Cytochemical Aspects of Chitin Breakdown During the Parasitic Action of a *Trichoderma* sp. on *Fusarium oxysporum* f. sp. *radicis-lycopersici*

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ABSTRACT

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A strain of *Trichoderma*, isolated from a sample of peat collected in New Brunswick, displays the ability to produce chitinases and inhibits growth of the pathogenic fungus, *Fusarium oxysporum* f. sp. *radicis-lycopersici*, in dual culture tests. Scanning electron microscope investigations of hyphal interactions show that growth inhibition of the host mycelium does not result from hyphal penetration by *Trichoderma*. The noticeable wall alterations along with the rapid collapse and loss of cell turgor of *F. o. radicis-lycopersici* in areas where *Trichoderma* was not in direct contact with the host mycelium indicates that extracellular metabolites could be responsible for the observed degradation. The cytochemical localization of *N*-acetylglucosamine residues in cell walls of *F. o. radicis-lycopersici* at different times after inoculation revealed that alteration of chitin molecules is an early event preceding wall disruption and leakage of cytoplasm. The observation that *N*-acetylglucosamine residues are released in the growing medium supports the idea that wall-bound chitin may be rapidly hydrolyzed by an extracellular chitinase produced by *Trichoderma*. Although it is likely that other lytic enzymes are involved in the complete degradation of mycelial walls of *F. o. radicis-lycopersici*, the present cytochemical investigation together with biochemical data on chitinase activity provides evidence for a major chitinolytic activity of *Trichoderma* and indicates that production of this enzyme may be of great significance in the antagonistic process.

Fusarium oxysporum f. sp. *radicis-lycopersici* Jarvis & Shoemaker is an important and widespread vascular wilt pathogen of tomato (13,14); its epidemiology (12) and geographical distribution (10,16,21,29) have been studied extensively. Although Fusarium wilt-resistant or -tolerant tomato cultivars have been developed (29,32), none of them have been widely used in disease control. For many years, control of Fusarium crown and root rot of tomato has been limited to the use of conventional soil sterilizing procedures together with the application of fungicides (24). However, complete eradication of *F. o. radicis-lycopersici* from soil by steam-sterilization and fumigation with chemicals could not be achieved mainly because of the rapid recolonization of sterile soil from airborne microconidia of the pathogen (15).

The failure of chemical control and the frequent pathogen development in sterilized soil led Jarvis (12) to suggest that the disease would be amenable to control by biological means. In addition to an interest in allelopathic control (17), the possibility of reducing the incidence of the disease by augmenting the soil with antagonists that could decrease or halt pathogen development in the early stages of soil reinfestation has received particular attention (18,19). In this context, an increasing number of researchers have convincingly shown the potential of some isolates of *Trichoderma* spp. as biocontrol agents of soilborne plant pathogens (1,27).

Hyphal interactions between *Trichoderma* spp. and some plant pathogenic fungi have been studied at the light and electron microscope levels (7,22). In addition, it has been convincingly demonstrated that a number of *Trichoderma* isolates are able to excrete hydrolytic enzymes, such as chitinases and β-1,3-glucanases, into the medium when grown in liquid media supplemented with laminarin or chitin (4,7,8,22,27). Because chitin and β-1,3-glucans are the main structural components of fungal cell walls (9), it has been suggested that the hydrolases produced by *Trichoderma* were likely involved in the degradation of pathogenic fungal cells through alteration of their wall components (26). However, it remains to be investigated whether or not the production of hydrolases by *Trichoderma* is related to an antifungal activity in vivo.

In an attempt to validate this assumption, the interaction between a recently isolated strain of *Trichoderma* and *F. o. radicis-lycopersici* was investigated by means of scanning electron microscopy (SEM) and transmission electron microscopy (TEM) with gold cytochemistry. In the present paper, we demonstrate that a *Trichoderma* sp., isolated from a commercially available sample of peat from New Brunswick, is able to produce several chitinases, to degrade cell wall chitin of *F. o. radicis-lycopersici*, and ultimately to induce pathogenic cell death. The possibility that the main mechanism involved in the antagonism between the isolated *Trichoderma* sp. and *F. o. radicis-lycopersici* is related to hydrolytic enzymatic activities is discussed.

MATERIALS AND METHODS

Fungal isolates and growth conditions. The isolate of *F. o. radicis-lycopersici* used in this study was supplied by P. O. Thibodeau (Service de la recherche en défense des cultures, MAPAQ). It was isolated from an infected tomato plant, periodically reinoculated and reisolated from ripe tomato fruits, and grown on Difco potato-dextrose agar (PDA) medium at 25 C.

The isolate of *Trichoderma* was selected among a collection of fungi isolated from a commercial peat produced in New Brunswick (Canada). The selection was based on the ability of the isolated fungus to inhibit development of *F. o. radicis-lycopersici* on PDA medium.

Growth of the isolate of *Trichoderma* in liquid culture. The fungus was grown in Erlenmeyer flasks containing 25 ml of a synthetic medium consisting of: $MgSO_4 \cdot 7H_2O$, 0.2 g/L; K_2HPO_4, 0.9 g/L; KCl, 0.2 g/L; NH_4NO_3, 1 g/L; Fe^{2+}, Mn^{2+}, Zn^{2+}, 0.002 g/L; in distilled water. The medium was supplemented with either glucose or cell walls of *F. o. radicis-lycopersici* as sole carbon sources at a concentration of 10 mg/ml^{-1}. Mycelium was collected after 2, 3, 5, and 7 days by centrifugation at 7,000 *g* for 15 min. The supernatants were filtered through a 0.2-μm Millipore filter paper and frozen at −20 C until use for enzyme assay by polyacrylamide gel electrophoresis (PAGE).

Cell walls of *F. o. radicis-lycopersici* were prepared according to the procedure of Chet et al (5), sonicated, lyophylized, and ground to a fine powder with a mortar and pestle. The cell wall preparation was autoclaved at 120 C for 60 min and stored in a desiccator until use.

