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Induction of Defense-Related Ultrastructural Modifications in Pea Root Tissues Inoculated with Endophytic Bacteria[1]

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The stimulation exerted by the endophytic bacterium *Bacillus pumilus* strain SE34 in plant defense reactions was investigated at the ultrastructural level using an in vitro system in which root-inducing T-DNA pea (*Pisum sativum* L.) roots were infected with the pea root-rotting fungus *Fusarium oxysporum* f. sp. *pisi*. In nonbacterized roots, the pathogen multiplied abundantly through much of the tissue including the vascular stele, whereas in prebacterized roots, pathogen growth was restricted to the epidermis and the outer cortex. In these prebacterized roots, typical host reactions included strengthening the epidermal and cortical cell walls and deposition of newly formed barriers beyond the infection sites. Wall appositions were found to contain large amounts of callose in addition to being infiltrated with phenolic compounds. The labeling pattern obtained with the gold-complexed laccase showed that phenolics were widely distributed in *Fusarium*-challenged, bacterized roots. Such compounds accumulated in the host cell walls and the intercellular spaces as well as at the surface or even inside of the invading hyphae of the pathogen. The wall-bound chitin component in *Fusarium* hyphae colonizing bacterized roots was preserved even when hyphae had undergone substantial degradation. These observations confirm that endophytic bacteria may function as potential inducers of plant disease resistance.

In recent years, the process of plant "immunization" or induced resistance to diseases has received increasing attention (Kùc, 1987; Uknes et al., 1992). A promising strategy that is gaining interest concerns the potential value of endophytic bacteria in promoting plant disease resistance (Chen et al., 1995; Fiddaman and Rossall, 1995; Tuzun and Kloepper, 1995). During the course of their co-evolution, plants and bacterial endophytes have developed an intimate relationship that has probably resulted from an extensive exchange of information at the cellular and molecular levels. Evidence has shown that these bacteria naturally occur in healthy plant organs, where they establish residence without causing visible host damage (Jacobs et al., 1985; Misaghi and Donndelinger, 1990). Understandably, most efforts have been concentrated on the pattern of tissue colonization by these bacteria and on their possible beneficial effects in increasing nutrient uptake and stimulating plant growth (Patriquin and Döbereiner, 1978). Although such studies have undoubtedly contributed to our knowledge of plant biology and microbial ecology, it is only recently that inoculation of a host plant with endophytic bacteria has been shown to reduce disease incidence and severity (Poon et al., 1977; Dimock et al., 1989). A clear indication that these bacteria had the potential of becoming a new class of biocontrol agents came from a recent study conducted by Chen et al. (1995), who demonstrated that six of the tested bacterial strains significantly reduced the expression of *Fusarium* wilt in cotton, and they concluded that bacterial endophytes were good candidates for the biological control of vascular pathogens.

In spite of renewed interest in exploiting bacterial endophytes as a method for controlling plant diseases, the exact mechanisms by which these microorganisms confer increased plant protection have not been fully investigated, although a number of hypotheses including production of siderophores, accumulation of antifungal metabolites, nutrient competition, and niche exclusion have been raised (Chen et al., 1995). In a recent report, Benhamou et al. (1996) provided evidence that root colonization by the endophytic bacterium *Pseudomonas fluorescens*, strain 63–28, involved a sequence of events that included bacterial attachment to the plant root, proliferation along the elongating root, and local penetration of the epidermis. Although there is no direct evidence for the involvement of hydrolytic enzymes in the process of root colonization by beneficial rhizobacteria, enzymatic hydrolysis of epidermal cell walls has been postulated as a possible mechanism in some cases where strains of these bacteria were considered to be opportunistic pathogens (Campbell et al., 1987). Thus, there are good reasons to believe that bacterial penetration of the epidermis may affect to some extent the plant physiology, ultimately leading to the activation of defense genes upon pathogen challenge. Support for this hypothesis is drawn by the recent observations that enhanced resistance in plants inoculated with PGPR was correlated with marked host metabolic changes culminating in a number of physical (Anderson and Guerra, 1985) and biochemical (Van Peer et al., 1991; Zdor and Anderson, 1992) responses.

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Abbreviations: AGL, *Aplysia* gonad lectin; PDA, potato dextrose agar; PGPR, plant growth-promoting rhizobacteria; Ri, root-inducing; TEM, transmission electron microscopy; WGA, wheat germ agglutinin.

At present, the situation with endophytic bacteria is not so clearly defined and additional research is obviously needed to confirm that the observed reduction of disease incidence is correlated with effective stimulation of the plant defense system.

A growing body of evidence indicates that ultrastructural investigation of the host response to pathogen attack can provide key information about the mechanisms underlying plant disease resistance (Benhamou, 1995). Recent advances in the isolation and purification of specific probes have provided opportunities to develop approaches that not only allow an accurate localization of various molecules in their respective cell compartments, but also help to elucidate their potential functions (Benhamou, 1992; Benhamou et al., 1994b). Thus, cyto- and immunocytochemical approaches have become an essential complement to biochemical analyses of plant defense molecules, and it is expected that improvements in probe specificity will extend the applicability of these methods to additional research areas in plant disease resistance. Although endophytic bacteria have been successfully detected in plant tissues by means of light microscopy (Patriquin and Döbereiner, 1978), little is known about the cellular and molecular events associated with pathogen challenge in bacterized plants.

The present paper reports the influence exerted by endophytic bacteria in stimulating plant defense mechanisms. Recently, Benhamou et al. (1994a, 1996) provided evidence that Ri T-DNA-transformed pea (*Pisum sativum* L.) roots were useful tools for delineating the cytologically visible consequences of cross-protection. Transformed roots, obtained by inoculating plant tissues with virulent strains of the soil bacterium *Agrobacterium rhizogenes* and then isolating the adventitious roots arising from the wound sites (Savary and Flores, 1994), offer the advantages of genetic and biochemical stability and faster growth than untransformed root systems. To enhance our understanding of the potential for bacterial endophytes to induce plant disease resistance, the present study was undertaken to determine whether *Bacillus pumilus*, strain SE34, a bacterium that protects cotton roots against *Fusarium oxysporum* f. sp. *vasinfectum* attack (Chen et al., 1995), was also effective in controlling infection by the pea root-rotting fungus *Fusarium oxysporum* f. sp. *pisi* (Van Hall) Snyd and Hans. Our results demonstrate that pea root bacterization with *B. pumilus* triggers a set of plant defense reactions that culminates in the elaboration of permeability barriers and in the creation of a fungitoxic environment that protects the roots by restricting pathogen growth and development to the outermost tissues.

