Evidence for Antibiosis and Induced Host Defense Reactions in the Interaction Between *Verticillium lecanii* and *Penicillium digitatum*, the Causal Agent of Green Mold

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ABSTRACT

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Chronological events of the intercellular interaction between *Verticillium lecanii* and the postharvest pathogen *Penicillium digitatum* were investigated by transmission electron microscopy and gold cytochemistry. Growth inhibition of *P. oligandrum* as a response to *V. lecanii* attack correlated with striking host changes including retraction of the plasma membrane and cytoplasm disorganization. Such changes were associated with the deposition on the inner host cell surface of a chitin- and cellulose-enriched material which appeared to be laid down as a structural

defense reaction. The accumulation of chitin in the newly formed material correlated with a decrease in the amount of wallbound chitin. However, the deposition of cellulose appeared to correspond to a de novo synthesis, as evidenced by the occurrence of cellulose-containing vesicles which released their content in the space between the invaginated plasma membrane and the host cell wall. Results of the present study provide the first ultrastructural and cytochemical evidence that antagonism, triggered by *V. lecanii*, is a multifaceted process in which antibiosis, with alteration of the host hyphae prior to contact with the antagonist, appears to be the key process in the antagonism against *P. digitatum*.

Additional keywords: biological control, elicitor, hydrolytic enzymes, toxin.

Increasing expectations are emerging in the area of plant disease management for new strategies that have the potential to be efficient, reliable, and safe for the environment. Among the avenues being explored, antagonism, mediated by nonpathogenic fungi such as *Trichoderma* spp. (10,11,12) and *Pythium oligandrum* Dreschler (10,15), is one of the biocontrol approaches that offers excellent prospects for protecting plants against microbial infections. One noted facet of this strategy concerns the potential of the hyphomycete, *Verticillium lecanii* (Zimm.) Viégas (1,3). Since the first demonstration that this well known entomopathogenic fungus (2,20,27) could also parasitize rusts (29), convincing evidence has been provided that *V. lecanii* is a promising biocontrol agent of rusts and powdery mildew pathogens (1,25,30). The rationale for such an interest towards the remarkable properties of *V. lecanii* was that not only could this fungus be a powerful candidate in an integrated arthropod pest management strategy, but it could also become a valuable option for plant disease management (3). Recent investigations have shown that the beneficial effect of *V. lecanii* of exploiting insects and fungal pathogens involved a series of coordinated events including recognition, antibiosis and production of hydrolytic enzymes such as chitinases (1,2). While some toxic metabolites with insecticidal activity have been clearly identified (13,18), the question as to what extent antibiotics and hydrolytic enzymes are key determinants in the antifungal effect exerted by *V. lecanii* has not yet been answered conclusively.

With the refinement of cytological and molecular techniques and the application of these approaches to biological systems, several unanswered questions regarding the relationships established be-

tween an antagonist and its target host can now be addressed. In a recent report, Askary et al. (1) provided evidence that chitin hydrolysis was one of the key mechanisms involved in the mycoparasitic process exerted by *V. lecanii* against *Sphaerotheca fuliginea*, the causal agent of cucumber powdery mildew. This observation led to the suggestion that the strain of *V. lecanii* under study (DAOM 198499) displayed the ability to synthesize chitinases which likely facilitated internal colonization of the host in addition to providing the antagonist with sugars essential for growth and development. Except for these pioneer cytological observations, little is known about the enzymatic activity of *V. lecanii* and additional research is needed to confirm the extent to which active production of cell wall-degrading enzymes is involved in mycoparasitism. The demonstration that *V. lecanii* produced toxins with insecticidal properties (18) has shown that the biocontrol activity exerted by this fungus is much more complex than was originally thought. The mechanisms by which a single strain of *V. lecanii* exploits hosts of different origin appear highly complex, probably because specific molecular events at the cell surface as well as differential production of biologically active molecules determine the outcome of a given interaction (1,3). At present, the involvement of hydrolases and toxic metabolites in the antagonistic process remains unclear. Both mechanisms may not be mutually exclusive and the possibility that they operate synergistically needs to be more thoroughly investigated at the cellular and molecular levels.

