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FUNGAL DISEASES

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Cell interactions between a nonpathogenic *Fusarium oxysporum* strain and root tissues of *Eucalyptus viminalis*

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Abstract Nonpathogenic isolates of *Fusarium oxysporum* can be successful antagonists of pathogenic forms of the same fungal species that commonly attacks crop plants. The characteristics that distinguish nonpathogenic from pathogenic forms are not well understood. In this study, the mode of root colonization of *Eucalyptus viminalis* seedlings by a nonpathogenic *F. oxysporum* strain is described at the ultrastructural level. Root systems of *E. viminalis* plants were inoculated with nonpathogenic *F. oxysporum* strain Fo47 in an in vitro model system. Changes in the occurrence of nonesterified and methyl-esterified pectins in colonized *E. viminalis* roots were evaluated by in situ immunolabeling using two monoclonal antibodies, JIM 5 and JIM 7. Modes of penetration and root colonization patterns in *E. viminalis* seedlings by the nonpathogenic fungus were similar to those described for pathogenic forms of *F. oxysporum*. However, root interactions differed in that the nonpathogenic fungus did not induce host tissue damage. No papilla-like appositions were observed in host cells in response to invading hyphae, which did not disrupt the host plasma membrane in many cases, suggesting that a biotrophic relationship was established. Root colonization by the nonpathogenic strain did not induce alteration in JIM 7 labeling of methyl-esterified pectin in *E. viminalis* cell walls, whereas nonesterified pectin was detected to a significantly greater extent in cell walls of roots colonized by the fungus. Pectin components decreased slightly only at points of hyphal contact with host cells. Because nonpathogenic strains utilize pectin in pure culture, host control over enzyme activity or production by the fungi may at least partly explain their compatible interactions with host tissues.

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Introduction

Pathogenic and nonpathogenic forms of *Fusarium oxysporum* Schlechtend.:Fr. species are found in agricultural soils throughout the world (Armstrong and Armstrong 1981). Some forms induce damping off, root rot, or vascular wilt, on crops of economic importance, whereas others are unable to induce disease in a given plant species. The pathogenic *F. oxysporum* strain Foeu1 colonizes the root system of *Eucalyptus viminalis* Labill., causing rapid tissue maceration, cell plasmolysis, and root tissue degradation, which results in the damping off of seedlings (Salerno et al. 2000). Nonpathogenic *F. oxysporum* Fo47 can infect the root tissues of several plants, including *E. viminalis*, without causing tissue disorganization (Mandel and Baker 1991; Olivain and Alabouvette 1997; Postma and Luttikholt 1996; Salerno et al. 2000), and it can protect eucalypt seedlings against a pathogenic strain of *F. oxysporum* (Salerno et al. 2000). The mechanisms by which nonpathogenic *F. oxysporum* controls disease are poorly understood. Three modes of action have been proposed to contribute to the protective activity of nonpathogenic strains: competition for nutrients in soil and the rhizosphere, competition for infection sites and root colonization, or induced systemic resistance (Mandel and Baker 1991; Salerno et al. 2000). Competition for eucalypt root colonization has been reported to be one possible mode of action of the nonpathogenic *F. oxysporum* strain Fo47 (Salerno et al. 2000). Light microscopic observations of the penetration and colonization patterns of *E. viminalis* root tissues by both pathogenic and nonpathogenic *F. oxysporum* revealed that there were no major differences between the strains. This suggests that differences between pathogenic and nonpathogenic *F. oxysporum* strains may reside at the cellular level of root interactions. In fact, tissue rapidly became disorganized in *E. viminalis* roots colonized by pathogenic *F. oxysporum*, whereas root tissues did not

show signs of damage with progression of the nonpathogenic strain even 30 days after inoculation (Salerno et al. 2000).