Polyacrylamide gel electrophoresis (PAGE) under denaturing conditions. Sodium dodecyl sulfate (SDS)-PAGE was performed according to the procedure of Trudel and Asselin (30) in 10–15% (w/v) polyacrylamide gradient gels containing 0.01% (w/v) glycol chitin and 0.1% (w/v) SDS. Samples from supernatants, collected 2, 3, 5, and 7 days from *Trichoderma* liquid medium containing either glucose or cell walls of *F. o. radicis-lycopersici*, were boiled for 5 min with 15% (w/v) sucrose and 2.5% (w/v) SDS in 125 mM Tris-HCl, pH 6.7. Gels were run at room temperature for 65 min at 20 mA. Protein molecular weight markers ranged from 16,000 to 110,000 Da.

Detection of chitinase activity. After electrophoresis, gels were incubated for 2 hr at 37 C in 100 mM sodium acetate buffer, pH 5.0 containing 1% (v/v) Triton X-100 according to the procedure described by Trudel and Asselin (30). Gels were stained with Calcofluor White M2R in 500 mM Tris-HCl, pH 8.9, and destained in distilled water. Lytic zones were visualized by examining the gels under a Chromato-Vue C-62 transilluminator (UV light). Photographs were taken on Polaroid type 55 film with UV-haze and 02 orange filters.

Scanning electron microscopy. The following procedure was used to study hyphal interactions between the isolated *Trichoderma* and *F. o. radicis-lycopersici*: An autoclaved Millipore filter paper was placed on the surface of the agar medium. Disks (6 mm), cut from actively growing colonies of each fungus, were placed 3 cm apart, on the Millipore filter. The plates were incubated at 25 C under continuous light. The antagonist grew radially and hyphal interactions were evident by 48 hr after inoculation. Mycelial samples from the interaction region were collected from 2 to 9 days after inoculation, vapor-fixed with 2% osmium tetroxide in double-distilled water, air-dried, and sputter-coated with gold. Samples were kept in a desiccator until examination with a Cambridge Stereoscan 5-150 microscope (Cambridge Scientific Industries, Cambridge, MD) at 20 kV. Micrographs were taken on Polaroid type 52, positive film.

Transmission electron microscopy and cytochemical labeling of 1 *N*-acetylglucosamine residues. Mycelial disks from both fungi were placed 3 cm apart on the surface of the agar medium. Samples (1 mm^3) were collected from 1 to 9 days after inoculation from four different areas identified as A, B, C, and D: A = portion of mycelium of *F. o. radicis-lycopersici* facing the antagonist; B = portion of *F. o. radicis-lycopersici* towards the outside; C = portion of *Trichoderma* colony facing *F. o. radicis-lycopersici*; and D = portion of the antagonistic mycelium towards the outside.

All samples were fixed with 3% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2, for 2 hr at 4 C, postfixed with 1% osmium tetroxide in the same buffer for 1 hr at 4 C, dehydrated in a graded ethanol series and embedded in Epon 812. Ultrathin sections were collected on Formvar-coated nickel grids and processed for cytochemical labeling.

The colloidal gold suspension was prepared as described by Grandmaison et al (11). Wheat germ agglutinin (WGA), a lectin with *N*-acetylglucosamine-binding specificity (2,11), was used in a two-step procedure for studying the distribution of chitin, a linear polysaccharide of β-1,4-linked *N*-acetylglucosamine unit, within cell walls of both fungi in interaction. Because of its low molecular weight, WGA could not be directly complexed to gold. Ovomucoid, a water soluble glycoprotein isolated from egg white, was chosen for binding to WGA because of its previous successful use in the affinity chromatography of this lectin (2). Thus, this glycoprotein bearing sugar specific for WGA was conjugated to colloidal gold at pH 5.4 and used as a second-step reagent.

Sections were first incubated on a drop of WGA (25 μg/ml in phosphate-buffered saline [PBS], pH 7.4) for 60 min at room temperature, rinsed with PBS, and transferred to a drop of ovomucoid-gold complex for 30 min at room temperature. After washing with PBS and rinsing with double-distilled water, sections were contrasted with uranyl acetate and lead citrate, and examined with a JEOL 1200 EX transmission electron microscope. Experiments were repeated five times on samples collected from five sets of inoculated plates.

Control tests included: 1) incubation with WGA to which was previously added *N,N',N"* triacetyl-chitotriose (1 mg/ml in PBS); 2) incubations with WGA, followed by unlabeled ovomucoid and finally by ovomucoid-gold complex; and 3) direct incubation with the ovomucoid-gold complex, the lectin step being omitted.

Reagents. All chemicals for electrophoresis, protein molecular weight markers, and protein assay dye reagent concentrate were purchased from Bio-Rad (Canada). PDA medium was purchased from Difco Laboratories, Detroit, MI. Glutaraldehyde and osmium tetroxide were obtained from JBEM Chemical Co., Pointe Claire, Quebec, Canada, and tetrachloroauric acid from BDH Chemicals, Montreal, Quebec, Canada. All other reagents including WGA and ovomucoid were purchased from Sigma Chemical Co., St. Louis, MO.

RESULTS

Detection of chitinase activity after SDS-PAGE. Denatured proteins from supernatants of *Trichoderma* cultures containing either cell walls of *F. o. radicis-lycopersici* or glucose as sole carbon sources were subjected to SDS-PAGE in gels containing glycol chitin as the substrate and analyzed for chitinase activity (30) after renaturation of the enzymes in buffered Triton X-100. Figure 1 illustrates the results obtained with filtrate samples collected from cultures of *Trichoderma* containing cell walls of *F. o. radicis-lycopersici* (Fig. 1A) or glucose (Fig. 1 B) as carbon sources. As soon as 2 days after growth of *Trichoderma* in liquid medium supplemented with cell walls of the pathogenic fungus, a significant chitinase activity was detected in culture filtrates, as judged by the occurrence of at least 3 lytic bands located between 33 and 40 kDa (Fig. 1A). The protein band at about 40 kDa was found to decrease rapidly from 3 to 7 days, whereas those at about 33 and 38 kDa remained stable (Fig. 1A). Interestingly, protein bands that were not detected by 2 days, started to appear by 3 days and showed a marked increase in activity as estimated by the substantial increase in intensity of lysis. Bands that appeared to be stimulated from 3 to 5 days corresponded to proteins at about 20 and 45 kDa. Results of this time-course study indicate that at least three major and five minor chitinase activities are found in cultures of *Trichoderma* grown in presence of cell walls. Whereas some chitinases are produced early after contact between the antagonist and the pathogen cell walls, others seem to be gradually excreted in the medium (Fig. 1A). When *Trichoderma* was grown in liquid medium supplemented with glucose, chitinase activities could not be detected even after 7 days of growth (Fig. 1B).