MATERIALS AND METHODS

Preparation of Ri T-DNA-Transformed Pea Roots

Transformed pea (*Pisum sativum* L.) roots were obtained from Dr Y. Piché (Faculté de Foresterie, Université Laval, Québec, Canada). They were prepared by infecting 1-month-old pea shoots with *Agrobacterium rhizogenes* (American Type Culture Collection no. 15834, Rockville, MD) as described by Bécard and Fortin (1988). Adventitious roots were transferred onto modified White's medium (Bécard and Fortin, 1988) solidified with 0.4% (w/v) gellan gum (ICN). Clonal lines were established as axenic cultures after several transfers of root tips to fresh medium. Stock cultures were maintained in Petri dishes at 24°C in the dark.

Bacterial Strain and Growth Conditions

Bacillus pumilus strain SE34, stored in nutrient broth with 10% glycerol at −80°C, was retrieved by streaking on nutrient broth agar at 24°C. Two days later, bacterial cells were suspended in 0.85% aqueous NaCl and pelleted by low-speed centrifugation. Bacterial pellets were resuspended in 0.85% aqueous NaCl and the density of the suspension was adjusted to 10^6 cells/mL by measuring A_{640} and comparing it with a standard curve.

Fungal Strain and Growth Conditions

The root pathogen *Fusarium oxysporum* f. sp. *pisi* (Van Hall) Snyd and Hans (kindly provided by Dr. C. Richard, Agriculture Canada, Ste.-Foy, Quebec, Canada), known to be virulent on pea, was grown on potato-dextrose agar (Difco Laboratories, Detroit, MI) at 26°C in the dark.

Root Inoculation with *P. pumilus* and *F. oxysporum* f. sp. *pisi*

Inoculation of Ri T-DNA-transformed pea roots was performed by depositing 2 mL of the bacterial suspension at 10^6 cells/mL along the main root using a sterile micropipet. Bacterized roots were kept at 24°C for 2 to 3 d prior to being either directly processed for electron microscopy or challenged with the fungal pathogen. Samples from bacterized roots were collected from five Petri dishes in three replicated experiments.

Two to 3 d after inoculation with *B. pumilus*, transformed pea roots were inoculated with the mycelium of 3-d-old colonies of *F. oxysporum* f. sp. *pisi*. Fungal inoculation was performed either by placing 3-mm mycelial discs at a 2-cm interval along the root surface or by using a hyphal and spore suspension (10^8 colony-forming units/mL) prepared by scraping the surface of a Petri plate and homogenizing in 15 mL of sterile, distilled water in a blender (Waring). 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 bacterized. The pathogen was then allowed to grow for 1 to 3 d in contact with the roots before sampling for electron microscopy. Root samples were collected from five Petri dishes per time period in three replicated experiments.

Tissue Processing for Light Microscopy and TEM

Samples (2 mm^3) were carefully excised from control and pathogen-inoculated pea roots at sites of potential pathogen penetration and pre-embedded in 2% aqueous Bactoagar (Difco, Detroit, MI) to preserve the rhizosphere mi-

crobial populations. They were then 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 prior to dehydration in a graded ethanol series and embedding in Epon 812 (JBEM Chemical, Pointe-Claire, Quebec, Canada). Thin sections (0.7 μm) cut from the Epon-embedded material using glass knives were mounted on glass slides and stained with 1% aqueous toluidine blue prior to examination with a microscope (Axioscope, Zeiss). Ultrathin sections (0.1 μm), collected on nickel grids, were either contrasted with uranyl acetate and lead citrate for immediate examination with a transmission electron microscope (1200 EX, JEOL) operating at 80 kV, or further processed for cytochemical labeling. For each treatment, an average of five samples from three different roots were investigated. For each sample, 10 to 15 ultrathin sections were examined under the electron microscope.

Preparation of the Gold-Complexed Probes

Colloidal gold with particles averaging 12 nm in diameter was prepared according to Frens (1973) using sodium citrate as a reducing agent.

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 (Benhamou et al., 1987).

The AGL, a lectin isolated from the gonads of the sea mollusk *Aplysia depilans*, was used for localizing polygalacturonic acid-containing molecules (pectin) (Benhamou et al., 1988). This lectin was complexed to colloidal gold at pH 9.5.

WGA, a lectin with GlcNAc binding specificity, was used for localizing GlcNAc residues (chitin) according to a previously described procedure (Benhamou, 1989). 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.

A β-1,3-glucanase, purified from tobacco reacting hypersensitively to tobacco mosaic virus, was used for localizing β-1,3-glucans according to a recently described method (Benhamou, 1992). The enzyme was conjugated to colloidal gold at pH 5.5.

Localization of lignin-associated phenolic compounds was performed by using a laccase (EC 1.10.3.2) purified from the white rot fungus *Rigidoporus lignosus* (Geiger et al., 1986). The enzyme was complexed to colloidal gold at pH 4.0, a pH value close to its reported pI of 3.83 (Benhamou et al., 1994b).

All gold complexes were stored at 4°C until use.

Cytochemical Labeling

For the direct labeling of cellulosic β-1,4-glucans, polygalacturonic acids, β-1,3-glucans, and phenolic compounds, ultrathin sections were first incubated on a drop of PBS containing 0.02% (w/v) of PEG 20,000 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, the β-1,3-glucanase, and the laccase; pH 8.0 for the AGL). Sections were thereafter 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 with uranyl acetate and lead citrate.

For the indirect labeling of GlcNAc residues, sections were first floated on a drop of 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 (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 above.

Cytochemical Controls

Specificity of the labelings was assessed by the following control tests: (a) incubation with the gold-complexed enzymes to which were previously added the corresponding substrates (β-1,4-glucans from barley for the exoglucanase, polygalacturonic acids from citrus for the AGL, laminarin or laminaribiose for the β-1,3-glucanase, and *p*-coumaric acid or ferulic acid for the laccase, 1 mg/mL in PBS, pH 7.2); (b) substitution of the protein-gold complex under study by the BSA-gold complex to assess the nonspecific adsorption of the protein-gold complex to the tissue sections; (c) incubation of the tissue sections with the protein-gold complexes under nonoptimal conditions for biological activity; (d) incubation with the WGA to which was previously added an excess of *N-N'-N''*-triacetylchitotriose (1 mg/mL in PBS); and (e) incubation with WGA followed by unlabeled ovomucoid and finally by the ovomucoid-gold complex.

Reagents

The exoglucanase was kindly provided by Dr. C. Breuil (Forintek, Ottawa, Canada), and the AGL was obtained from Dr. N. Gilboa-Garber (Bar Ilan University, Ramat-Gan, Israèl). The laccase was obtained from Dr. M. Nicole (Organisation pour la Recherche Scientifique dans les Territoires d'Outre Mer, Montpellier, France). Tetrachloroauric acid was purchased from BDH Chemicals (Montreal, Canada). All other reagents for electron microscopy were obtained from JBEM Chemical.