Over the past few years, much effort has been directed towards elucidating the mechanisms of host specificity and pathogenicity of *V. lecanii* against insects (21,22). By contrast, the host range of *V. lecanii* among plant pathogens has not been the subject of intensive studies and, except for rusts and powdery mildew fungi, no other fungal species has been reported as vulnerable to *V. lecanii*. Our preliminary data indicate that *V. lecanii* has the capacity to parasitize a number of other pathogenic fungi through differential

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modes of action according to the target species involved (Benhamou and Brodeur, unpublished results). In light of these results, we became interested in delineating the mechanisms involved in the antagonistic process, and, more precisely, the extent to which antibiosis and mycoparasitism could account for the observed host fungal disturbances.

In an attempt to address these questions, the mechanisms by which *V. lecanii* strain DAOM 198499 could parasitize *Penicillium digitatum* Saccardo, the causal agent of green mold, an important postharvest disease, were investigated. The objectives of the present research were, first, to determine the pattern of *P. digitatum* colonization by *V. lecanii*, second, to ultrastructurally delineate the series of events leading to successful antagonism, and, third, to cytochemically investigate the relative importance of antibiosis in the process.

We provide evidence that, in spite of its spectacular ability to elaborate structural defense reactions, *P. digitatum* is highly vulnerable to *V. lecanii* mainly through synthesis of antibiotic compounds. To our knowledge, this is the first report providing evidence for a key role of *V. lecanii*-produced toxins in fungal antagonism.

MATERIALS AND METHODS

Fungal culture and growth conditions. *V. lecanii* (strain DAOM 198499), collected from codling moths in Quebec, was kindly provided by Dr W. R. Jarvis, Harrow Research Station Agriculture and Agrifood Canada, Ontario. *P. digitatum* (isolate SRSF707, known to be highly virulent on citrus fruits) was obtained from the collection maintained at Agriculture and Agrifood Canada, Sainte-Foy, Québec. Both fungi were grown on Difco potato dextrose agar (PDA, Difco Laboratories, Detroit, MI). Plates were incubated at 24°C in the dark and subcultured weekly.

Dual culture tests. The interaction between hyphae of *V. lecanii* and hyphae of *P. digitatum* was studied according to the following procedure. A mycelial disk (5-mm in diameter), collected from the margin of an actively growing *V. lecanii* culture, was placed 1 cm from the edge of a 9-cm petri dish containing freshly prepared PDA medium. Seven days later, a disk of *P. digitatum* mycelium was deposited in a diametrically opposed position, 3 cm away from the *V. lecanii* disk (Fig. 1B). Plates were maintained at 24°C under continuous light. Since contact between both fungi did not occur, mycelial samples were collected from the edge of the *Penicillium* colony in the area facing *V. lecanii*. Samples, taken at 2, 3, 4, and 5 days after inoculation of *P. digitatum*,

were processed for electron microscope investigation. The experiment was repeated twice using three petri dishes per sampling time.

In order to determine whether the observed growth inhibition of *P. digitatum* in dual cultures was due to a diffusible metabolite, agar disks from the fungus-free interaction region were collected 3 and 4 days after inoculation of *P. digitatum* and deposited at the margin of a freshly prepared *P. digitatum* culture. Controls included agar disks taken from noninoculated PDA plates (Fig. 1C). Mycelial samples were collected 2 days after exposure to the PDA disks and further processed for electron microscopy.

Tissue processing for histological and ultrastructural observations. Mycelial samples (2 mm³), collected as described above, were fixed by immersion in 3% (vol/vol) glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2, overnight at 4°C, and post-fixed with 1% (wt/vol) osmium tetroxide in the same buffer for 1 h at 4°C. Samples were dehydrated in a graded ethanol series and embedded in Epon 812 (IBEM Chemicals, Pointe-Claire, Quebec, Canada). Thin sections (0.7 µm), cut from the Epon-embedded material with glass knives, were mounted on glass slides and stained with 1% aqueous toluidine blue prior to examination with a Zeiss Axioscope microscope (Carl Zeiss Canada, Don Mills, Ontario, Canada). Ultrathin sections (0.1 µm), collected on nickel grids using a diamond knife, were either contrasted with uranyl acetate and lead citrate for immediate examination with a 1200 EX transmission electron microscope (JEOL Ltd., Tokyo, Japan) operating at 80 kV or further processed for cytochemical labeling. An average of five samples from three different plates per sampling time was investigated. For each sample, 10 to 15 ultrathin sections were examined under the electron microscope.