In a study of interactions between *E. viminalis* and pathogenic *F. oxysporum*, the fungus greatly reduced the pectin component of cell walls during colonization of roots and ruptured the host plasma membrane, causing rapid host tissue and cell damage (Salerno et al. 2004). The objectives of this study were to (1) investigate interactions between *E. viminalis* root tissues and nonpathogenic *F. oxysporum* strain Fo47 at the ultrastructural level and (2) elucidate possible reasons for the lack of disease development by studying distribution patterns of nonesterified (NE) and methyl-esterified (ME) pectin in *E. viminalis* root tissues in situ during root colonization.

Material and methods

Fungal strain

Nonpathogenic *F. oxysporum* strain Fo47 was kindly provided by C. Alabouvette (INRA, Dijon, France); it was recovered from horticultural soil in Chateaurenard (France) where *Eucalyptus* species have never grown. The strain was maintained on potato dextrose agar (PDA) (Difco, Detroit, MI, USA).

Plant growth and inoculation with *F. oxysporum* strain Fo47

The surfaces of *Eucalyptus viminalis* Labill. seeds were disinfested with sodium hypochlorite (NaOCl) (12% active chloride) for 20 min, rinsed with sterile distilled water, and pregerminated on water agar (0.6% w/v) for 3 days at 27°C in the dark. When the roots were 0.5 cm long, seedlings were transferred to Crush and Hay medium (containing 5 g of active carbon) (Salerno et al. 2000) in petri dishes and incubated in a growth chamber. After 6 days of seedling growth, when roots were 1.5 cm long, they were inoculated with mycelium plugs of the nonpathogenic Fo47 strain as described by Salerno et al. (2000). Noninoculated seedlings were used as controls. Seedlings were sampled 6 days after inoculation and 5 mm long root pieces were cut 0.5 cm behind the apex. Root pieces were fixed in 2% glutaraldehyde, dehydrated through an ethanol gradient, and embedded in LR White resin (Oxford Instrument, Orsay, France), as described by Gianinazzi and Gianinazzi-Pearson (1992).

Ultrathin (98 nm) sections of four or five resin-embedded root fragments per treatment were cut on a Reichert-Jung Ultracut ultramicrotome, collected on gold grids, and stained with 1% uranyl acetate (Valentines 1961) for 20 min followed by lead citrate (Reynolds 1963) for 10–15 min. Sections were examined using a Hitachi 600 transmission electron microscope (TEM) at 75 kV.

Immunogold detection of pectin

Pectin was visualized in situ with the monoclonal mouse antibodies (immunoglobulin G) JIM 5 and JIM 7 (provided by P. Knox, John Innes Centre, Norwich, UK), which recognize nonesterified (NE) and high methyl-esterified (ME) pectins, respectively (Knox et al. 1990; VandenBosch et al. 1989). Ultrathin sections were incubated with the primary antibodies diluted 1:4000 in TBS/Tween/BSA buffer (Gianinazzi and Gianinazzi-Pearson 1992) followed by incubation for 1 h at room temperature with a 15-nm gold-conjugated secondary anti-mouse antibody (Biocell, Cardiff, UK) diluted 1:20 in buffer. Sections were stained with 1% uranyl acetate (Valentines 1961) and observed with the TEM at 75 kV. Control sections were prepared either by omitting the primary antibodies, incubating sections with 0.1 N HCl for 10 min before immunolabeling, or incubating in an unrelated polyclonal antibody from the fungus *Acaulospora laevis* (Lemoine et al. 1995).