Macroscopic observations of fungal growth in dual culture. A time-course study of fungal growth in dual cultures revealed that colonization of the agar medium by the peat-isolated *Trichoderma* occurred earlier and to a much higher extent than that caused by *F. o. radicis-lycopersici*. The first apparent contact between both fungi was usually observed within 48 hr after inoculation. By 3 days after inoculation, an intense and nearly complete colonization of the agar medium by *Trichoderma* occurred. The mycelium of the pathogen was almost totally encircled by the fast-growing colony of *Trichoderma*. In the following days,

hyphae of *Trichoderma* overgrew the pathogen colony and began to sporulate abundantly.

SEM observations. Mycelial samples from single cultures of *Trichoderma* and *F. o. radicis-lycopersici* as well as samples from the interaction region of dual cultures were observed by SEM. Both fungi were composed of a dense, regularly septate mycelium. The average diameter of hyphae of both *F. o. radicis-lycopersici* and *Trichoderma* was highly similar (2–3 μm), but the two fungi could be differentiated on the basis of conidial morphology.

In the interaction region of dual cultures, it was difficult to differentiate both fungi during the first hours after the inoculation, although apparent hyphal coiling was sometimes observed (Fig. 2A). As early as 3 days after inoculation, significant alterations of conidia and hyphae of *F. o. radicis-lycopersici* were readily discernible (Fig. 2B and C). Pronounced collapse and loss of turgor of the pathogen mycelium were observed frequently (Fig. 3E) together with severe cell wall disintegration. Hyphae of the pathogen that were not affected by the antagonist retained a normal elongated shape with an average diameter of 2 μm. From 5 to 7 days after inoculation, the mycelium of *F. o. radicis-lycopersici* consisted of highly distorted and collapsed filaments (Fig. 2D) that ultimately were entirely covered by hyphae and conidia of the mycoparasite (Fig. 2E).

Hyphal and conidial alteration of the pathogen within 3 to 5 days after inoculation occurred also in areas where the mycoparasite was not detected. This indicated that intimate contact between both fungi was not a major determinant of the antagonistic activity of the *Trichoderma* spp. under study. In addition, in all examined samples, the mycoparasite never penetrated hyphae of *F. o. radicis-lycopersici*. These SEM observations needed, however, to be further complemented by a more accurate investigation of the interaction at the cellular and molecular levels.

Cytochemical localization of *N*-acetylglucosamine residues in fungal cell walls. After incubation of ultrathin sections from single colonies of *F. o. radicis-lycopersici* (Fig. 3A) and *Trichoderma* spp. (Fig. 3B) with the WGA/ovomucoid-gold complex, an intense and specific labeling was observed over hyphal walls. Gold particles appeared preferentially associated with the innermost wall layers (Fig. 3B). Cytoplasm and organelles including nucleus, mitochondria, and lipid bodies were nearly free of labeling.

Observations of ultrathin sections from samples collected at 1-day intervals from different areas of dual cultures showed striking variations in the intensity and distribution of labeling over cell walls of *F. o. radicis-lycopersici* (Figs. 3 and 4). By contrast, from 1 to 9 days after inoculation, no differences in wall labeling were observed over *Trichoderma* as judged by the intense and even distribution of gold particles (Fig. 5C and D).

In the portion of the colony of *F. o. radicis-lycopersici* facing the antagonist, alterations in cell wall labeling were easily discernible within 2 days after inoculation (Fig. 3C). Such alterations usually occurred in well-delineated wall areas (Fig. 3C, arrows) as judged by the intense and regular labeling observed over adjacent wall portions (Fig. 3C, arrowheads). These restricted wall-labeling alterations always were associated with an abundant presence of gold particles in the agar medium (Fig. 3C, double arrows). Labeling also was found to accumulate in both the cytoplasm and the vacuoles (Fig. 3D), which were unlabeled in cells grown in single cultures. At this stage after inoculation, obvious signs of damage at the cellular level were not observed. All cells exhibited a good preservation of their protoplasm, and the occurrence of numerous organelles indicated that they were still metabolically active. Thus, within 2 days after inoculation, changes occurred only at the molecular level without any visible ultrastructural modifications.