RESULTS

Light Microscope Observations

Light microscopy of control and bacterized roots showed striking differences in the rate and extent of pathogen colonization upon challenge with *F. oxysporum* f. sp. *pisi* (Fig. 1). In control roots grown in the absence of *B. pumilus*, hyphae of the pathogen proliferated abundantly through much of the cortex, the endodermis, and the paratracheal parenchyma cells, and radiated rapidly toward the vascu-

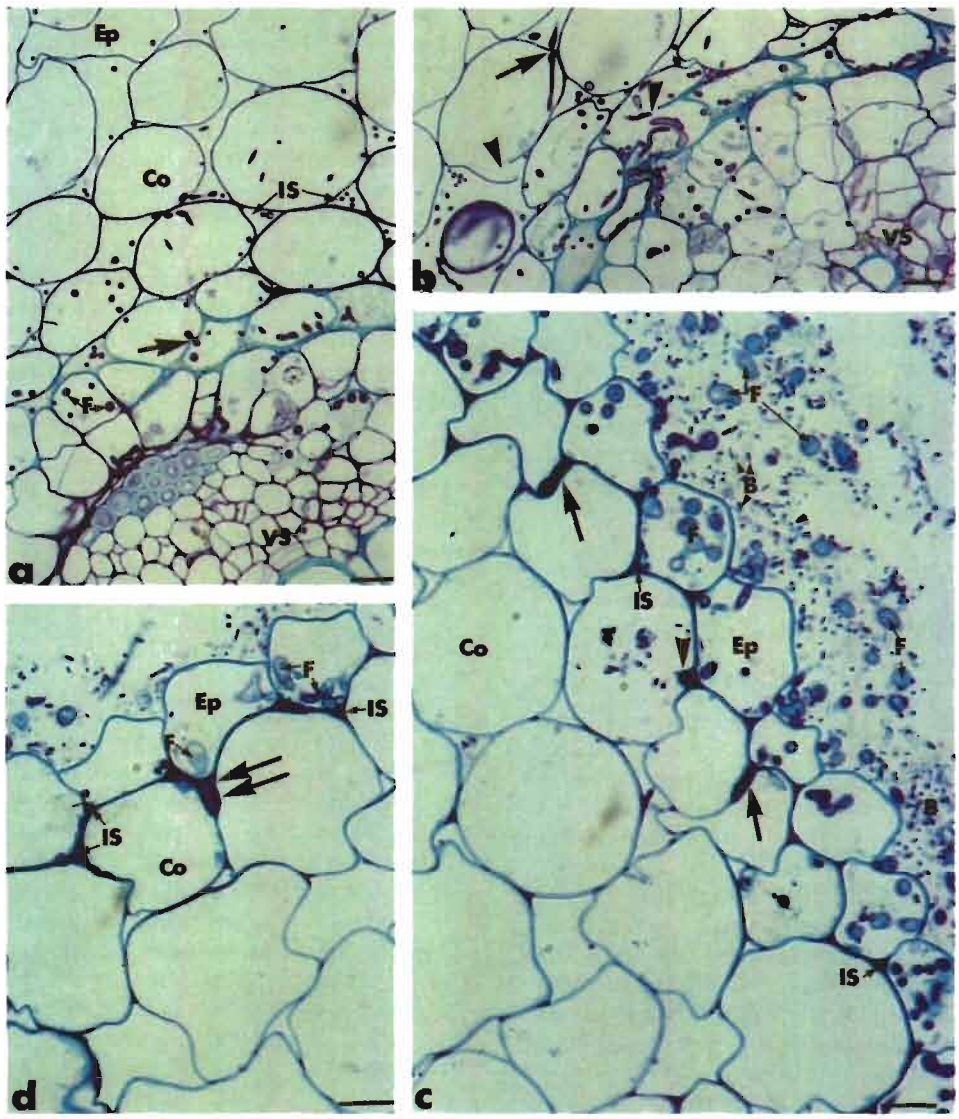

Figure 1. Light micrographs of samples from Ri T-DNA-transformed pea roots. a and b, Samples from nonbacterized (control) pea roots collected 2 d after inoculation with *Fusarium oxysporum* f. sp. *pisi*. Hyphae of the pathogen (F) multiply abundantly in the epidermis (Ep) and the cortex (Co) and reach the vascular stele (VS) by centripetal growth. Fungal growth occurs intra- and intercellularly. Direct penetration into the host wall by means of constricted hyphae is frequently observed (arrows). Pathogen ingress toward the vascular stele coincides with local cell-wall alterations (arrowheads). IS, Intercellular space. Bars = 20 μm. c and d, Samples from *B. pumilus*-inoculated pea roots collected 2 d after inoculation with *F. oxysporum* f. sp. *pisi*. Fungal growth is mainly restricted to the outermost root tissues, including the epidermis (Ep) and the first outer cortical (Co) layers. Most fungal cells (F) appear swollen and less intensely stained with toluidine blue than those seen in the control roots. Pathogen penetration in the epidermis coincides with the formation of wall thickenings that stain densely and that vary in shape from elongated deposits along a large portion of the cell wall (c, arrows) to hemispherical protuberances (c, arrowhead). An unsuccessful attempt of the pathogen to penetrate a papilla is observed (d, double arrows). Intercellular spaces (IS) are occluded by a material that stains densely with toluidine blue. B, Bacterial cells. Bars = 10 μm.

lar stele by centripetal growth (Fig. 1, a and b). Fungal growth occurred intra- and intercellularly (Fig. 1a). Direct penetration into the host wall by means of constricted hyphae was frequently observed (Fig. 1, a and b, arrows). Pathogen ingress toward the vascular stele usually coincided with local cell-wall alterations as judged by the presence of localized wall disruptions in places (Fig. 1b, arrowheads). Typical features of host defense reactions such as formation of wall thickenings and accumulation of intercellular deposits were not detected.

In bacterized roots, the pattern of colonization by *F. oxysporum* f. sp. *pisi* differed markedly from that observed in control roots (Fig. 1, c and d). Although extensive fungal multiplication was seen at the root surface, fungal growth in planta was mainly restricted to the outermost root tissues, including the epidermis and the outer cortex (Fig. 1c).