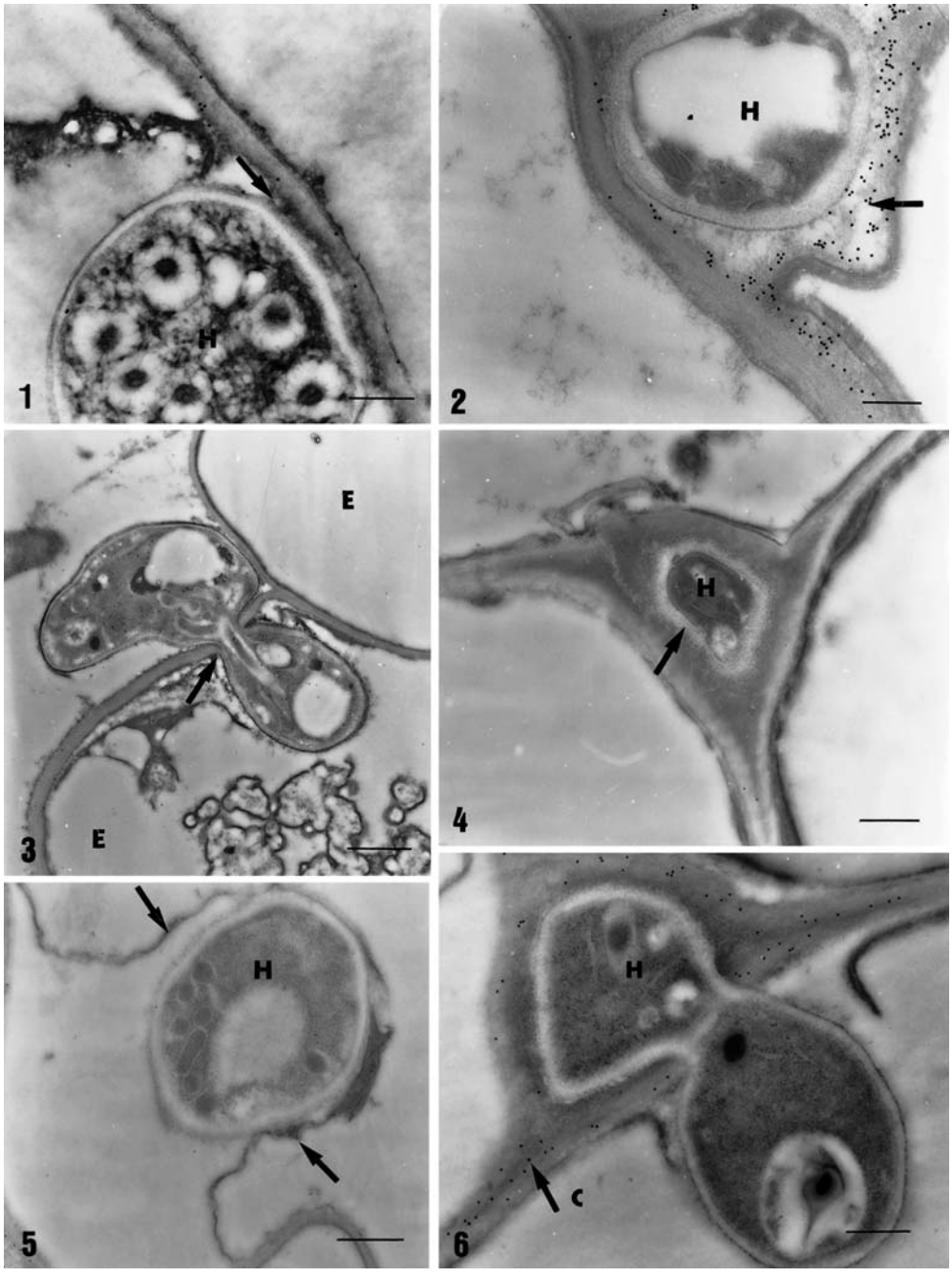
The number of gold particles present in the epidermal, cortical, and phloem cell walls was evaluated per unit area (square micrometers) at five random locations on five sections from each of three plants per treatment, at 20 000-fold magnification, with the aid of the image analysis system SAMBA (Samba Technologies, Grenoble, France) coupled to a video CCD camera (Tokina Optical Co. Ltd., Tokyo, Japan) equipped with an AF Nikkor 20-mm lens. Data were subjected to analysis of variance (ANOVA), and the treatment means that corresponded to each root tissue were compared separately by the Student-Newman-Keuls test ($P < 0.05$) using the SAS program (SAS Institute 1996).

