attachment of fluorescent pseudomonads to root surfaces, although a number of hypotheses involving fimbriae (38), outer membrane proteins (15), and lipopolysaccharides (37) have been raised. However, these explanations are still a matter of speculation, and the exact role of cell-surface molecules in the adhesion process remains obscure, although there is strong evidence that cell-surface interactions provide the initial binding that determines the host-recognition response in a number of host-microbe interactions (16).

When bacterized pea roots were challenged with either *P. ultimum* or *F. oxysporum* f. sp. *pisi*, strong differences in the extent of fungal damage were observed by SEM. The rapid collapse and loss of turgor of *P. ultimum* hyphae was taken as an indication that the bacteria produced antifungal metabolites that could play an important role in controlling the pathogen population in the rhizosphere. However, the finding that *Fusarium* hyphae were not affected in the same manner by the presence of bacteria corroborates the current concept that a large number of PGPR strains, which enhance plant protection against a broad range of pathogens, produce metabolites that have very specific effects and target selected microorganisms only (41).

In line with these SEM observations, our cytological investigations of the root surface confirmed that the interaction between *Pseudomonas fluorescens* and *F. oxysporum* f. sp. *pisi* did not correlate with cell disturbances (cytoplasm disorganization, plasmalemma retraction, or loss of protoplasm) similar to those seen with *Pythium* hyphae (8). This suggests that *F. oxysporum* f. sp. *pisi* is much less sensitive than *P. ultimum* to the action of the secondary metabolites produced by *Pseudomonas fluorescens* strain 63-28, indicating that direct antimicrobial activity is not an operable mechanism in biological control of this fungal pathogen.

This idea has been substantiated recently by the observation that direct antibiosis and competition are not involved in biological control of *Colletotrichum orbiculare*, the agent of cucumber anthracnose (39). Support for this hypothesis also was provided by experiments in which the pathogen and the PGPR strain were applied at spatially separated locations (2,40). In these studies, which excluded direct antagonism, the activation of the plant defense strategy, leading to systemic protection, was considered the only mechanism responsible for the observed disease suppression. Because of the first indication by Kempe and Sequeira (23) that plant disease resistance possibly could be stimulated by beneficial *Pseudomonas*, an increasing number of studies have contributed to the demonstration of the key role played by the plant in the biological control exerted by PGPR (34). However, in spite of renewed interest in PGPR-induced resistance, information on the nature, magnitude, and spatial distribution of plant defense reactions is limited and warrants further investigation.

Restriction of *Fusarium* growth and development to the epidermis and outer cortex and a marked decrease in pathogen viability were features observed only in bacterized pea roots. In controls, rapid necrotrophic colonization of epidermal, cortical, and stelar tissues correlated with the formation of widespread, macroscopically