Hyphae of the pathogen were seldom seen in the inner cortex and they were never detected in the endodermis or the vascular stele. In addition, most fungal cells appeared to be swollen and less intensely stained with toluidine blue than those in the nonbacterized, control roots (Fig. 1d). Pathogen penetration in the root epidermis always coincided with striking cytological changes, mainly characterized by the elaboration of structural barriers in the regions proximal to potential fungal penetration (Fig. 1, c and d). The wall thickenings formed in the reacting epidermal and outer cortical host cells were intensely stained and varied in size and shape that ranged from elongated deposits along a large portion of the cell wall (Fig. 1c, arrows) to hemispherical protuberances resembling papillae (Fig. 1c, arrowhead). Unsuccessful attempts of the pathogen to penetrate papillae were frequently recorded (Fig. 1d, double arrows). Beside the formation of wall appositions, another typical host reaction in the colonized area was the plugging of most intercellular spaces with a material that stained densely with toluidine blue (Fig. 1, c and d). It is interesting that such host reactions remained localized in the area of potential fungal spread. Wall appositions and intercellular plugging were not detected in the uncolonized root tissues beneath the invaded cell layers. Bacterial cells could be seen in some intercellular spaces, but it was usually difficult to delineate them clearly by light microscopy.

These observations provided evidence that prior inoculation of pea roots with *B. pumilus* correlated with the formation of structural barriers that likely shielded the inner root tissues from pathogen invasion. Thus, a more precise investigation at the TEM level was essential in order to bring further insights into the functional significance of the host reactions in restricting pathogen growth and development.

Ultrastructural and Cytochemical Observations

Nonbacterized, Control Pea Roots

Examination of *Fusarium*-infected samples from nonbacterized pea roots at the TEM level confirmed the extensive fungal proliferation seen by light microscopy (Fig. 2a). Fungal cells multiplied actively by intra- and intercellular modes of growth and rapidly reached the xylem vessels.

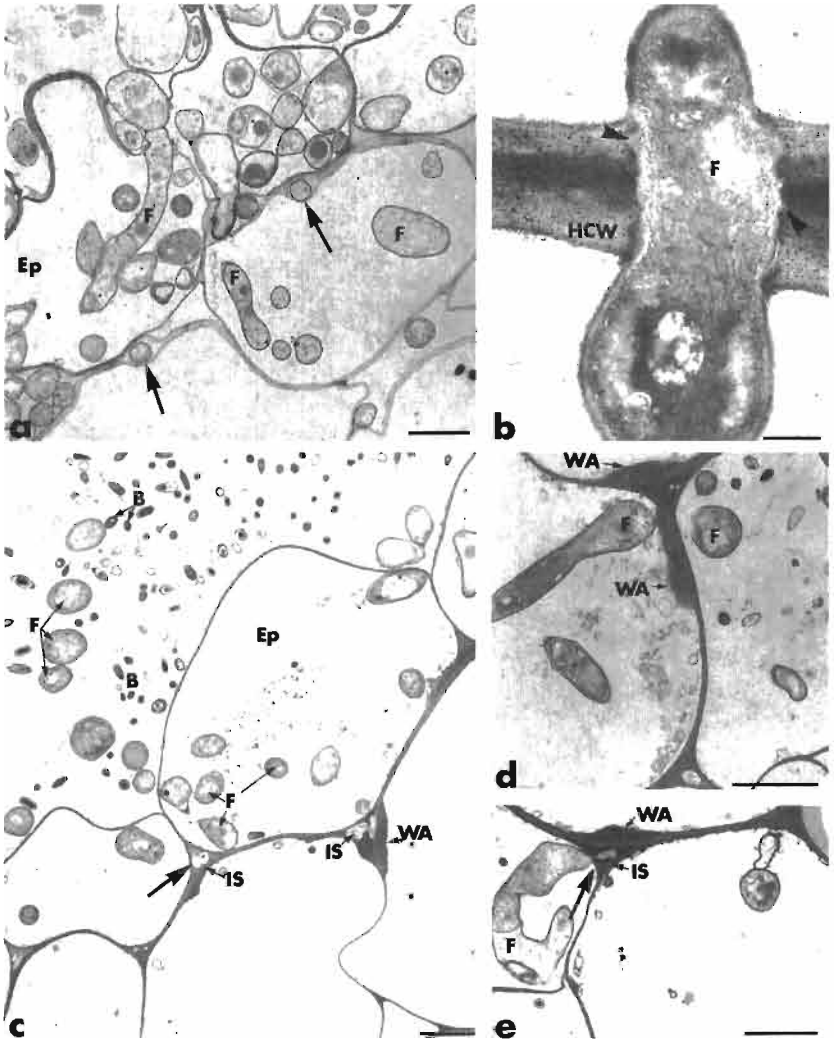

Figure 2. Transmission electron micrographs of *B. pumilus*-inoculated pea root tissues collected 2 d after challenge with *F. oxysporum* f. sp. *pisi*. a and b, In control roots grown in the absence of *B. pumilus*, *Fusarium* hyphae (F) multiply abundantly at the root surface and rapidly colonize the root tissues by centripetal growth. Fungal growth occurs intra- and intercellularly and even within host cell walls (a, arrows). Cell invasion occurs through direct host cell wall (HCW) penetration. Incubation with the gold-complexed exoglucanase for the localization of cellulosic compounds results in a regular deposition of gold particles over the host cell wall (b). Small lysis zones are seen along the fungus pathway (b, arrowheads). a, Bar = 3.0 μm; b, bar = 1.0 μm; c to e, in bacterized roots, fungal cells (F) are essentially found in the epidermis (Ep). Pathogen ingress in the root epidermis is associated with the deposition of electron-opaque wall appositions (WA) at sites of potential host cell-wall penetration and with the occlusion of most intercellular spaces (IS) with electron-dense substances. Altered penetration pegs are seen in the occluded intercellular spaces (c and e, arrows). Bars = 3.0 μm.

They also grew within host cell walls, splitting them apart (Fig. 2a, arrows). Cell invasion through host-wall penetration was commonly observed (Fig. 2b). Channels of penetration were usually narrower than the average hyphal diameter. Upon incubation with the gold-complexed exoglucanase, labeling of the host cell wall appeared regular even in areas adjacent to the channel of pathogen penetration. Close examination, however, revealed that small lysis zones, characterized by a decrease in electron density and by the absence of gold particles, occurred along the fungus pathway (Fig. 2b, arrowheads). Most fungal cells showed a typical ultrastructure with a dense cytoplasm closely appressed against the thin cell wall. In these nonbacterized roots, pathogen penetration failed to stimulate plant cell-wall responses as judged by the absence of typical wall appositions or intercellular space occlusions.

Bacterized Pea Roots

As recently reported in *Pseudomonas fluorescens* (Benhamou et al., 1996), observation of pea root samples inoculated with *B. pumilus* revealed that a large number of bacteria had grown on the root surface and displayed the ability to colonize some intercellular spaces in the epidermis and the outer cortex (not shown). This colonization of the outermost root tissues was usually restricted to a few bacterial cells and did not correlate with the induction of visible host defense reactions.

