

Parasitism of Sclerotia of *Sclerotium rolfsii* by *Trichoderma harzianum*: Ultrastructural and Cytochemical Aspects of the Interaction

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ABSTRACT

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The interaction between *Trichoderma harzianum* and sclerotia of the soilborne plant pathogen *Sclerotium rolfsii* was studied by scanning and transmission electron microscopy (SEM and TEM, respectively) to assess the potential role of enzymatic hydrolysis in the antagonistic process. SEM investigations revealed that hyphae of *T. harzianum* grew abundantly on the sclerotial surface and formed a dense branched mycelium that appeared to establish contact with the outer host cells through a thin mucilage. Observation of cross-sections of parasitized sclerotia by light microscopy showed that hyphae of the antagonist multiplied on the sclerotial surface and displayed the ability to penetrate the rind. Growth of the antagonist in the rind layer was mainly intracellular, and host-wall penetration was achieved by means of constricted hyphae. Ultrastructural observations showed that *Trichoderma* growth and development coincided with extensive host cell alterations, such as retraction and aggregation of the cytoplasm and vacuole breakdown. In the invaded outer rind cells, host cell walls apparently were not altered, as judged by their preserved structure. In contrast, cell breakdown due to host cell-wall disruption was observed more frequently in inner rind cells adjacent to medullary cells. Ingress of *T. harzianum* hyphae in the medulla was characterized mainly by a change in the mode of growth from intra-

intercellular. *Trichoderma* hyphae did not penetrate the medullary cells, although the latter showed pronounced alterations, such as cytoplasm disorganization and aggregation. The use of wheat germ agglutinin/ovomucoid-gold complex for localization of chitin monomers resulted in regular labeling of both host and antagonist cell walls, even when sclerotia were massively colonized. Chitinolytic degradation at a distance from the site of *Trichoderma* penetration was never observed. There was no indication of cell-wall disorganization in either the host or the antagonist, as shown by the regular distribution of labeling, even in zones of penetration by constricted *Trichoderma* hyphae. In the medulla, gold labeling was regularly distributed over the thick host cell walls, even when the medullary cells showed obvious signs of disorganization. When ultrathin sections of parasitized sclerotia were incubated with the gold-complexed β -1,3-glucanase for localization of β -1,3-glucans, a regular distribution of gold particles was observed over the walls of both outer and inner rind cells, even when these exhibited a disorganized cytoplasm that was reduced to a few remnants of aggregated material. Incubation with gold-complexed lipoprotein lipase yielded a pattern of labeling similar to that obtained with gold-complexed β -1,3-glucanase. Gold particles were evenly distributed over the host cell walls in the rind layer.

Additional keywords: antibiosis, biological control, gold cytochemistry, hydrolytic enzymes.

In the past few years, biocontrol research on rhizosphere-specialized microorganisms has been greatly influenced by the idea that biocontrol agents could be a potential alternative to the use of chemicals for management of plant diseases caused by soilborne pathogens (9,10,16,26). Since the pioneering work of Weindling and Fawcett (33) on the use of *Trichoderma* strains to control damping-off caused by *Rhizoctonia solani* in citrus, considerable attention has been focused over the last 20 years on the isolation and identification of fungal antagonists that could be as effective as chemicals in repressing fungal pathogens (7,29). Today, there is accumulating evidence that *Trichoderma* spp., which are easily isolated from soil and readily grown (9), are among the most promising biocontrol agents in terms of large-scale applications.

Sclerotium rolfsii Sacc., a septate filamentous fungus, remains one of the most widely distributed and destructive pathogens of several economic crops (1,30). Management of the diseases caused

by this soilborne plant pathogen with chemical measures has proved impractical, mainly because the fungus displays the ability to survive in soil through the formation of dark-brown spherical sclerotia that have strong resistance to both chemical and biological degradation (11,31). As a result, research scientists and industries have focused on developing new strategies that can provide safe and reliable means for improving crop protection against plant infection by *S. rolfsii* (20,28). Interest in the potential of *Trichoderma* spp. to control sclerotial fungi has increased steadily since the first demonstration by Wells et al. (34) that prior infestation of the soil with *T. harzianum* Rifai grown on an autoclaved mixture of ryegrass seeds and soil resulted in a significant reduction of the symptoms caused by *S. rolfsii*.