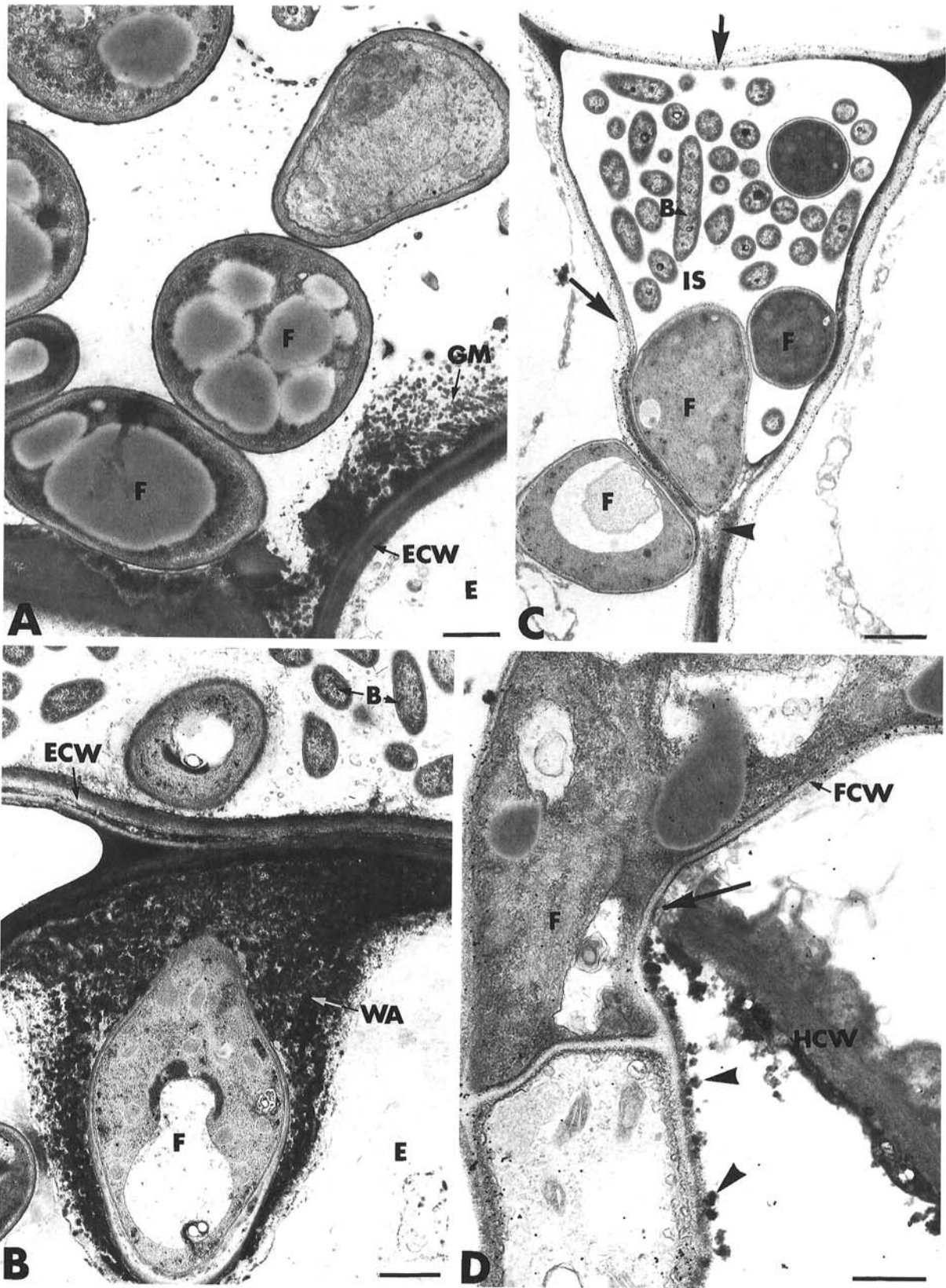


Fig. 4. Transmission electron micrographs of *Pseudomonas fluorescens*-inoculated pea root tissues collected 2 days after challenge with *Fusarium oxysporum* f. sp. *pisi*. **A**, Penetration of *Fusarium* hyphae (F) through localized epidermal cell wall (ECW) alterations characterized by an apparent disruption of the fibrillar network at the junction between epidermal (E) cells and by the accumulation of electron-dense granular material (GM). 18,000 \times ; bar = 0.5 μ m. **B**, Formation of a wall apposition (WA) along the epidermal cell wall (ECW) in which a fungal cell (F) is trapped. E = epidermis. 10,000 \times ; bar = 1 μ m. **C**, An intercellular space (IS) in the outer root cortex is colonized by both the bacteria (B) and *Fusarium* hyphae (F). Some host wall alterations are detected, as exemplified by the apparent decrease in the electron density of the primary walls (arrows) and by the punctual disruption of middle lamellae in places (arrowhead). 10,000 \times ; bar = 1 μ m. **D**, A marked decrease in labeling intensity over the pathogen cell walls (FCW) is observed after treatment with the wheat germ agglutinin/ovomucoid-gold complex. Labeling is nearly absent over the fungal cell wall areas neighboring the host cell wall (arrow). Electron-opaque flecks lining the host cell walls frequently extend toward the inside and interact with the fungal cell surface (arrowheads). 25,000 \times ; bar = 0.5 μ m.