Results

Noninoculated *E. viminalis* roots, which had a typical structure with compact epidermal, cortical, and vascular tissues, have been described in detail previously (Salerno et al. 2000). Epidermal cell walls tended to have a loose fibrillar surface (see Fig. 7, below), and fibrillar material was also observed frequently in the intercellular spaces of the parenchymal cortex (see Fig. 8, below).

Root penetration and colonization

Hyphae of the nonpathogenic *F. oxysporum* strain Fo47 growing along the surface of *E. viminalis* roots were frequently enclosed by a film of electron-opaque material. When they came into contact with the host cells, their walls appeared to be closely attached to the outer epidermal walls by fibrillar material (Fig. 1). Hyphae frequently grew along the junctions between epidermal cells and were embedded in a loose fibrillar matrix before penetration (Fig. 2). This matrix was labeled by the JIM 5 antibody but not by JIM 7, indicating that it contained a nonesterified pectin component (Fig. 2). The most common point of entry of the nonpathogenic strain in roots was via root hairs or between two



Figs. 1–6. Electron micrographs of infection of *Eucalyptus viminalis* roots by nonpathogenic *Fusarium oxysporum* strain Fo47. **1** Hypha (*H*) of *F. oxysporum* adhering to the external epidermal cell wall of an *E. viminalis* root by fibrillar material (*arrow*). *Bar* 0.5 μm. **2** Section immunolabeled with JIM 5 monoclonal antibody showing a hypha of *F. oxysporum* surrounded by extracellular material containing nonesterified pectin (*arrow*) adjacent to the epidermal cell wall of an *E. viminalis* root. *Bar* 0.3 μm. **3** Hyphal penetration (*arrow*) by *F. oxysporum* directly through the outer epidermal cell (E) wall of an *E. viminalis* root. *Bar* 1.7 μm. **4** Hypha of *F. oxysporum* developing in intercellular spaces containing electron-opaque material (*arrow*) in the parenchymal cortex of an *E. viminalis* root. Control section without primary antibody. *Bar* 0.5 μm. **5** Hypha of *F. oxysporum* causing invagination of the host plasma membrane (*arrows*) in a parenchymal cortex cell from an *E. viminalis* root. *Bar* 0.7 μm. **6** Immunogold detection (*arrow*) with JIM 7 antibody of methyl-esterified pectin in the penetrated points of cortical cell walls (*C*) and a hypha of the nonpathogenic *F. oxysporum*. *Bar* 0.5 μm

adjacent epidermal cells (not shown). However, hyphae were able to penetrate directly through the outer epidermal cell walls and develop inside the epidermal cells (Fig. 3). *Fusarium oxysporum* sometimes formed an ill-defined appressorium-like structure before infection (Fig. 3) and infected epidermal tissue without causing signs of damage, as previously reported (Salerno et al. 2000).

Fusarium oxysporum developed along the middle lamella in the parenchymal cortex, and hyphae in intercellular spaces were generally embedded in an electron-opaque

material (Fig. 4). When hyphae invaded host cells, they were frequently surrounded by the host plasma membrane and remained at the cell periphery; the cell protoplast was not invaded (Fig. 5). The host membrane of living parenchymal cells was only occasionally ruptured (Fig. 6). Host cells did not become disorganized and resembled cells in a noninoculated parenchymal cortex. Papilla-like appositions were not observed in the presence of the nonpathogenic *F. oxysporum* strain. When hyphae invaded the central cylinder of roots in the region 0.5 cm behind the apex (Salerno et al. 2000), the phloem was rarely colonized and did not show any apparent disorganization.

Visualization of methyl-esterified and nonesterified pectin

Control sections without primary antibodies or incubated in anti-*A. laevis* antibody were not immunolabeled (Fig. 4). Using the JIM 7 antibody, ME pectins were easily visualized throughout the primary cell walls of the parenchymal cortex and, to a greater extent, the phloem walls of noninoculated *E. viminalis* roots; epidermal cell walls had slightly weaker immunolabeling (Table 1). Neither the radial walls of hypodermal and endodermal cells nor xylem vessels were immunolabeled. In roots colonized by *F. oxysporum* strain Fo47, the ME pectin content in cell walls of the epidermis, the parenchymal cortex, or phloem did not change significantly compared to the noninoculated control roots (Table 1). Only a slight, highly localized decrease in immunolabeling with JIM 7 was observed in cell walls at fungal penetration points (Fig. 7). Pretreatment of root sections with 0.1 N HCl did not affect these results; therefore, this decreased immunolabeling was not due to masking of antigenic sites.