Cytochemical labeling. The colloidal gold suspension (BDH Chemicals, Montréal, Canada), with particles averaging 15 nm in diameter, was prepared as described by Benhamou (1989). Wheat germ agglutinin (WGA), a lectin with N-acetylglucosamine binding specificity was used for localizing N-acetylglucosamine residues (chitin) according to a previously described procedure (4). Because of its low molecular weight, this lectin could not be directly complexed to colloidal gold. It was used in a two-step procedure with ovomucoid as a second step reagent. Ovomucoid was conjugated to colloidal gold at pH 5.4 (4). 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 kindly provided by C. Breuil,

Fig. 1. Effect of *Verticillium lecanii* and its metabolites on the growth of *Penicillium digitatum*, 3 days after inoculation of the pathogen. **A**, *P. digitatum* (P) grown in single culture; **B**, *P. digitatum* grown in the presence of *V. lecanii* (V); a marked growth inhibition is noticeable (arrow). **C**, Growth of *P. digitatum* is halted in the presence of an agar disk taken from the interaction region with *V. lecanii* (3); no effect is observed with agar disks from uninoculated plates (1 and 2).

Forintek, Canada. It was complexed to colloidal gold at pH 9.0 and used in a one-step procedure (7).

For the indirect labeling of chitin, sections were first floated on a drop of phosphate-buffered saline (PBS), pH 7.4, for 5 min, then transferred to a drop of WGA (25 µg/ml in PBS, pH 7.4) for 60 min at room temperature in a moist chamber. After washing with PBS, pH 7.4, sections were incubated on a drop of the ovomucoid-gold complex for 30 min at room temperature. Sections were washed with PBS, rinsed with distilled water and contrasted with uranyl acetate and lead citrate.

For the direct labeling of cellulosic β-1,4-glucans, sections were first incubated on a drop of PBS, pH 6.0, containing 0.02% (wt/vol) of polyethylene glycol (PEG) for 5 min at room temperature. Sections were transferred to a drop of the gold-complexed exoglucanase for 30 min at room temperature in a moist chamber. After washing with PBS and rinsing with distilled water, grids were contrasted as described above.

Cytochemical controls. Specificity of the labelings was assessed with the following control tests: (i) incubation with the WGA including a previously added excess of *N-N'-N''*-triacetylchitotriose (1 mg/ml in PBS); (ii) incubation with WGA, followed by unlabeled ovomucoid and finally by ovomucoid-gold complex; (iii) incubation with the gold-complexed exoglucanase to which were previously added β-1,4-glucans from barley, 1 mg/ml in PBS); and (iv) incubation with stabilized or unstabilized gold suspensions.

Quantification of labeling. The density of labeling obtained with the WGA/ovomucoid-gold complex and the exoglucanase-gold complex over *Penicillium* cell walls and wall appositions was determined by counting the number of gold particles per micrometer squared. Area determinations were carried out by the point counting method established by Weibel (30) using negatives of electron micrographs projected on a lattice. Amount of labeling over specified wall areas (Sa) was estimated by counting the number of gold particles (Ni) on a photographic enlargement. Density of labeling (Ns) was calculated as follows: $Ns = Ni/Sa$, where Ns represents the number of gold particles per unit surface.

RESULTS

Dual culture assays. In single cultures, *P. digitatum* grew actively and colonized the entire agar surface within 3 days following inoculation (Fig. 1A). In dual cultures, contact between *V. lecanii* and *P. digitatum* did not occur by 6 days after inoculation. As early as 3 days following inoculation, *Penicillium* growth was

halted and changes in the hyphal morphology at the edge of the colony were observed visually (Fig. 1B, arrow). When agar samples from the interaction region between both fungi were deposited at the margin of an actively growing *Penicillium* colony, mycelial growth was halted (Fig. 1C-3). By contrast, the fungus easily overgrew agar disks taken from uninoculated PDA (Fig. 1C-1 and 2).

Histological observations. At the light microscope level, *Penicillium* hyphae grown in dual cultures were markedly altered (Fig. 2B and C). In addition to exhibiting morphological changes mainly characterized by hyphal distortions (Fig. 2C), most hyphae of *Penicillium* (nearly 90%) stained more intensely with toluidine blue (Fig. 2B) than those that were grown in single cultures stained (Fig. 2A). The host hyphal wall appeared well-preserved and, in many cases, thickened by the deposition of a thick material which surrounded the aggregated cytoplasm (Fig. 2B and C, arrows).