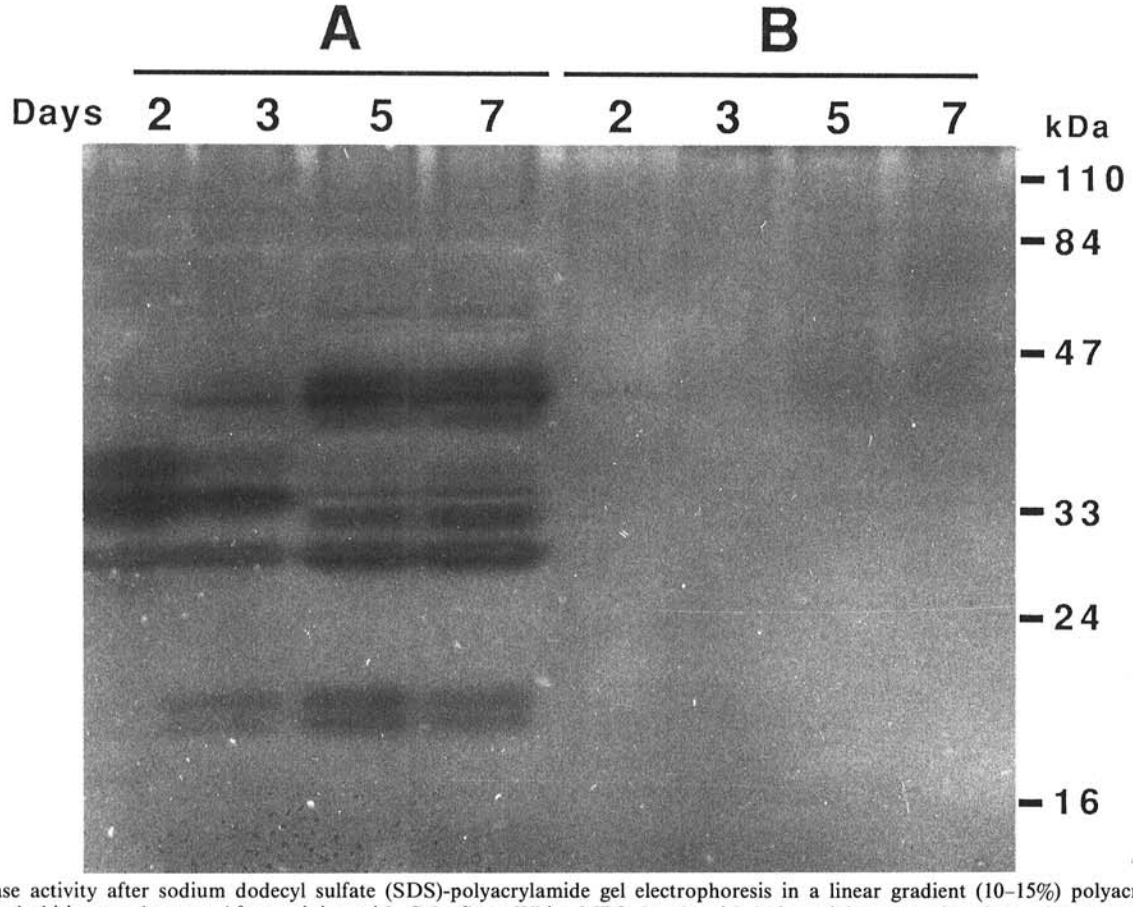

Fig. 1. Chitinase activity after sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis in a linear gradient (10–15%) polyacrylamide gel containing glycol chitin as substrate. After staining with Calcofluor White MR2, bands with lytic activity were visualized with a transilluminator (UV light). Culture supernatant samples that were subjected to SDS-PAGE consisted of: **A,** Supernatant samples collected from cultures of *Trichoderma* containing cell walls of *Fusarium oxysporum* f. sp. *radicis-lycopersici* as carbon source. Samples were collected after 2, 3, 5, and 7 days of growth; **B,** Supernatant samples collected from cultures of *Trichoderma* containing glucose as carbon source. Samples were also collected after 2, 3, 5, and 7 days of growth. Molecular mass markers are indicated on the right.

By 3 days after inoculation, labeling alterations were noticed over the entire cell walls (Fig. 4A) and an increasing number of gold particles was released. Although most cells of *F. o. radicis-lycopersici* displayed an apparently well-preserved cytoplasm (Fig. 4A), some of them appeared depleted of their protoplasm (Fig. 4B). Cytoplasm and organelles were severely altered and the few gold particles located at the periphery of these cells were the only indication of a previously living entity (Fig. 4B). Most fungal cells appeared severely damaged within 4 days after inoculation (Fig. 4C). The main characteristic features of these cells was a partial to complete wall disintegration associated with a release of the cytoplasm content. Scattered gold particles were seen over the surrounding agar medium. From 5 to 7 days after inoculation, cells of *F. o. radicis-lycopersici* exhibited pronounced damage, as judged by the total loss of their protoplasm and their nearly complete wall disintegration (Fig. 4D). In most cases, these cells were reduced to traces that could only be identified by the presence of gold particles in remaining wall debris. At this stage after inoculation, conidia and hyphae of *Trichoderma* were seen in all examined sections and did not show any visible alteration (not shown).

Observations of ultrathin sections from the outside portion of colonies of *F. o. radicis-lycopersici* revealed that alterations in wall labeling started to appear by 3 days after inoculation, thus later than for cells facing the antagonist. From 3 to 9 days after inoculation, wall degradation, cytoplasmic disintegration, and ultimately cell death were similar to those observed with cells of zone A (Fig. 5A and B). Examination of ultrathin sections from samples of colonies of *Trichoderma* either facing the pathogen or located towards the outside revealed an excellent structural preservation of conidia (Fig. 5C) and hyphae (Fig. 5D) during the entire interaction process. The intense and specific wall labeling observed over *Trichoderma* cells from 1 to 9 days after inoculation indicated the absence of wall alterations. Similarly, cytoplasm and organelles did not show any sign of degradation (Fig. 5D). Specificity of the labeling pattern was assessed by the negative results obtained with all control tests including the previous adsorption of the WGA with *N,N′,N″*-triacetylchitotriose (Fig. 5E).

DISCUSSION

Since the initial demonstration that *Trichoderma* sp. produce antifungal substances (31) several investigators have suggested that this fungus may serve as a biocontrol agent against soilborne plant pathogenic fungi (4). During recent years, the possible mechanisms involved in *Trichoderma* antagonism have been studied intensively in terms of antibiotic and enzyme production as well as hyphal interactions (6,7). The ability of *T. harzianum* to produce extracellular β-1,3-glucanases and chitinases into the