Examination of ultrathin sections of samples from *Fusarium*-inoculated, bacterized pea roots showed that the intensity of fungal colonization was appreciably reduced compared with that observed in nonbacterized roots. Fungal cells were essentially found in the epidermis and more occasionally in the first outer cortical cell layers (Fig. 2c). Bacterial cells were also restricted to these outer tissues and occurrence of both microorganisms could be detected in some intercellular spaces (Fig. 3a). Although at first sight the fungus appeared to display a normal ultrastructure, close examination of the invaded areas showed that most hyphae suffered from some damage characterized by increased vacuolation and/or cytoplasm disorganization and plasmalemma retraction (Figs. 2e and 3d).

The interaction between *B. pumilus* and *F. oxysporum* f. sp. *pisi* at the root surface did not correlate with substantial fungal alterations. Pathogen ingress in the root epidermis was associated with both the deposition of an electron-opaque material at sites of potential host cell-wall penetra-

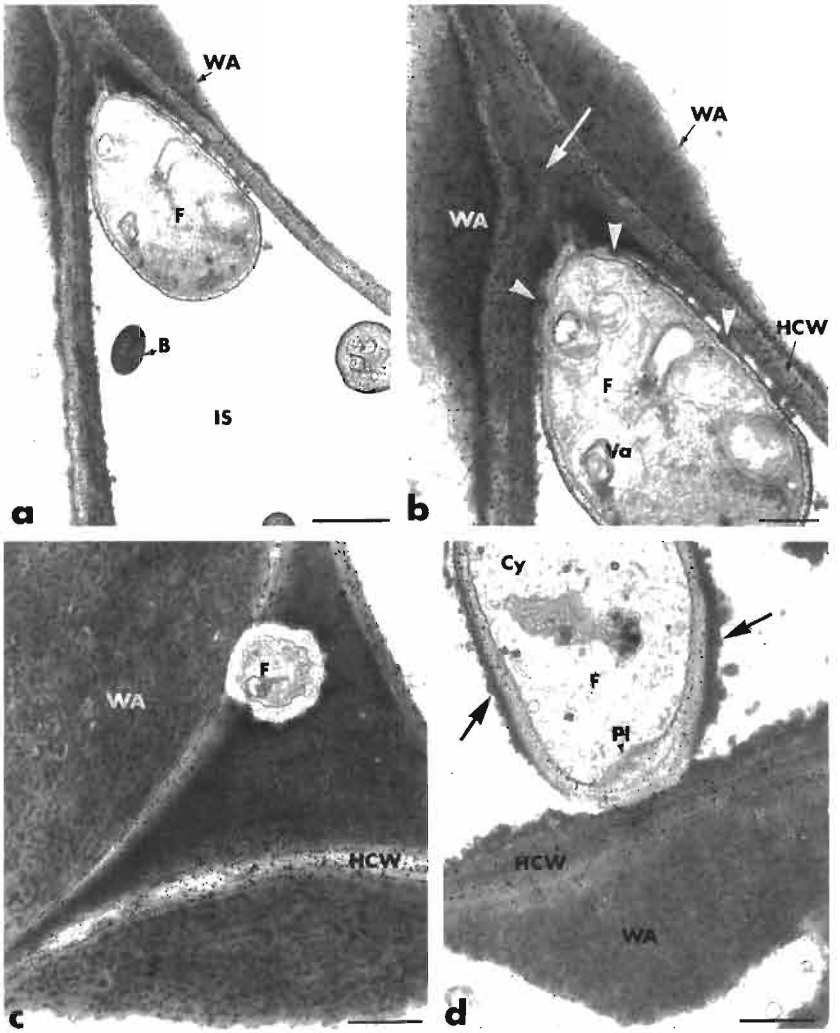

Figure 3. Transmission electron micrographs of *B. pumilus*-inoculated pea root tissues collected 2 d after challenge with *F. oxysporum* f. sp. *pisi*. a to d, The wall appositions (WA) formed in the reacting host cells are apparently made of an amorphous matrix that is impregnated by osmiophilic substances. The host cell wall (HCW) displays a higher electron density than normal (b, white arrow). An electron-dense material is lining the primary walls in nearly all infected intercellular spaces (IS) and extends toward the inside to form either small deposits that interact with the fungal cell (b, white arrowheads) or a continuous coating band at the fungal cell surface (d, arrows). Hyphal cells show various degrees of alteration, including distortion and retraction of the plasmalemma (Pl) and pronounced disorganization of the cytoplasm (Cy) with involution of vacuole membranes (Va). Labeling with the gold-complexed exoglucanase results in a specific deposition of gold particles over the host cell walls, whereas the wall appositions and the osmiophilic coating material are free of significant labeling. a, Bar = 1.0 μm; b to d, bars = 0.5 μm.

tion, and the occlusion of most intercellular spaces with electron-dense substances (Fig. 2, c and e). Attempts to progress from one cell to another through the filled intercellular spaces was usually aborted as illustrated by the presence of highly altered penetration pegs within the deposited material (Fig. 2, c and e, arrows). These host reactions always occurred in the first tissue layers underlying the invaded epidermis and were not seen in the inner tissues. At a higher magnification, the wall appositions formed in the reacting host cells were greatly varied in their appearance from hemispherical or dome-like protuberances formed at the junction of adjacent host cells (Fig. 3, a and b) to multitextured deposits along a large portion of the host cell wall (Fig. 3c). These appositions were usually made from an amorphous matrix that was impregnated by osmiophilic substances and was often delimited by a loosely arranged layer of fine fibrillo-granular material (Fig. 3c). The host cell wall itself displayed a higher electron density than normal, thus indicating the probable infiltration of structural molecules (Fig. 3b, white arrow). Both the impregnated host cell wall and the wall appositions were efficient in preventing fungal ingress, since successful hyphal penetration of these structures was seldom observed. Beside the formation of structural barriers, another typical host reaction was the deposition of an electron-dense material lining the primary walls in nearly all infected intercellular spaces (Fig. 3, b and d). This material usually extended toward the inside to form either small, polymorphic deposits that frequently interacted with the wall of invading hyphae (Fig. 3b, white arrowheads), or a continuous coating band at the fungal cell surface (Fig. 3d, arrows). Hyphal cells trapped by this material showed various degrees of alteration, including distortion and retraction of the plasma membrane (Fig. 3c), as well as pronounced disorganization of the cytoplasm, which was frequently accompanied by the involution of vacuole membranes (Fig. 3b) and the formation of polymorphic vesicles (Fig. 3c).