Time-course investigation of the cellular events involved in the *V. lecanii*-*P. digitatum* interaction. When grown in single cultures, *Penicillium* hyphae showed a typical ultrastructure. Cells were delimited by a thin cell wall and contained a dense poly-ribosome-enriched cytoplasm in which a large number of organelles, including mitochondria, nuclei, and vacuoles were found (Fig. 3A).

When mycelial samples, collected from the margin of the *Penicillium* colony facing *V. lecanii* in dual cultures, were examined by transmission electron microscopy, early signs of alteration were easily discernible as soon as 2 days after inoculation of the pathogen (Fig. 3B). The main features of this cell disorganization included a slight densification of the cytoplasm accompanied by an increased vacuolation and a local retraction of the plasma membrane. Interestingly, the space between the cell wall and the invaginated plasma membrane appeared filled with a fibrillar wall-like material (Fig. 3B). After 3 days of dual culture, host cell disorganization was markedly amplified, as evidenced by the extent of plasma membrane retraction which usually correlated with an early stage of cytoplasm aggregation (Fig. 3C). A close examination of these reacting host cells revealed that plasma membrane retraction was, most often, accompanied by the sequential deposition of a fibrillo-granular network in the paramural space (Fig. 3C). The newly-formed wall appositions displayed a more loosened texture towards the inside of the cell, indicating that they were sequentially formed. At a more advanced stage (4 days) of the interaction, a substantial increase in the extent and magnitude of the host cellular changes was observed (Fig. 3D). These amplified changes were essentially characterized by a com-

Fig. 2. Light micrographs of sectioned mycelial samples, collected at the margin of a *Penicillium digitatum* colony, 2–3 days after inoculation. Staining with toluidine blue. **A**, *P. digitatum* grown in single culture; bar = 10 µm. **B**, and **C**, Aspect of *P. digitatum* hyphae (P) grown in dual cultures with *Verticillium lecanii*. Hyphae stain intensely with toluidine blue. The host hyphal wall appears thickened by the deposition of a thick material which surrounds the aggregated cytoplasm (arrows); bars = 10 µm.

Fig. 3. Transmission electron micrographs of mycelial samples, collected at the margin of a *Penicillium digitatum* colony, 2–4 days after inoculation. **A**, *P. digitatum* grown in single culture. The hypha is delimited by a thin cell wall (PW) and contains a dense polyribosome-enriched cytoplasm (Cy) in which mitochondria (M), nuclei (N), and vacuoles (Va) are found; bar = 1 μ m. **B**, *P. digitatum* grown in dual culture with *Verticillium lecanii*, 2 days after inoculation. A slight increase in cytoplasm (Cy) density accompanied by an increased vacuolation and a local invagination of the plasma membrane (PM) from the cell wall (PW) are visible. A wall apposition (WA) is formed between the cell wall and the invaginated plasma membrane; bar = 0.5 μ m. **C**, *P. digitatum* grown in dual culture with *V. lecanii*, 3 days after inoculation. The cytoplasm (Cy) is aggregated. A wall apposition (WA) is formed in the paramural space between the plasma membrane (PM) and the cell wall (PW); bar = 0.5 μ m. **D**, *P. digitatum* grown in dual culture with *V. lecanii*, 4 days after inoculation. The plasma membrane is completely retracted and the cytoplasm (Cy) is reduced to undefined, aggregated remnants. A considerable enlargement of the stratified wall apposition (WA) is noticeable; bar = 1 μ m.

plete retraction of the plasma membrane and by a strong aggregation of the cytoplasm. Such changes correlated with a considerable enlargement of the stratified wall appositions deposited onto the inner cell wall surface. By 5 days, spectacular host changes were detected (Fig. 4). In such cells, the entire space between the cell wall and the tiny aggregated cytoplasmic remnants was filled with a heterogeneous material forming a tight network (Fig. 4B). A close examination of the wall appositions revealed that they were most often made of a material varying in texture and electron density (Fig. 4A). Some electron-opaque inclusions could be seen in the heterogeneous layer neighboring the aggregated cytoplasm (Fig. 4A, arrows).