Laboratory experiments (17,18) as well as biocontrol field trials (20,27) have shown convincingly that some species of *Trichoderma* display the ability to attack the mycelium and sclerotia of *S. rolfsii*, reducing pathogen inoculum in soil. However, the exact mechanisms by which *Trichoderma* spp. contribute to biological control of *S. rolfsii* are not understood fully, although a growing body of evidence from a number of reports indicates that mycoparasitism involving cell-wall hydrolytic enzymes accounts for the observed process (17,18,25).

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The remarkable ability of sclerotia to survive for long periods in soil has been associated with the occurrence of an outer cell layer, the rind, composed of empty melanized cells (8). The absence of the melanin-like pigment in hyphae of *S. rolfsii* led Chet et al. (12) to suggest that this compound is involved in the resistance of sclerotia to chemical and biological degradation. However, the finding that *T. harzianum* can penetrate the rind and colonize the inner cell layers of sclerotia (17) provides support for the concept that the extraordinary ability of *T. harzianum* to attack resistant resting structures is related to the synergistic action of lytic enzymes, including proteases, lipases, glucanases, and chitinases. Although both possibilities may not be mutually exclusive, the uncertainty concerning the exact mode of action of *T. harzianum* suggested that further information on the sequence of events taking place during the parasitism of sclerotia of *S. rolfsii* was needed. One approach toward this end was a thorough in situ analysis of the relative contribution of enzymatic hydrolysis during the antagonistic process.

Using scanning and transmission electron microscopy (SEM and TEM, respectively), the objectives of the present study were to study the cytology of parasitism in terms of both the potential benefit and disadvantage to the antagonist and host, respectively, and to delineate the potential role of enzymatic hydrolysis by following the pattern of chitin and β -1,3-glucan labeling in the sclerotial cell walls with highly specific gold-complexed probes.

MATERIALS AND METHODS

Fungal isolates and growth conditions. The isolate of *T. harzianum* (T-203) used in this study was isolated by Elad et al. (20) and is as an effective mycoparasite of *R. solani* and *S. rolfsii* (19). It was grown on potato dextrose agar (PDA) at 23 to 25°C. The type A strain of *S. rolfsii*, ATCC 26325 (11), was grown either on PDA or a synthetic medium (28) at 24 \pm 1°C. Sclerotia were collected from 4- to 5-week-old culture plates.

Dual culture tests. The interaction between sclerotia of *S. rolfsii* and hyphae of *T. harzianum* was studied according to the following procedure. Mycelial plugs (5 mm diameter) collected from actively growing colonies of both fungi were placed 3 cm apart on the surface of the agar and allowed to grow at 25°C under continuous light. The antagonist and its host grew toward each other, and overgrowth of *S. rolfsii* mycelium by hyphae of *T. harzianum* occurred by 4 to 5 days after inoculation. Two weeks later macroscopic observations of the plates showed that the antagonist multiplied abundantly and established close contact with sclerotia of the pathogen. Parasitized sclerotia were carefully removed and processed for SEM and TEM.

SEM studies. Sclerotia of *S. rolfsii* were vapor-fixed with 2% (wt/vol) aqueous osmium tetroxide for 20 h at room temperature, air-dried, and sputter-coated with gold palladium in a Polaron E 500 sputter coater Nanotech, Sempreps, England. The sclerotia were kept in a dessicator until examination with a Cambridge Stereoscan S-150 scanning electron microscope (Cambridge Scientific Industries, Cambridge, MA) operating at 20 kV. Micrographs were taken on Polaroid type 52 film (Polaroid Corp., Cambridge, MA) with UV haze and orange filters. The experiment was repeated twice. For each replicate, 10 sclerotia were examined under SEM.