Application of the gold-complexed exoglucanase to sections of these bacterized roots for localization of cellulosic β-1,4-glucans resulted in a specific deposition of gold particles over the electron-dense host cell walls, whereas the intercellular spaces, the wall appositions, and the osmiophilic coating material lining the invading fungal cells were free of significant labeling (Fig. 3). Control tests, including incubation with the gold-complexed enzyme to which were previously added β-1,4-glucans from barley, were negative (not shown).

Upon incubation with the gold-complexed AGL for localization of pectic compounds, a few scattered gold particles were detected over the host cell walls (Fig. 4a). Such a slight gold deposition contrasted with the more extensive labeling pattern usually obtained with this probe (Benhamou et al., 1988). Considering the unusually high electron density of the host cell walls, this observation suggested that deposition of additional material over wall-bound pectin likely prevented free access of the gold-complexed probe to its target molecules. Gold particles were also distributed over the wall appositions without any preferential localization (Fig. 4a). All control tests performed to assess labeling specificity resulted in a near absence of labeling (not shown).

Incubation of sections with the tobacco β-1,3-glucanase-gold complex for callose localization resulted in the accumulation of a considerable number of gold particles over the outermost wall layers at sites of potential papilla formation (Fig. 4b, arrow) as well as over mature wall appositions (Fig. 4c). A few scattered gold particles were also detected over the middle lamella matrices (Fig. 4c). By contrast, the electron-opaque material surrounding fungal cells in some intercellular spaces was unlabeled (not shown). Control tests, including incubation of the enzyme-gold complex with laminarin prior to section labeling, yielded negative results (not shown).

When sections were incubated with the gold-complexed laccase for localization of phenolics, a specific deposition of gold particles was detected over both the wall appositions (Fig. 4, d and e) and the material filling some intercellular spaces (Fig. 5a). In mature appositions, characterized by a high compactness of their content, gold particles were irregularly distributed (Fig. 4d), whereas in younger ones, they appeared predominantly associated with the electron-opaque flecks embedded in the amorphous underlying matrix (Fig. 4e). The host cell walls were also labeled, but the intensity of labeling was found to vary from a few scattered gold particles in some areas to massive accumulation of particles in areas adjacent to the formation of new appositions (Fig. 4e). The electron-opaque material lining the host cell walls in intercellular spaces and releasing polymorphic deposits found to interact frequently with hyphae was also specifically labeled (Fig. 5a). Gold particles were detected not only over the dense material accumulating in intercellular spaces, but also at the surface or even inside fungal cells (Fig. 5a). In all cases, these hyphal cells showed obvious signs of alteration mainly associated with increased vacuolation and cytoplasm aggregation.

It is interesting that laccase-gold labeling was also detected in inner, uninvaded endodermal and parenchyma host cells characterized by the presence of a large, centrally located vacuole surrounded by a layer of dense cytoplasm appressed against the cell wall (Fig. 5b). In such cells gold particles were specifically deposited over polymorphic, amorphous structures accumulating in the vacuole (Fig. 5b, arrows). 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, the wall appositions, and the dense material (not shown).

When the WGA-ovomucoid-gold complex was applied to sections of bacterized pea roots that were inoculated with *F. oxysporum* f. sp. *pisi* for localization of chitin, gold labeling was evenly distributed over the walls of invading hyphae even when those were substantially altered (Fig. 6a). The wall appositions as well as the coating material interacting with fungal cells were unlabeled (Fig. 6b, arrow). All control tests, including previous adsorption of the WGA with *N*-*N*′-*N*″ triacetylchitotriose, yielded negative results (not shown).