visible lesions that led eventually to the death of the root system. Support for the close association between the presence of bacterial cells and induced resistance came from the observation that host cell wall damage in advance of invading hyphae, a typical feature

of invasion monitored in nontreated plants, was absent from bacterized roots. In pretreated roots, the apparent preservation of the cell wall architecture as well as the massive accumulation of structural barriers at sites of attempted penetration indicated that

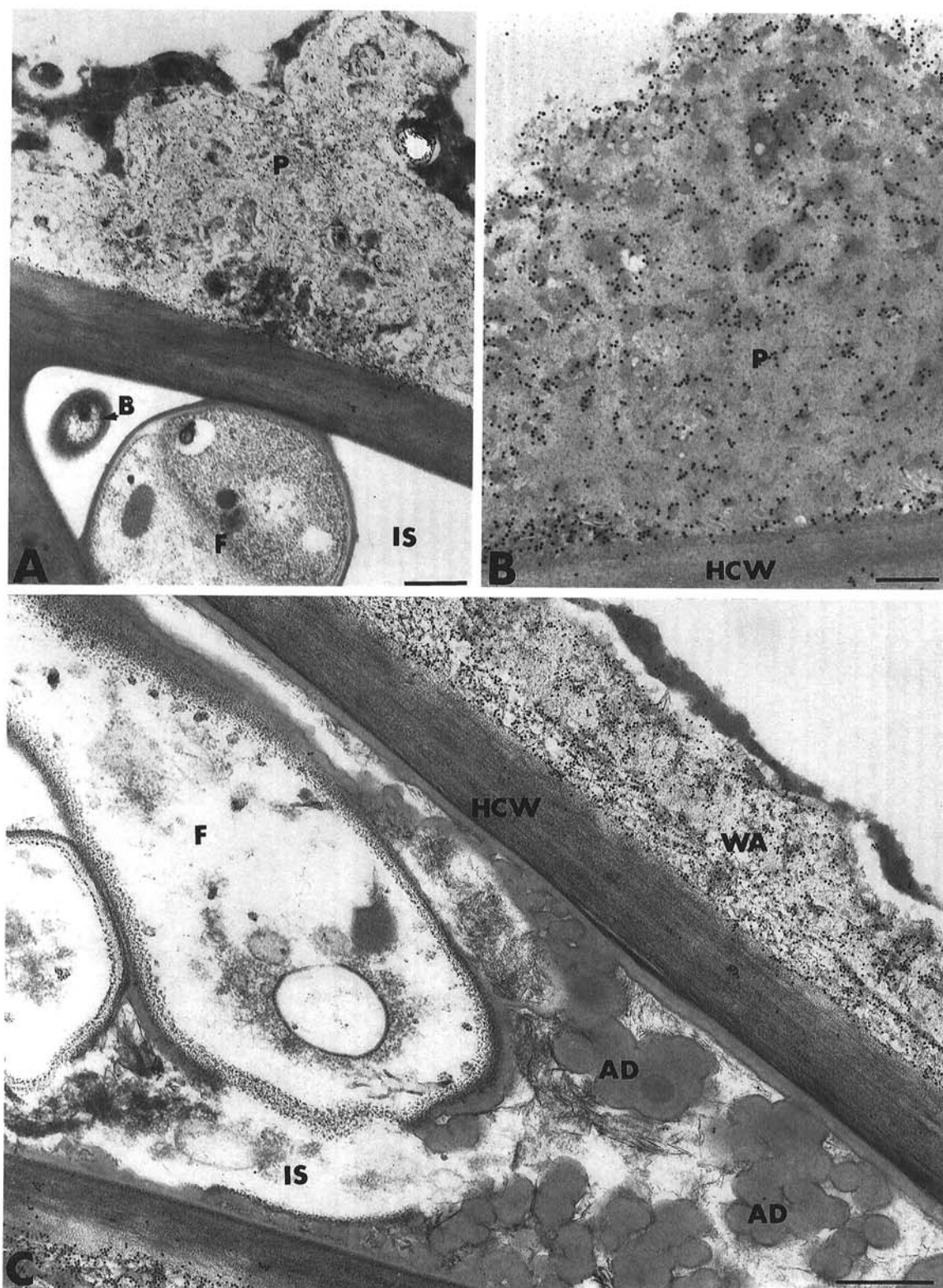


Fig. 5. Transmission electron micrographs of *Pseudomonas fluorescens*-inoculated pea root tissues collected 2 days after challenge with *Fusarium oxysporum* f. sp. *pisi*. **A and B**, Hemispherical or domelike protuberances, often delimited by a band of osmiophilic material (**A**), are seen in areas of potential fungal penetration. These papillae (**P**) are heterogeneous in size, shape, and texture and are made of an amorphous matrix in which small electron-dense structures are embedded. Incubation with gold-complexed tobacco β -1,3-glucanase for the localization of callose results in the deposition of numerous gold particles over the papillae. **B** = bacteria; **IS** = intercellular space. **B**, A few scattered gold particles are detected over the host cell walls (**HCW**). **A**, 20,000 \times ; bar = 0.5 μ m. **B**, 40,000 \times ; bar = 0.25 μ m. **C**, A callose-enriched, elongated wall apposition (**WA**) is formed along the host cell wall in an area adjacent to an invaded intercellular space where aggregated deposits (**AD**) interacting with *Fusarium* hyphae (**F**) are detected. 30,000 \times ; bar = 0.5 μ m.