JIM 5 antibody labeling confirmed that in noninoculated roots NE pectin was mainly located in the external epidermal cell walls (Fig. 7), the middle lamella and contents of intercellular spaces in the parenchymal cortex (Fig. 8), and phloem cell walls. Intercellular spaces in the parenchymal cortex (Fig. 7) and cell junctions in hypodermal, endodermal, and phloem tissues were also labeled. In root tissues colonized by *F. oxysporum* strain Fo47, NE pectin epitopes persisted and even increased in cell walls of the epidermis (Fig. 9, Table 1). JIM5 binding to NE pectin epitopes occurred in the middle lamella and intercellular spaces of the colonized parenchymal cortex tissue (Fig. 10) and to a greater extent than in the control tissues (Table 1). Only a slight, highly localized decrease in immunolabeling was observed in host walls adjacent to hyphae in the parenchymal cortex (Fig. 10). The amount of NE pectin detected in the phloem tissue in these roots increased greatly even though it was not colonized (Table 1).

Table 1. Density of immunolabeling of nonesterified (JIM 5 antibody) and methyl-esterified (JIM 7 antibody) pectin in cell walls of noninoculated control *Eucalyptus viminalis* and roots infected with *Fusarium oxysporum* Fo47

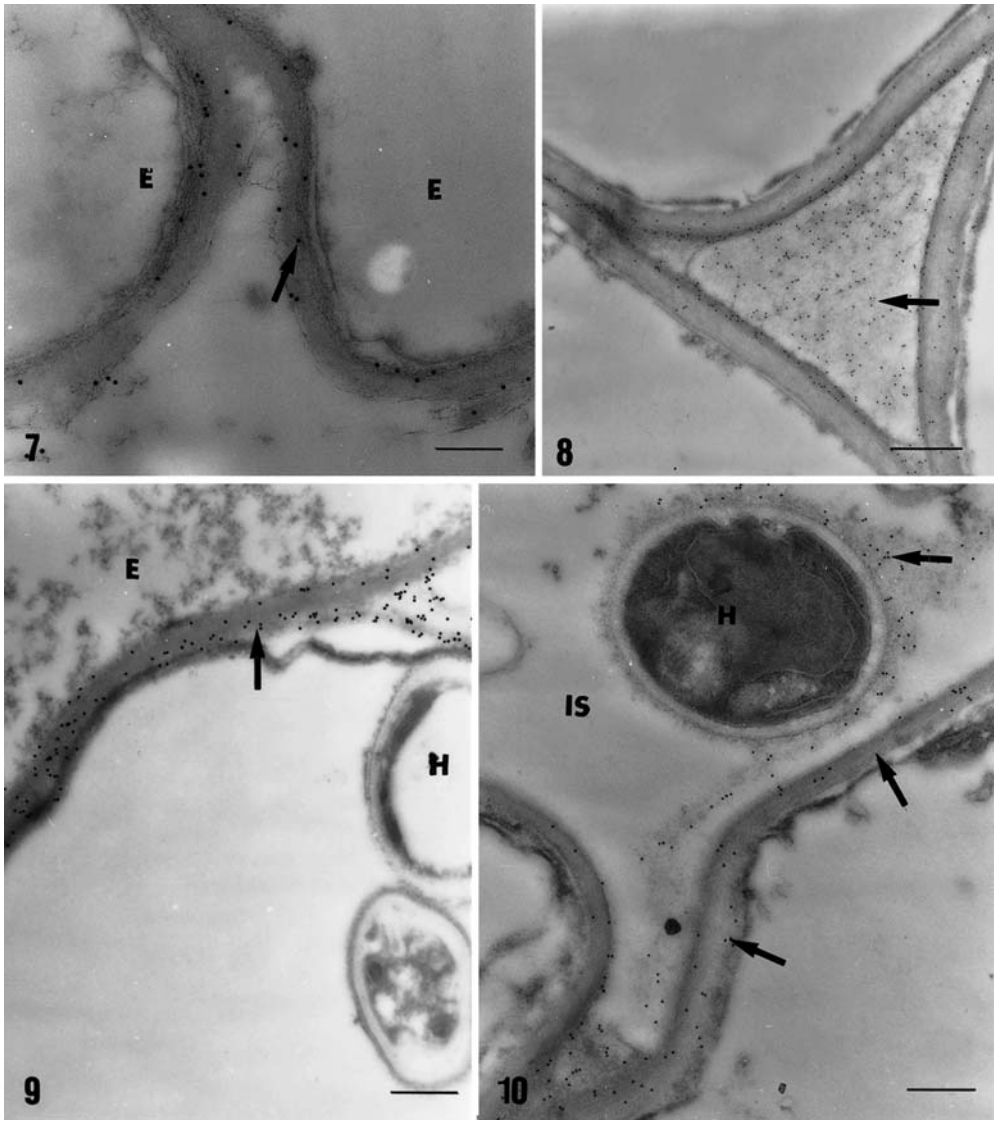
Antibody	Tissue	No. of gold particles per unit area (μm^2)	
		Noninoculated control	Nonpathogenic *F. oxysporum*
JIM 5	Epidermis	1759 ± 255 A	6010 ± 906 B
	Cortex	2017 ± 724 A	4347 ± 987 B
	Phloem	1745 ± 855 A	13164 ± 3101 B
JIM 7	Epidermis	632 ± 342 A	906 ± 397 A
	Cortex	1006 ± 232 A	1213 ± 549 A
	Phloem	5145 ± 1161 B	6543 ± 1828 B