Cytochemical labeling of chitin. Incubation of ultrathin sections from individual colonies of *P. digitatum* with the WGA/ovo-mucoid-gold complex resulted in an intense labeling of the cell wall (Fig. 5A). Gold particles were found to be distributed over all wall layers with a preferential localization over the inner wall layers (Fig. 5A). Control tests, including previous adsorption of WGA with *N*-*N*'-*N*''-triacylchitotriose, did not reveal significant wall labeling (not shown).

After 2 days of dual culture, only a few dispersed gold particles were detected throughout the material deposited between the labeled cell wall and the locally retracted plasma membrane (Fig. 5B). At 3 days, labeling of the wall appositions significantly

increased while retraction of the plasma membrane was amplified (Fig. 5C). Quantification of labeling indicated a slight decrease in labeling intensity of the host cell wall as compared to that found in cells grown in single cultures (Table 1). By day four, a considerable increase in labeling density of the wall appositions was observed (Fig. 5D, Table 1). Gold particles appeared to closely follow the gradual invagination of the plasma membrane (Fig. 5D). Examination of sections at a high magnification revealed that the underlying matrix of wall appositions was crossed by numerous, branched channels which were specifically labeled (Fig. 5E, arrows). A close examination of these areas indicated that gold particles, apparently released from the loosened host cell wall (Fig. 6A, arrow), were conveyed towards the inside via the elongated channels which frequently infiltrated in the aggregated cytoplasm (Fig. 6A, double arrows). At that time, the host cell wall was significantly less labeled than the control (Fig. 6A, Table 1). Labeled vesicles were never seen in the cytoplasm (Fig. 5E; 6A). At an advanced stage of the interaction (5 days), the tight network of newly-formed material was intensely labeled (Fig. 6B, Table 1). However, the pattern of labeling distribution was not uniform. Indeed, labeling intensity decreased towards the inside, reduced to a few gold particles in the immediate vicinity of the aggregated cytoplasmic remnants (Fig. 6B). This gradient of labeling was observed in all examined sections.

Fig. 4. Transmission electron micrographs of mycelial samples, collected at the margin of a *Penicillium digitatum* colony grown in dual culture with *Verticillium lecanii*, 5 days after inoculation. **A**, and **B**, The entire space between the cell wall (PW) and the reduced aggregated cytoplasmic (Cy) remnants is filled with heterogeneous wall appositions (WA). Electron-opaque inclusions are seen in the heterogeneous layer neighboring the aggregated cytoplasm (**A**, arrows). **A**, and **B**, bars = 0.5 μ m.

Cytochemical localization of cellulose. Incubation of ultrathin sections from individual colonies of *P. digitatum* with the exoglucanase-gold complex resulted in a labeling specifically localized over the innermost wall layers the cell wall (Fig. 7A). Control tests,

including preincubation of the gold-complexed exoglucanase with β -1,4-glucans from barley, yielded negative results (not shown).

In dual cultures, labeling with the gold-complexed enzyme resulted in a massive deposition of gold particles over the host cell

Fig. 5. Transmission electron micrographs of mycelial samples, collected at the margin of a *Penicillium digitatum* colony, 2–4 days after inoculation. Labeling of chitin with the wheat germ agglutinin/ovomucoid-gold complex. **A**, *P. digitatum* grown in single culture. The cell wall (PW) is evenly labeled while the cytoplasm (Cy) is free of labeling; bar = 0.5 μ m. **B**, *P. digitatum* grown in dual culture with *Verticillium lecanii*, 2 days after inoculation. Only a few dispersed gold particles are seen over the wall apposition (WA) deposited between the labeled cell wall (PW) and the locally retracted plasma membrane; bar = 0.5 μ m. **C**, *P. digitatum* grown in dual culture with *V. lecanii*, 3 days after inoculation. Labeling of the wall apposition (WA) is substantial while retraction of the plasma membrane is amplified; bar = 0.5 μ m. **D**, and **E**, *P. digitatum* grown in dual culture with *V. lecanii*, 4 days after inoculation. The wall apposition (WA) is intensely labeled. Gold particles appear to follow the gradual invagination of the plasma membrane (PM). The underlying matrix of the wall apposition (WA) is crossed by numerous, branched channels which are specifically labeled (**E**, arrows). **D**, bar = 0.5 μ m; **E**, bar = 0.25 μ m.