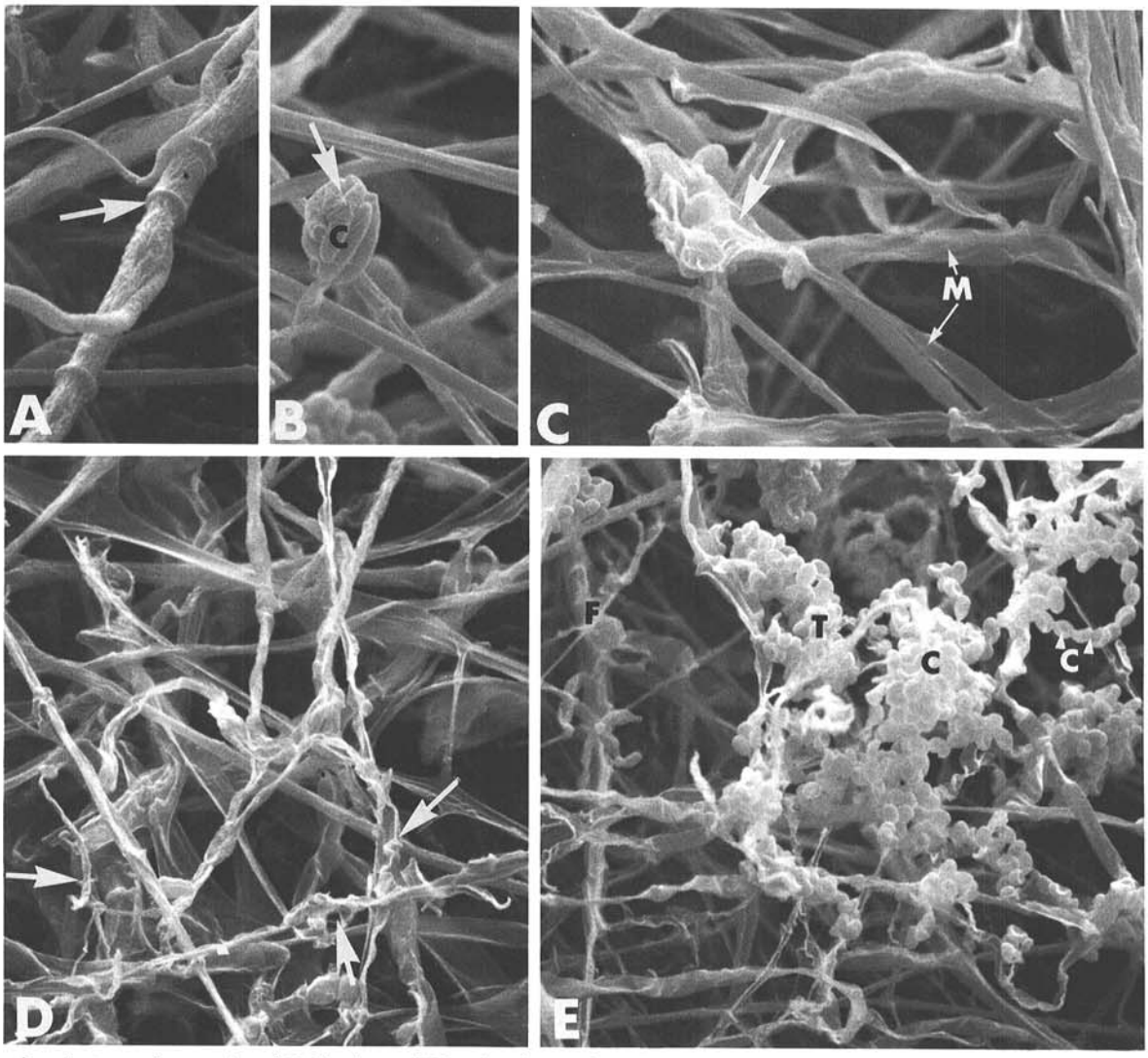

Fig. 2. Scanning electron micrographs of *Trichoderma* (T) hyphae interacting with those of *Fusarium oxysporum* f. sp. *radicis-lycopersici* (F) in dual cultures. **A,** Apparent hyphal coiling is observed (arrow). ×3,000. **B,** Three days after inoculation, alterations of conidia are noticeable (arrow). ×2,500. **C,** From 3 to 5 days after inoculation, pronounced collapse and loss of turgor of mycelial cells of *F. o. radicis-lycopersici* occur (arrow). ×3,000. **D,** As early as 4 days after inoculation, wall breakdown is easily discernible (arrows). ×2,000. **E,** By 7 days after inoculation, *Trichoderma* mycelium, recognizible by the size and shape of conidia (C), overgrows the highly altered mycelium of *F. o. radicis-lycopersici*. ×2,000.

medium when grown in presence of laminarin and chitin (8) led several authors to postulate that the released enzymes were actively involved in microbiological control (4). However, conclusive evidence on the role played by these hydrolases could not be reached in the absence of data concerning precise in situ localization of sites of lytic activity. In an attempt to address this question, Elad et al (7) studied the interaction between *T. harzianum* and some pathogenic fungi by using SEM and fluorescence techniques. Although this study provided strong support for the assumption that *Trichoderma* hydrolases were active determinants in the antagonistic process, it did not allow an accurate demonstration of a lytic activity at the molecular level. The present cytochemical study was undertaken to gain a better insight into the role of chitinases in the outcome of *F.*