Each value represents five replicate counts on five sections
For each antibody, data followed by different letters are significantly different ($P = 0.05$)

Discussion

In previous observations, nonpathogenic *F. oxysporum* strain Fo47 did not cause disease symptoms in *E. viminalis* seedlings (Salerno et al. 2000). The present study elucidates in more detail root colonization by the strain Fo47 and the responses of *E. viminalis* root tissues at the ultrastructural level. Nonpathogenic *F. oxysporum* strain Fo47 produced ill-defined appressoria-like structures on the root surface, confirming previous observations that *Fusarium* strains only slightly modify root morphology before penetration (Mendgen et al. 1996; Olivain and Alabouvette 1997, 1999). This nonpathogenic strain did penetrate root tissues between epidermal cells, which had been shown earlier only for pathogenic strains. Papilla formation was not observed in *E. viminalis* root tissues in response to infection by *F. oxysporum* strain Fo47, but electron-dense deposits in intercellular spaces of the colonized root cortex may indicate the diffusion of phenols from cells (Kosuge 1969; Mueller and Beckman 1974), as observed by Benhamou et al. (2002) in interactions between *F. oxysporum* strain Fo47 and *Pythium ultimum*-infected cucumber seedlings. Nonpathogenic *Fusarium* species have been reported to induce more phenols than pathogenic forms in tomato plants (Matta et al. 1969). Phenolic compounds and their oxidized products may play an active role in the enhanced resistance of plants to pathogenic *Fusarium* species (Carrasco et al. 1978). *Eucalyptus viminalis* root tissues infected by the nonpathogenic *F. oxysporum* strain had a high degree of cell integrity, even though hyphae breached plant cell walls. *Fusarium oxysporum* strain Fo47 does not appear to penetrate the host protoplast in most cases of cell colonization, and hyphae develop in the peripheral periplasmic space.