wall and the wall appositions (Fig. 7B). The intensity of cell wall labeling was markedly magnified when compared to that of *Penicillium* hyphae grown in single cultures (Table 2). Interestingly, specifically labeled, electron-opaque vesicles were frequently seen at the periphery of the multilobed, condensed cytoplasm (Fig. 7B and C, arrows). A close examination revealed that some of the labeled vesicles could also be enclosed in the aggregated cytoplasm (Fig. 7D, arrows) prior to release of their content in the wall appositions (Fig. 7E, arrowheads). At more advanced stages of the

TABLE 1. Density of labeling obtained with the wheat germ agglutinin/ovomucoid-gold complex (labeling of chitin) over cell walls and wall appositions of *Penicillium digitatum* during the interaction with *Verticillium lecanii*

Time (days) after inoculation in dual cultures	Gold particles per $\mu\text{m}^2 \pm \text{SD}^a$	
	Cell wall	Wall apposition
Control ^b	41.1 $\pm$ 12.5	...
2	38.2 $\pm$ 9.3	2.5 $\pm$ 0.1
3	31.4 $\pm$ 8.1	18.2 $\pm$ 4.3
4	27.8 $\pm$ 6.2	45.5 $\pm$ 9.5
5	14.6 $\pm$ 5.2	52.3 $\pm$ 5.5

^a Densities were determined by counting the number of gold particles over randomly selected areas of cell walls and wall appositions on 20 micrographs.

^b Single culture of *P. digitatum* at 4 days.

interaction, the stratified wall appositions were intensely labeled while the cytoplasm was reduced to a polymorphic, undefined structure in which organelles were no longer discernible (Fig. 8, Table 2).

Effect of diffusible metabolites on the ultrastructure of *P. digitatum*. When agar disks taken from the fungus-free interaction region between *V. lecanii* and *P. digitatum* were deposited at the margin of a *Penicillium* colony (see Fig. 1C), host cell reactions, similar to those noticed in dual cultures, were observed (Fig. 9A). Retraction of the plasma membrane associated with a gradual disorganization and condensation of the cytoplasm were typical features of reaction (Fig. 9A). The formation of wall appositions was also a typical host cell response. Upon incubation with the WGA-gold complex, the wall appositions appeared less intensely labeled than in *Penicillium* cells grown in dual cultures (Fig. 9B). By contrast, the pattern of labeling obtained with the gold-complexed exoglucanase was similar, in most respects, to that noticed in dual cultures (Fig. 9C). Labeled vesicles, releasing their content over the wall appositions, were always seen in the condensed cytoplasm (Fig. 9D, arrows).

DISCUSSION

Results of the present study provide the first evidence that the antagonistic properties of *Verticillium lecanii* are not restricted to

Fig. 6. Transmission electron micrographs of mycelial samples, collected at the margin of a *Penicillium digitatum* colony grown in dual culture with *Verticillium lecanii*, 5 days after inoculation. Labeling of chitin with the wheat germ agglutinin/ovomucoid-gold complex. **A**, and **B**, The wall appositions (WA) are heavily labeled and channels are infiltrating the cytoplasm (Cy) (**A**, double arrows). Release of gold particles from the cell wall (PW) is seen in some places (**A**, arrow). Labeling intensity decreases towards the inside, reduced to a few gold particles in the immediate vicinity of the aggregated cytoplasmic (Cy) remnants (**B**). **A**, bar = 0.25 μm ; **B**, bar = 0.5 μm .

rusts and powdery mildews (1,3), but extend to other fungal pathogens such as *P. digitatum*, highlighting the remarkable and atypical potential of this fungus as a promising biocontrol agent of a wide array of pathogens. These results shed more light into the

differential modes of action of *V. lecanii* and demonstrate that antibiosis is a key determinant of the antagonistic process operating against *P. digitatum*. In addition, our observations confirm earlier reports that induced resistance is a defense strategy that

Fig. 7. Transmission electron micrographs of mycelial samples, collected at the margin of a *Penicillium digitatum* colony grown in dual culture with *Verticillium lecanii*, 3–4 days after inoculation. Labeling of cellulose with the exoglucanase-gold complex. **A**, *P. digitatum* grown in single culture. Labeling is preferentially associated with the innermost wall layers the cell wall (PW); bar = 0.5 µm. **B–E**, In dual cultures, a massive deposition of gold particles is seen over the host cell wall and the wall appositions (WA). Specifically labeled, electron-opaque vesicles are found at the periphery of the multilobed, condensed cytoplasm (**B**, and **C**, arrows). Some of the labeled vesicles are enclosed in the aggregated cytoplasm (Cy) (**D**, white arrows) prior to releasing their content in the wall appositions (**D**, and **E**, arrowheads). **B**, bar = 0.5 µm; **C**, bar = 0.25 µm; **D**, bar = 0.5 µm; **E**, bar = 0.25 µm.

may also occur in fungi (5,8), in a way similar to that abundantly documented in plants.