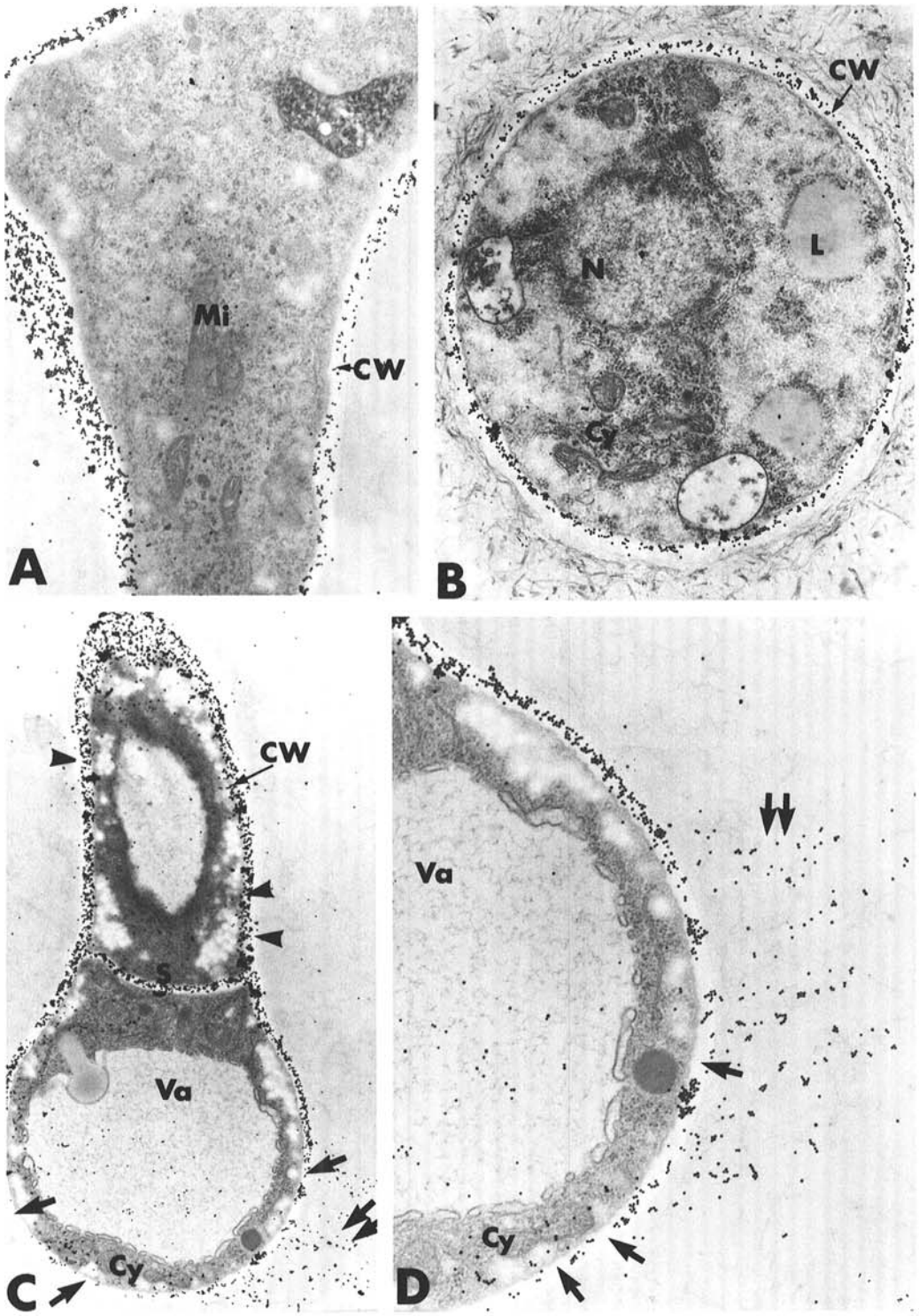

Fig. 3. Transmission electron micrographs of single cultures (A and B) and dual cultures (C and D) between *Trichoderma* and *Fusarium oxysporum* f. sp. *radicis-lycopersici*. Incubation of sections with the WGA/ovomucoid-gold complex. **A,** *F. o. radicis-lycopersici*. An intense and regularly distributed labeling occurs over cell walls (CW). Cytoplasm (Cy) and organelles including mitochondria (Mi) are nearly free of labeling. ×24,000. **B,** *Trichoderma* spp. Gold particles are evenly distributed over cell walls (CW) and preferentially associated with the innermost wall layers (arrow). Cytoplasm (Cy), nucleus (N), lipid bodies (L), and other organelles are devoid of significant labeling. ×24,000. **C and D,** Two days after inoculation in dual cultures. Labeling alterations occur in well-delineated wall areas (arrows). Gold particles are released in the agar medium (double arrows). They occur also in the cytoplasm (Cy) and vacuoles (Va) of a cell of *F. o. radicis-lycopersici*. Labeling is regularly distributed over other wall areas as well as over the septum (S). C. ×20,000. D. ×35,000.