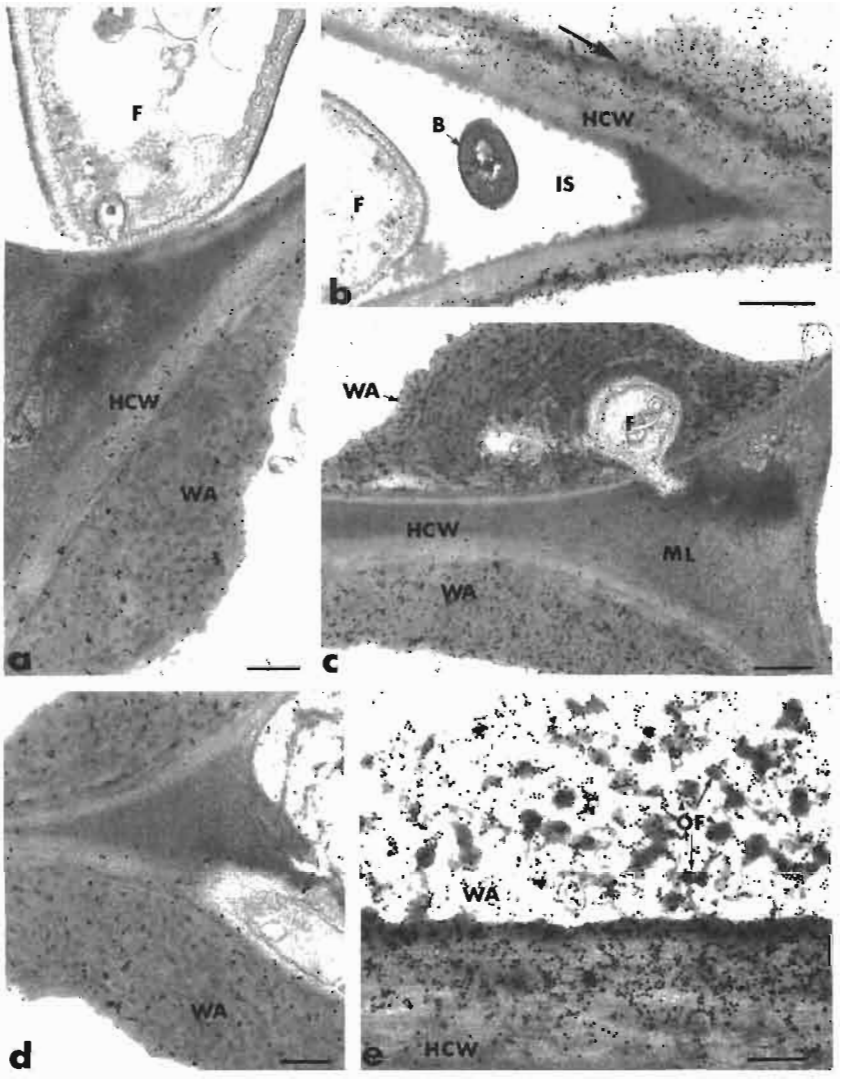

Figure 4. Transmission electron micrographs of *B. pumilus*-inoculated pea root tissues collected 2 d after challenge with *F. oxysporum* f. sp. *pisi*. a, Labeling of pectin substances with the gold-complexed AGL. Gold particles are randomly distributed over the host cell wall (HCW) and over a wall apposition (WA). The fungal cell (F) is unlabeled. Bar = 0.25 μm. b and c, Labeling of β-1,3-glucans with the gold-complexed tobacco β-1,3-glucanase. A heavy deposition of gold particles occur over the host cell wall (HCW) at sites of potential pathogen penetration (b, arrow) as well as over mature wall appositions (WA). A slight labeling is seen over the middle lamella (ML); B, bacterial cell; F, fungal cell. Bars = 0.5 μm. d and e, Labeling with the gold-complexed fungal laccase for the localization of phenolic compounds. In mature wall appositions (WA), gold particles are irregularly distributed (d), whereas in younger ones, they appear predominantly associated with the electron-opaque flecks (OF) embedded in the amorphous underlying matrix. The host cell wall (HCW) is intensely labeled in areas adjacent to the formation of new appositions. Bars = 0.25 μm.

DISCUSSION

Results of the present study demonstrate that transformed pea roots, preinoculated with the endophytic bacterium *B. pumilus*, strain SE34, afford increased resistance against the fungal pathogen *F. oxysporum* f. sp. *pisi*. This induced protection was associated with the accumulation of newly formed structural compounds at sites of attempted fungal penetration. Such structural plant defense reactions were not seen in *B. pumilus*-infected pea roots, whereas they were easily detected upon inoculation with *F. oxysporum* f. sp. *pisi*. This observation supports the hypothesis that fungal challenge is essential for the expression of a prominent structural response in prebacterized plants (Benhamou et al., 1996). Defense responses expressed at the perimeter could efficiently contain the infection, since the pathogen was seldom seen in the inner tissues. These observations confirm that endophytic bacteria, known to be natural inhabitants of plant cells, influence the plant physiology in such a way that increased resistance is conferred upon pathogen attack (Tuzun and Kloepper, 1995).

When pea roots were challenged with *F. oxysporum* f. sp. *pisi*, strong differences in the rate and extent of tissue invasion were observed, whether or not the roots were bacterized. The observation that *Fusarium* hyphae, interacting with cells of *B. pumilus* at the root surface, maintained active growth and displayed the ability to massively penetrate the root epidermis indicated that the mechanisms of biocontrol against *F. oxysporum* f. sp. *pisi* did not rely on a strong antagonistic activity in the rhizosphere. This finding, together with the recent observation that direct antimicrobial activity apparently did not correlate with the biological control of *Fusarium* wilt by several bacterial endophytes (Chen et al., 1995), provides support to the notion that antifungal metabolites, known to be produced by most *Bacillus* strains grown in vitro (Priest, 1977; Fiddaman and Rossall, 1993), do not necessarily operate in vivo. In a recent study, Leifert et al. (1995) reported that *Bacillus pumilus*, strain CL27, found to produce anti-*Botrytis* antibiotics in liquid media in vitro, had no in vivo activity against *Botrytis cinerea*. Thus, there are good reasons to believe that bacterial endophytes produce and/or induce

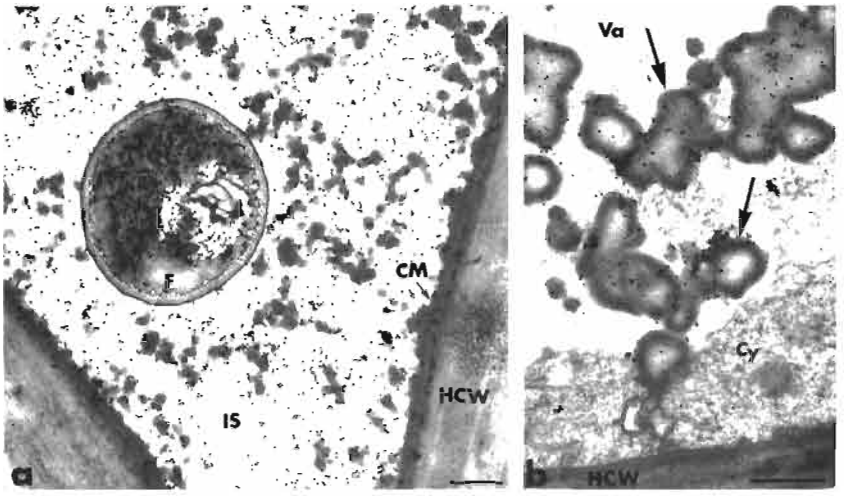

Figure 5. Transmission electron micrographs of *B. pumilus*-inoculated pea root tissues collected 2 d after challenge with *F. oxysporum* f. sp. *pisi*. Labeling of phenolic compounds with the gold-complexed laccase. a, The coating material (CM) lining the host cell wall (HCW) in an invaded intercellular space (IS) and the released aggregates are intensely labeled. Gold particles are also detected within the invading fungal cell (F). Bar = 0.25 μm. b, Gold particles are specifically deposited over amorphous structures (arrows) accumulating in the centrally located vacuole (Va) of an uninvaded parenchyma cell. Cy, Cytoplasm; HCW, host cell wall. Bar = 0.5 μm.

metabolites that target only selected microorganisms. Evidence is now accumulating to suggest that biocontrol activity exerted by some soil bacteria is more than a simple process mediated by antimicrobial metabolites. Perhaps one of the most cogent examples demonstrating the key role of the plant itself in the protection induced by soil bacteria emerged from the experiments in which the pathogen and the bacterial strain were applied at spatially separated locations (Wei et al., 1994). In such studies, which excluded antagonistic interactions, reduction of disease incidence was correlated with an activation of the plant's defense strategy, ultimately leading to systemic protection. This correlation is further supported by data from the present study, in which new insights into the mechanisms by which bacterized roots respond to pathogen attack are provided.

Restriction of fungal growth to the outermost root tissues together with striking modifications of the epidermal and cortical cell walls and accumulation of newly formed barriers beyond the infection sites were the main visible features of the cellular response to *Fusarium* attack in bacterized pea roots. This massive deposition of unusual structures at sites of attempted fungal entry clearly indicated that bacterized root cells were signaled to mobilize a number of defense strategies for preventing the spread of the pathogen in the inner tissues. Considering that fungal nutrition and ingress toward internal tissues are essential prerequisites for successful pathogenesis, host cell-wall degradation is conceivably one of the most harmful events associated with the infection process by pathogenic fungi (Collmer and Keen, 1986). It is not surprising that, in turn, plants have developed the potential to prevent effective pathogen penetration by producing an array of substances for reinforcing the cell walls (Ride, 1983) and protecting them from the deleterious action of enzymes and toxins (Hahlbrock and Scheel, 1987). However, several lines of evidence have clearly shown that strengthening of the host cell walls could confer resistance only if accumulation of structural compounds occurred rapidly and in advance of the ingressing fungal hyphae (Mattern et al., 1995).