Light and electron microscope investigations revealed that growth inhibition of *P. digitatum* as a response to *V. lecanii* attack correlated with marked cellular changes including invagination of

TABLE 2. Density of labeling obtained with the exoglucanase-gold complex (labeling of cellulose) over cell walls and wall appositions of *Penicillium digitatum* during the interaction with *Verticillium lecanii*

Time (days) after inoculation in dual cultures	Gold particles per $\mu\text{m}^2 \pm \text{SD}^a$	
	Cell wall	Wall apposition
Control ^b	10.7 $\pm$ 2.3	...
2	18.2 $\pm$ 5.5	9.6 $\pm$ 1.2
3	25.4 $\pm$ 8.1	22.4 $\pm$ 5.3
4	26.5 $\pm$ 3.1	25.2 $\pm$ 9.5
5	25.5 $\pm$ 7.9	34.5 $\pm$ 9.2

^a Densities were determined by counting the number of gold particles over randomly selected areas of cell walls and wall appositions on 20 micrographs.

^b Single culture of *P. digitatum* at 4 days.

the plasma membrane, condensation and aggregation of the cytoplasm, as well as organelle breakdown. Because such cell reactions were induced in the absence of intimate contact between both fungi, the possibility that diffusible toxic metabolites may be responsible for the observed disturbances appears realistic. Similar disturbances in the overall fungal organization have often been reported in the case of fungal cells exposed to either antibiotics (19,23) or sterol-binding fungicides (17). Because the plasma membrane has been reported to be a potential target for antibiotics (24), changes in plasma membrane permeability may have promoted internal osmotic imbalances, leading to alterations such as cytoplasm disorganization and aggregation. Although antifungal metabolites have not been identified during the course of this study, strong support for the concept of antibiosis as the main mechanism by which *V. lecanii* operates against *P. digitatum* is provided by the finding that agar disks, collected from the fungus-free intermediate region between both fungi, could trigger the same sequence of events, ultimately leading to host cell alteration. While toxin involvement in insect infection by *V. lecanii* has been suggested (14,18), little is known about pathogen

Fig. 8. Transmission electron micrographs of mycelial samples, collected at the margin of a *Penicillium digitatum* colony grown in dual culture with *Verticillium lecanii*, 5 days after inoculation. Labeling of β -1,4 glucans with the exoglucanase-gold complex. A, and B, the stratified wall appositions (WA) are intensely labeled while the cytoplasm (Cy) is reduced to a polymorphic undefined structure; bars = 0.5 μm .

cell degradation mediated by *Verticillium*-produced toxins. In the past, most efforts have been concentrated on the role of hydrolytic enzymes in the antagonistic process against rusts and powdery mildews (26) and not on the possible involvement of antibiotics.

Askary et al. (1) were the first to suggest that antibiosis, causing cytoplasm disorganization, preceded parasitism and subsequent internal colonization of the cucumber powdery mildew pathogen, *S. fuliginea*. Antifungal synergisms between enzymes and toxic

Fig. 9. Effect of metabolites from *Verticillium lecanii* on the ultrastructure of *Penicillium digitatum*. **A**, In the presence of an agar disk taken from the fungus-free interaction region between *V. lecanii* and *P. digitatum*, *Penicillium* cell reactions include invagination of the plasma membrane (PM), condensation of the cytoplasm (Cy), and formation of enlarged deposits (D); bar = 1 μ m. **B**, and **C**, Labeling of chitin with the wheat germ agglutinin /gold complex. The deposits (D) corresponding to wall appositions (WA) are slightly labeled. **B**, bar = 0.5 μ m; **C**, bar = 1 μ m. **D**, Labeling of β -1,4 glucans with the gold-complexed exoglucanase. Labeled vesicles (white arrows), releasing their content over the wall appositions, are seen in the condensed cytoplasm (Cy); bar = 0.25 μ m.