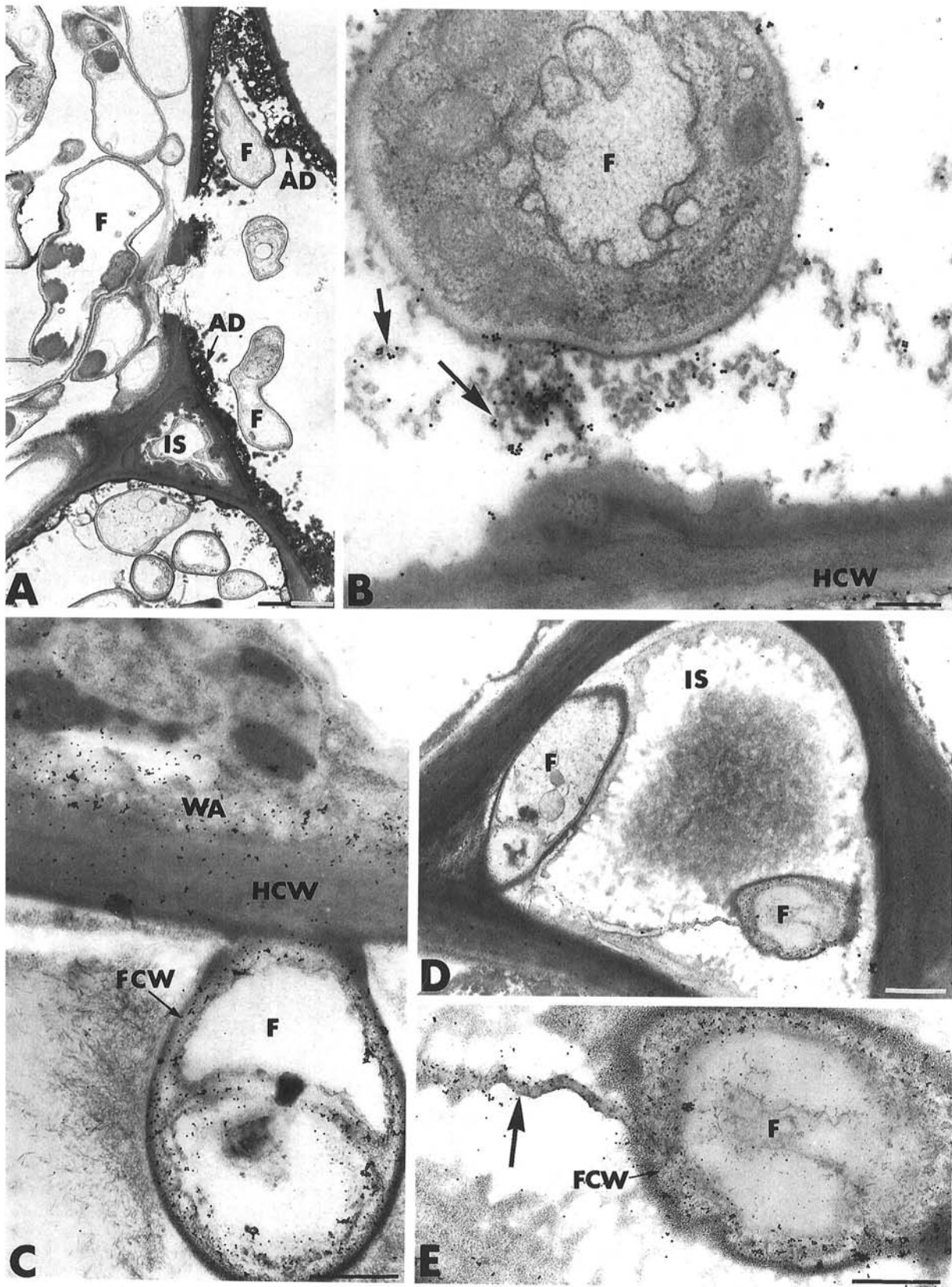


Fig. 6. Transmission electron micrographs of *Pseudomonas fluorescens*-inoculated pea root tissues collected 2 days after challenge with *Fusarium oxysporum* f. sp. *pisii*. **A**, In some areas of the outer root cortex, an abundant accumulation of aggregated deposits (AD) is detected in both the host cells and the intercellular spaces (IS). F = fungus. 6,000X; bar = 2 μ m. **B through E**, Labeling with the gold-complexed laccase for the localization of phenolic or lignin-like compounds results in deposition of gold particles over **B**, the host cell walls (HCW), **C**, the wall appositions (WA), and **D and E**, the material filling some intercellular spaces (IS and arrow, respectively). Although no labeling is detected over the aggregates lining the host cell walls, probably because of the high compactness of this material, gold particles are specifically associated with the fine electron-opaque flecks likely released from the aggregated material and interacting with the fungal cell surface (**B**, arrows). **C and E**, Gold particles also occur over the walls of altered fungal cells (FCW). **B**, 40,000X; bar = 0.25 μ m. **C**, 30,000X; bar = 0.5 μ m. **D**, 10,000X; bar = 1 μ m. **E**, 30,000X; bar = 0.5 μ m.

host cell walls were likely protected against both physical and biochemical contact with the pathogen.

In line with earlier reports (10,12), it was clear that fungal challenge was essential for the expression of a prominent structural response in bacterized roots. In the absence of *Fusarium* infection, the cytological consequences of *Pseudomonas* infection were limited to the colonization of a few intercellular spaces without any visible expression of host cell reactions (data not shown). Similar observations have been reported recently in a study dealing with the influence of a mycorrhizal infection on the rate and extent of pathogen colonization in carrot roots. It was found that defense reactions were expressed at a much higher magnitude when contact with the pathogen was established (10).

Because *Pseudomonas*-treated plants responded more efficiently to *Fusarium* infection than did nonbacterized plants, it seems reasonable to assume that the bacteria evoked the transcriptional activation of defense genes, the expression of which was likely transient until a pathogen-produced signal could restimulate the posttranscriptional process. These results are of particular relevance because they provide further insights into the concept that certain agents of a chemical (14) or biological (30) nature are capable of evoking biochemical events characteristic of the natural plant disease resistance process upon pathogen infection.

Recent papers have shown that PR protein genes are coordinately induced to high levels of expression during the onset of PGPR-induced resistance in various plants (35,42). Although these observations suggest that an increase in PR protein synthesis is one of the main determinants involved in the expression of resistance triggered by *Pseudomonas* strains, one may argue that effective resistance is the result of the coordinated action in space and time of several defense reactions. In this context, the current study is the first to provide a clear image of the drastic structural changes occurring at the onset of PGPR-induced resistance in pea.