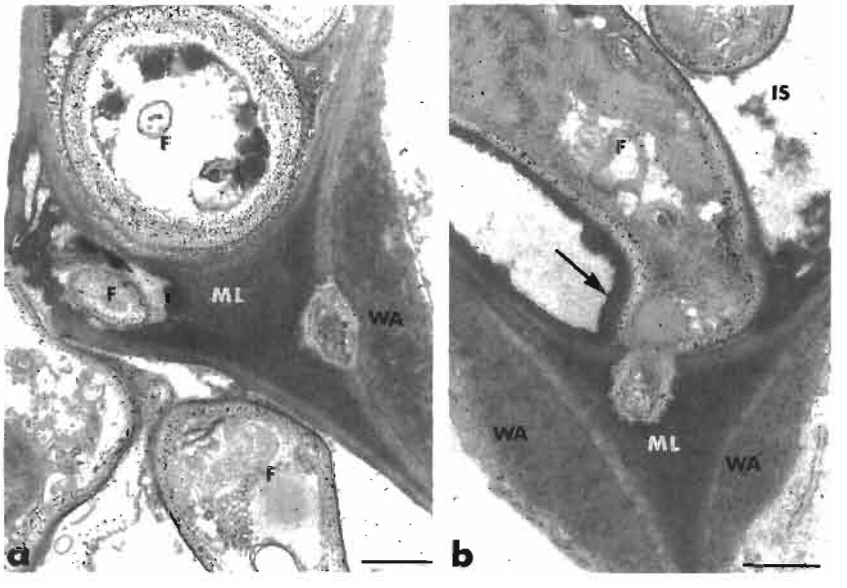

Figure 6. Transmission electron micrographs of *B. pumilus*-inoculated pea root tissues collected 2 d after challenge with *F. oxysporum* f. sp. *pisi*. Labeling of GlcNAc residues (chitin) with the WGA/ovomucoid-gold complex (a and b). The cell walls of invading *Fusarium* hyphae (F) are specifically labeled. The host cell wall, the wall appositions (WA), and the coating material interacting with the fungal cell surface (arrow) are free of labeling. IS, Intercellular space; ML, middle lamella. Bars = 0.5 μm.

A prominent facet of this rapid, induced plant response is clearly illustrated in the present study, in which the deposition onto the inner surface of the cell walls of a considerable number of heterogeneous wall appositions occurred only in root tissues that were preinoculated with *B. pumilus*. Support for the close association between bacterial treatment and induced resistance also came from the observation that intercellular spaces, known to be strategic sites for pathogen ingress, were filled or even occluded by a phenolic-enriched material. Such host reactions were not seen in control roots in which the pattern of fungal colonization was similar in many respects to that known to occur in other compatible *Fusarium*-host interactions (Benhamou et al., 1994b; Benhamou, 1995). These results are of particular relevance because they bring further insights to the concept that bacterial endophytes are capable of evoking biochemical events characteristic of the natural plant disease resistance process.

With regard to the functional significance of the cellular changes detected in bacterized roots upon pathogen challenge, our data provide evidence that the newly formed wall appositions are more than inert barriers laid down to prevent mechanical penetration of plant tissues and to impair the diffusion of toxic molecules. The observation that fungal hyphae appressed against wall appositions or trapped in the osmiophilic material formed in intercellular spaces had frequently undergone cellular disorganization was indicative of a fungicidal environment. This correlation was further supported by the detection of phenolic-like substances in the appositions and within fungal cells. According to our observations, it is likely that the direct effect exerted by phenolics acts as a second defensive line against fungal cells that have escaped the first barriers imposed by the reinforced cell walls and the wall appositions.

The present cytochemical results showed that callose and pectin occurred in close association with phenolic compounds in the newly formed wall appositions. According to the patterns of labeling obtained with the probes under study, these appositions appeared to be made of a polysaccharidic matrix composed mainly of callose, on which phenolic compounds (likely lignin) were deposited. Although the origin of the accumulating pectic material is still unknown, the structural integrity of the host cell walls adjacent to newly formed appositions as well as the absence of cellulosic compounds preclude a cell-wall release but rather suggest a de novo synthesis. Several recent reports agreed on the key role of pectin as a template for the subsequent deposition of lignin (Lewis and Yamamoto, 1990). It has been convincingly shown that lignification could render wall structures highly resistant to mechanical and enzymatic disruption mainly because lignin polymerization coincided with the formation of peroxidase-mediated cross-links with wall carbohydrates such as hemicellulose, pectin, and callose (Minor, 1991). In line with our results, the reported increase in peroxidase activity in roots colonized by rhizosphere bacteria (Albert and Anderson, 1987) supports the concept that effective restriction of fungal growth and spread in bacterized pea roots may, for the most part, correlate with the formation of impervious composites in which lignin and pectin are covalently bound through the action of peroxidases.

In an attempt to determine whether enzyme-mediated wall hydrolysis was associated with the frequent disorganization of fungal hyphae colonizing the outer tissues in bacterized roots, the ultrastructural localization of chitin was performed. From the examination of the labeling pattern obtained, it was clear that the fungal cytoplasm underwent pronounced disorganization at a time when chitin still occurred in the cell walls. This observation suggests that production of plant chitinases is not a primary determinant in the expression of plant resistance. It is more likely that synthesis of toxic substances such as phenolics and phytoalexins precedes the production of chitinases and other pathogenesis-related proteins that probably contribute to a more complete disintegration of the fungal cells. Studying the influence of root-colonizing bacteria on the defense response of bean, Zdor and Anderson (1992) could not detect a substantial increase in the level of mRNAs encoding for chitinases, and they concluded on an apparent selective induction of plant defense strategies upon bacterial root colonization. This conclusion, however, should be viewed with caution, since the possibility that chitinases may be produced as a late process in the sequence of events leading to resistance establishment cannot be ruled out.

In summary, evidence is provided in this study that pea root bacterization with *B. pumilus*, strain SE34, confers increased protection against infection by *F. oxysporum* f. sp. *pisi* by stimulating a number of plant defense reactions that culminate in the elaboration of permeability barriers and in the creation of a fungitoxic environment. As the mechanisms underlying the biological functions of bacterial endophytes are revealed, the possibility of sensitizing a plant to respond more rapidly to pathogen attack by prior inoculation with selected plant-associated bacteria can be considered one of the most encouraging options for effective management of plant diseases in the near future.

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