metabolites have been frequently reported over the past few years (28,31), one of the best examples probably being the fungal antagonist, *Gliocladium virens*. While gliotoxin, a fungitoxic metabolite, has long been thought to be the main mechanism of biocontrol by this fungus, recent studies have shown that cell wall-degrading enzymes were also involved in the process (16). The synergistic effect of the enzymes was likely due to partial wall hydrolysis which facilitated rapid diffusion of the toxin. Whether such a synergism occurs in the interaction between *V. lecanii* and *P. digitatum* deserves to be more fully investigated. The observed reduction of chitin labeling over *Penicillium* cells grown in dual culture supports the idea that both antibiotics and hydrolytic enzymes may be involved in the antagonistic process mediated by *V. lecanii*. However, these results may be viewed with caution since the possibility cannot be excluded that such a decrease in labeling intensity reflects inaccessibility of the WGA probe to its corresponding receptors.

Structural changes induced in *P. digitatum* hyphae were found to correlate with the abnormal deposition of a heterogeneous material between the invaginated plasma membrane and the host cell wall. Even though the precise biological function played by the newly formed wall appositions is not clearly defined, one may presume that the massive accumulation of structural compounds at unusual sites reflects a defense strategy elaborated by *Penicillium* hyphae to halt or restrict ingress of the antagonist, and to shield the cell surface from the action of deleterious compounds such as hydrolytic enzymes and toxins. While the elaboration of structural barriers as a response of resistant plant cultivars to fungal attack is a well documented phenomenon (6,9), little information is available on the role played by defense-related structural responses in fungi. Furthermore, the mechanisms by which the fungal cell perceives and responds to a stress signal originating from the antagonist are not understood. In that context, the nature of the elicitation stimulus remains a great challenge. While it is clear that toxins may act as stress inducers by triggering a series of alteration events at the membrane level, the possibility that oligosaccharides, derived from the host cell wall, may themselves be potent inducers of defense reactions has to be taken into consideration. In plants, chitin as well as β -1,3-glucan fragments are involved in the induction of resistance through binding to specific membrane receptors (14). Because the amount of wall-bound chitin in *P. digitatum* was found to decrease during the course of the interaction, one may hypothesize that chitinases may have allowed the release of chitin oligomers that, in turn, may have operated as elicitors of a structural defense response. Whatever the origin of this material, evidence was provided from our observations that the gradual decrease in the amount of wallbound chitin correlated with a progressive increase in chitin deposition over the underlying matrix of the newly formed wall appositions. Whether this phenomenon reflects a response designed to reinforce the altered host cell wall and protect the cell from the diffusion of toxic compounds deserves to be more deeply investigated.

Another prominent and unexpected facet of the host response was the massive accumulation of β -1,4-glucans over the wall appositions. Interestingly, labeling with the gold-complexed exoglucanase revealed that cellulose, together with chitin, occurred in *Penicillium* cell walls. However, the observation that cellulose was confined to the innermost wall layers indicated that it was a minor compound compared to chitin which was more widely distributed. Considering the low amount of wallbound cellulose in *P. digitatum*, it is obvious that the considerable accumulation of cellulose over the wall appositions does not originate from the cell walls. Support for this concept came from the finding that labeled vesicles were conveyed from the cytoplasm towards the appositions where they released their content (Fig. 7). Such a de novo synthesis of wallbound polymers in response to stress has seldom been reported in fungi (8). This finding raises the question of how

an altered fungal cell can regulate the synthesis or the mobilization of cellulose precursors while the cytoplasm is disorganized and the organelles are no longer discernible. Whatever the role played by the chitin and cellulose-enriched material, *P. digitatum* did not survive *Verticillium* attack since the fungus could not be recovered once reinoculated to fresh PDA medium (unpublished results).

The present results provide the first ultrastructural and cytochemical evidence that antagonism, triggered by *V. lecanii*, is a multifaceted process. While mycoparasitism, mediated by intimate hyphal interactions and enzymatic action, was the main mechanism involved in the antagonism against the cucumber mildew pathogen (1), antibiosis, with alteration of the host hyphae prior to contact with the antagonist, appears to be the key process in the antagonism against *P. digitatum*. Biochemical analyses are presently being undertaken to isolate and characterize toxins produced by *V. lecanii* in order to delineate their involvement in the antagonistic process against pathogenic fungi.

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