Numerous studies have attempted to implicate pectic enzymes in events associated with colonization of susceptible tissue by invading fungi that overcome host resistance and induce disease symptoms (Hahn et al. 1989). In the present study, the polygalacturonate substrates for enzymes were localized to follow the various modifications that occur during the infection process by the nonpathogenic *F. oxysporum* strain. Nonesterified pectin (NE) is equally localized in external epidermal walls, the middle lamella, and walls at cell junctions of noninoculated *E. viminalis* roots,



Figs. 7–10. Ultrastructural localization of nonesterified pectin in *Eucalyptus viminalis* roots. **7** Immunolabeling (*arrow*) of nonesterified pectin with the JIM 5 antibody in cell walls of the epidermis (*E*) of a noninoculated *E. viminalis* root. *Bar* 0.2 μm. **8** Immunolabeling (*arrow*) of nonesterified pectin with the JIM 5 antibody in cell walls and intercellular spaces of the parenchymal cortex in a noninoculated *E. viminalis* root. *Bar* 0.7 μm. **9** Immunolabeling (*arrow*) of nonesterified pectin with JIM 5 antibody in an external epidermal cell wall (*E*) of an *E. viminalis* root inoculated with *Fusarium oxysporum* strain Fo47 (*H*). *Bar* 0.2 μm. **10** Immunolabeling (*arrow*) with the JIM 5 antibody in the cell wall of the parenchymal cortex (*C*) and of intercellular space material (*IS*) of an *E. viminalis* root colonized by *Fusarium oxysporum* strain Fo47. Slight, localized decrease in immunolabeling in the plant cell wall close to the hypha (*arrow*). *Bar* 0.5 μm

whereas methyl-esterified (ME) pectin is detected to a much greater extent in phloem tissues. The apparent lack of ME degradation in root tissues colonized by the nonpathogenic *F. oxysporum* strain Fo47 may explain why the integrity of the infected tissues was maintained after inoculation. Furthermore, the increased accumulation of NE pectin in cell walls across infected roots may be indicative of cell wall strengthing in the tissues. These observations suggest that pectinolytic enzymes may only be weakly active when *F. oxysporum* strain Fo47 develops in *E. viminalis* roots, in contrast to those reported for root interactions with a pathogenic *F. oxysporum* strain that causes tissue disorganization and where pectin degradation appears to occur (Salerno et al. 2004). Because both pathogenic and nonpathogenic strains of *F. oxysporum* can utilize pectin in pure culture (Steinberg et al. 1999), differences in tissue degradation between *Fusarium* strains may be attributable to differential host control over their enzymatic activity, so enzyme repression occurs in one case but not in the other. In the same way, the differences in the pattern of cell wall alteration by the pathogenic and nonpathogenic strains suggests that the enzymes have preferential sites of action.

Conclusions

Although the molecular basis of nonpathogenicity in the bioprotective Fo47 strain of *F. oxysporum* is not known, its

effects on tissue integrity of *E. viminalis* roots differ greatly from those of a pathogenic *F. oxysporum* strain (Salerno et al. 2004). This diversity does not strictly affect fungal virulence in *E. viminalis* seedlings because both fungi are able to intensely colonize *E. viminalis* roots (Salerno et al. 2000). However, differences in pathogenicity do appear to be linked to differences in polysaccharide degradation capacities between the *F. oxysporum* strain in planta. Production of pectin-degrading enzymes is correlated quantitatively to the extent of disease development in the case of *F. oxysporum* f. sp. *dianthii* (Bayen et al. 1997). Consequently, pathogenic *Fusarium* strains may be able to produce the large quantities of multienzymic complexes (Paquin and Coulombe 1962) required for tissue degradation and pathogenicity, and secretion of a smaller amount of enzymes by nonpathogenic strains such as Fo47, or their inactivation within host tissues, may explain tissue conservation during root colonization. The use of labeling techniques similar to those reported by Benhamou et al. (2002) to localize specific cell wall-degrading enzymes in eucalypt tissues infected by *F. oxysporum* would help us better understand the mechanisms by which pathogenic and nonpathogenic *F. oxysporum* strains operate to cause different symptoms in *E. viminalis* roots.

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