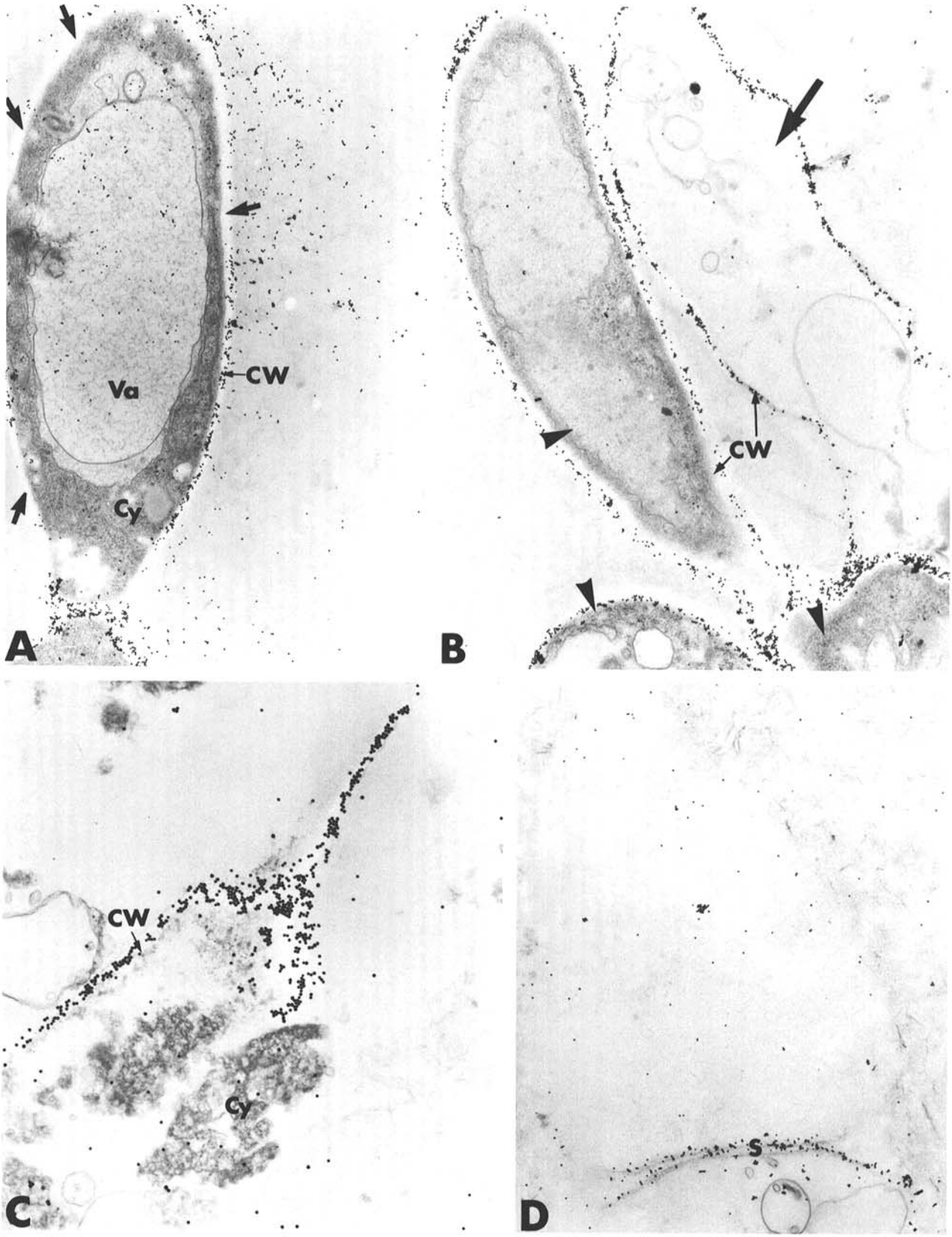

Fig. 4. Transmission electron micrographs of cells of *Fusarium oxysporum* f. sp. *radicis-lycopersici* in dual cultures. Labeling with the WGA/ovomucoid-gold complex. **A,** By 3 days after inoculation, labeling alterations occur over several wall areas (arrows). Numerous gold particles are released outside. Labeling also occurs over both cytoplasm (Cy) and vacuoles (Va). ×24,000. **B,** A cell (arrow) appear depleted of its protoplasm, whereas others (arrowheads) are apparently well preserved. Labeling alterations are visible over some cell wall (CW) areas. ×24,000. **C,** By 4 days after inoculation, cell wall (CW) breakdown is noticeable and is associated with the release of the aggregated cytoplasm (Cy). ×45,000. **D,** From 5 to 7 days after inoculation, cells of *F. o. radicis-lycopersici* are severely damaged. Labeling occurs as scattered gold particles except in some areas, such as the septum. Such cells appear as traces often difficult to distinguish. ×24,000.

o. radicis-lycopersici–Trichoderma interactions. Our results based on ultrastructural observations and cytochemical localization of *N*-acetylglucosamine residues provide indirect evidence for a major chitinolytic activity of *Trichoderma* and indicate that production of this enzyme may have a direct significance in the parasitism of *Trichoderma* on *F. o. radicis-lycopersici.*

The recent introduction of detection techniques for chitinase activity in gels (30) has opened new avenues for an accurate evaluation of enzyme production in various biological systems. In the present investigation, such techniques were used to identify the chitinases produced by the isolate of *Trichoderma* under study. As pointed out by Trudel and Asselin (30), glycol chitin, when

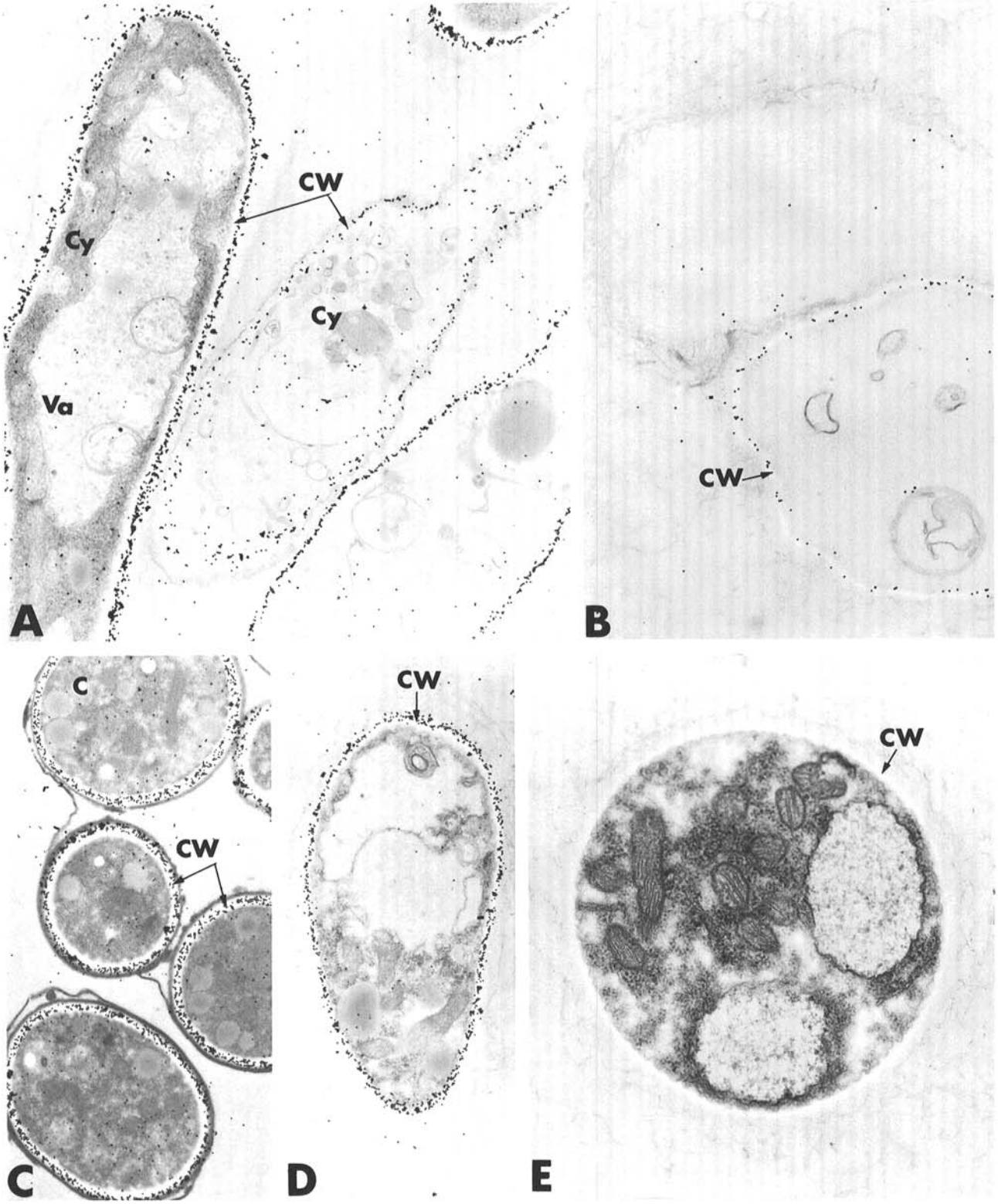

Fig. 5. Transmission electron micrographs of cells of *Fusarium oxysporum* f. sp. *radicis-lycopersici* (A and B) and of *Trichoderma* cells (C-D) in dual cultures. **A,** Three days after inoculation, alterations in wall labeling start to be visible. Some cells show an intense cytoplasm (Cy) disintegration. ×24,000. **B,** Cell wall (CW) breakdown as well as protoplasm disintegration are frequently observed. ×30,000. **C and D,** Conidia (C) and hyphae (D) of *Trichoderma* display an excellent ultrastructural preservation even 7 days after inoculation. Cell wall (CW) labeling is intense and regularly distributed. C. ×24,000; D. ×24,000. **E,** Control test. Preadsorption of WGA with *N,N',N''*-triacetylchitotriose before section labeling results in the absence of gold particles over the wall of a *Trichoderma* cell grown in single culture. ×24,000.

incorporated to the gels, was found to be the ideal substrate for chitinases because of its solubility in water.