The success of a plant in warding off invading pathogens relies primarily on its ability to rapidly build a line of defense for protecting cell walls against pathogen spread (7). Results from the current study demonstrate that striking modifications of the epidermal and cortical cell walls as well as deposition of newly formed barriers are triggered in *Pseudomonas*-treated plants in response to *Fusarium* infection. Such cellular changes, characterized by deposition onto the inner surface of the cell walls of callose-enriched wall appositions, were apparently efficient in preventing fungal ingress toward the vascular stele and probably also in shielding the inner root tissues from phytotoxic, diffusible products such as hydrolytic enzymes and toxins. Fungal cells in the vicinity of wall appositions frequently appeared disorganized, suggesting the occurrence of a fungitoxic environment. Support for this speculation came from the observation that aggregated material, enriched in phenolic compounds, as shown by laccase-gold labeling, often interacted with invading hyphae.

In agreement with a previous report (12), the current cytochemical study confirmed that laccases, the enzymes known to play a key role in lignin breakdown (13) in addition to being involved in the oxidation and polymerization of endogenous plant phenols (31), were valuable tools for localizing phenolic-like compounds in plant tissues. The labeling pattern obtained with the gold-complexed laccase showed that phenolic compounds were widely distributed in *Fusarium*-challenged, bacterized roots. If one considers that phenolic substances can confer strong rigidity to cell wall structures through peroxidase-mediated cross-linking with constitutive (i.e., hemicellulose and pectin) and newly formed (i.e., callose) wall carbohydrates (19), it is tempting to speculate that these compounds contribute to the elaboration of physical barriers restricting pathogen spread. Support for this speculation is provided by earlier observations that indicated plant root colonization by PGPR promoted peroxidase activity (1) and enhanced lignin accumulation (3) in bean. In addition to their infiltration at strategic sites of potential penetration, phenolic compounds also

were detected in the host cells as amorphous aggregates often interacting with the fungal cell surface. This abnormal accumulation of phenolic-enriched deposits in the fungal cell walls was associated with morphological changes and cytological alterations of the invading hyphae.

Although the possibility that the detected phenolic compounds may originate from the fungus itself as a stress reaction cannot be ruled out, the current observations suggest that these compounds were laid down by the plant and may restrict pathogen growth through fungitoxic activity. It is well known that such secondary metabolites and the free radicals formed during oxidative polymerization reactions are highly toxic and disturb fungal metabolism by causing deregulation of enzyme production (33). According to their wide pattern of distribution, phenolic compounds may play a key role in PGPR-mediated induced resistance by directly inhibiting fungal growth and indirectly protecting plant cell walls from the deleterious effect of microbial toxins and enzymes. This confirms and extends earlier results suggesting that the increased accumulation of phytoalexins triggered by *Pseudomonas fluorescens* in carnation was, at least partly, responsible for the enhanced resistance of the plants to *Fusarium* infection (36).

Evidence was provided through the use of the WGA/ovomucoid-gold complex that the wall-bound chitin component in *Fusarium* hyphae colonizing bacterized roots was disrupted. The decrease in labeling intensity over *Fusarium* cell walls suggests that the root cells were signaled to produce chitinases that likely accumulated at sites of fungal penetration, as previously shown in the interaction between tomato roots and *F. oxysporum* f. sp. *radicis-lycopersici* (11). However, the finding that some chitin molecules still occurred over cell walls at a time when hyphae had undergone substantial degradation suggests that production of chitinases is not an early process in the cascade of events leading to the expression of plant resistance. It is more likely that structural barriers and toxic substances such as phenolics and phytoalexins are the first defense mechanisms preceding the synthesis and accumulation of other molecules, including chitinases and other hydrolytic enzymes, such as β -1,3-glucanases, that probably contribute to complete fungal cell breakdown.

The results reported here show that pea root bacterization with *Pseudomonas fluorescens* strain 63-28 induces a set of plant defense reactions that culminates in the elaboration of physical barriers and the creation of a fungitoxic environment that adversely affects pathogen growth and development. These observations are consistent with the current concept that PGPR may function as potential inducers of plant disease resistance and shed more light on the mechanisms by which this resistance is expressed at the cellular level.

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