Analysis of the pattern of chitinase activity revealed that the isolate of *Trichoderma* was able to excrete several chitinases when grown on cell walls of the pathogenic fungus. Our time course study showed that some chitinases were produced early while others were gradually excreted. In recent years, it has been convincingly demonstrated that chitin was enzymatically vulnerable and that, in turn, degradation products could have diverse effects among which the induction of chitinase production (23). In view of these considerations, one can speculate that the isolate of *Trichoderma* under study first produces the amount and the type of enzymes necessary for the release of some chitin fragments which, in turn, may act as signaling substances capable of mediating the synthesis of new lytic enzymes that would account for a complete pathogen wall disintegration. Although evidence was presented that the isolate of *Trichoderma* under study displayed the ability to produce chitinases, these biochemical data needed to be supported by further investigations of the spatial and temporal distribution of chitin in cells of *F. o. radicis-lycopersici* during pathogen-antagonist interaction.

The time-course investigation study of fungal development in dual cultures clearly showed that the isolate of *Trichoderma* under study caused inhibition of growth of the colony of *F. o. radicis-lycopersici* even before contact between the hyphae occurred. Estimation of growth inhibition was made visually by comparing daily the extent of colonies of *F. o. radicis-lycopersici* in single vs. dual cultures. In agreement with previous reports (6,26), this observation supports the idea that diffusible substances may be active in advance of the fungus itself.

SEM investigations of *F. o. radicis-lycopersici–Trichoderma* interaction at different times after inoculation showed that inhibition of growth of the host mycelium did not result from hyphal penetration by *Trichoderma*. Instead, the noticeable wall alterations together with the rapid collapse and loss of cell turgor of *F. o. radicis-lycopersici* in areas where *Trichoderma* was not in direct contact with the host mycelium provided supporting evidence to the concept that extracellular metabolites could be responsible for the observed degradation events. These observations are in line with those reported earlier by Dennis and Webster (6) on *Trichoderma*-fungal pathogens interactions. The intense labeling noted over the walls of *F. o. radicis-lycopersici* after treatment with the WGA 1 ovomucoid complex indicates that *N*-acetylglucosamine residues are one of the major components of the wall structure. This observation is in agreement with the well-documented occurrence of such polysaccharides in cell walls of Ascomycetes (9). Since chitin is a polymer of interlinked *N*-acetylglucosamine residues, it is likely that WGA binding sites are associated with chitin (2,11).

TEM observations of sections from *F. o. radicis-lycopersici* in dual cultures revealed that local alteration of chitin macromolecules in mycelium was an early event preceding visible wall disruption and leakage of cytoplasm. The observation that, as early as 48 hr after inoculation, *N*-acetylglucosamine residues were released in the growing medium, correlates well with the idea that wall-bound chitin may be locally hydrolyzed by extracellular chitinases produced by *Trichoderma*. Whereas the alteration of chitin occurred first in well-delineated wall areas, it appeared to be more generalized later on, thus indicating an increased excretion of chitinase by *Trichoderma*. This assumption is supported by our biochemical data, which clearly showed a considerable increase in chitinase activity from 2 to 7 days after contact between the isolate of *Trichoderma* and cell walls of *F. o. radicis-lycopersici*. Whether or not chitin oligomers released from the host cell walls may stimulate enzyme biosynthesis in a way similar to fungal elicitors of plant hydrolases (22) remains to be further investigated.

If one considers that most fungal cell walls are mainly composed of chitin and β-1,3-glucans embedded in a matrix of amorphous material (9), it is to be expected that successful wall degradation may result from the activity of more than one enzyme. In most fungi, including *F. o. radicis-lycopersici* (3), chitin is buried in β-glucans, thus not readily accessible to chitinase. It is, thus, likely that chitinase activity is preceded by or coincides with the hydrolytic activity of other enzymes, especially β-1,3-glucanase. In *Schizophyllum commune*, it has been convincingly demonstrated that the synergistic action of both chitinase and β-1,3-glucanase was required for cell wall lysis (25). Similarly, in a recent study, Mauch et al (20) found that plant chitinases and β-1,3-glucanases worked in concert in the inhibition of fungal growth. However, the possibility that other enzymes including proteases and lipases may also be involved in the wall degradation process has to be taken into consideration. In a recent work, Sivan and Chet (27) reported the failure of two isolates of *T. harzianum* to effectively degrade *F. o. vasinfectum* and *F. o. melonis*, although they were able to excrete chitinase and β-1,3-glucanase into the medium when grown in liquid cultures containing chitin and laminarin. The authors suggested that some proteins and lipids might hinder access of chitinase and β-1,3-glucanase to their corresponding substrates. In light of these observations, it can be speculated that the coordinated action of polysaccharidases, lipases, and proteases is an important determinant in the antagonistic process.

In summary, evidence has been presented in this study that a strain of *Trichoderma*, isolated from a sample of peat collected in New Brunswick, is capable of producing several chitinases that likely are involved in chitin breakdown of the pathogenic fungus *F. o. radicis-lycopersici*. However, it is likely that a coordinated action of chitinases and β-1,3-glucanases is a prerequisite to an effective wall disruption and dissolution. It will now be interesting to evaluate the synergistic action of isolated and purified chitinases and β-1,3-glucanases on the outcome of *F. o. radicis-lycopersici*.

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