TEM studies. Ten sclerotia of *S. rolfsii*, collected either from pure cultures (single-culture tests) or dual-culture tests were cut into two halves and immediately fixed by immersion in 3% (vol/vol) glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2, for 2 h at room temperature. Samples were postfixed with 1% (wt/vol) osmium tetroxide in the same buffer for 1 h at 4°C and dehydrated in a graded ethanol series prior to being embedded in Epon 812. Thin sections (0.7 μ m) 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, Thornwood, NY). Ultrathin sections (0.1 μ m) collected on nickel grids were either contrasted with uranyl acetate and lead citrate for immediate examination with a JEOL 1200 EX transmission electron microscope (JEOL, Tokyo) operating at 80 kV or further processed for cytochemical labeling. For each sclerotium sampled, about 15 ultrathin sections were examined under the electron microscope.

Preparation of gold-complexed probes. Colloidal gold with particles averaging 12 nm in diameter was prepared according to Frens (21) with sodium citrate as a reducing agent. 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 (3). Because of its low molecular weight, this lectin could not be complexed directly to colloidal gold; it was used in a two-step procedure with ovomucoid as a second-step reagent. Ovomuroid was conjugated to gold at pH 5.4 (3). A β -1,3-glucanase, purified from tobacco reacting hypersensitively to tobacco mosaic virus, was used for localizing β -1,3-glucans according to a recently described method (4). The enzyme was conjugated to colloidal gold at pH 5.5. A lipoprotein lipase from wheat germ (Sigma Chemical Company, St. Louis) was complexed to colloidal gold at pH 5.2 and used for localizing lipids (5).

Cytochemical labeling. Labeling with gold-complexed β -1,3-glucanase and lipoprotein lipase was performed by incubating the ultrathin root sections for 5 to 10 min on a drop of 0.01 M sodium phosphate-buffered saline (PBS) containing 0.02% of polyethylene glycol 20,000 (PEG 20,000) (PBS-PEG) at the pH corresponding to optimal protein activity (6.0 for both enzymes). Sections were transferred to a drop of the protein-gold complex for 30 min at room temperature in a moist chamber. After careful washing with PBS, pH 7.2, and rinsing with distilled water, sections were contrasted with uranyl acetate and lead citrate and observed with the JEOL 1200 EX transmission electron microscope.

For indirect labeling of chitin, sections were incubated on a drop of PBS, pH 7.2, transferred to a drop of WGA diluted 1:30 in PBS, pH 7.2, and incubated on a drop of the ovomucoid-gold complex diluted 1:20 in PBS-PEG, pH 6.0 (26). Sections were contrasted as described above.

Cytochemical controls. Specificity of the different labelings was assessed by the following control tests:

Direct labeling: (i) addition of the corresponding substrate to each protein-gold complex for a competition experiment: laminarin (1 mg ml⁻¹) for β -1,3-glucanase and triacetin (1 mg ml⁻¹) for lipoprotein lipase; (ii) substitution of the protein-gold complex under study by bovine serum albumin-gold complex to assess the nonspecific adsorption of the protein-gold complex to the tissue sections; (iii) incubation of the sections with the protein-gold complexes under nonoptimal conditions for biological activity; and (iv) incubation of the sections with colloidal gold alone to assess the nonspecific adsorption of the gold particles to the tissue sections.

Indirect labeling: (i) incubation with WGA to which was previously added an excess of *N*-*N*'-*N*''-triacetylchitotriose (1 mg ml⁻¹ in PBS); (ii) incubation with WGA followed by unlabeled ovomucoid and ovomucoid-gold complex; and (iii) incubation with stabilized or unstabilized gold suspension.

Reagents. WGA, ovomucoid, and lipoprotein lipase were purchased from Sigma. PDA was obtained from Difco Laboratories (Detroit), and tetrachloroauric acid was purchased from BDH Chemicals (Montreal). All other reagents for electron microscopy were obtained from JBEM Chemical Company (Pointe-Claire, Québec).

RESULTS

SEM observations. Using SEM, nonparasitized sclerotia of *S. rolfsii* appeared as spherical structures characterized by their rough surface, on which marked depressions were easily discernible (Fig.

1A). At a higher magnification, the sclerotial surface had a pitted, fractured appearance due to pronounced collapse and loss of turgor of the outermost rind cells (Fig. 1B). Colonization by *T. harzianum* apparently did not affect the size and shape of sclerotia (Fig. 1C). A close examination of these parasitized sclerotia revealed that hyphae of the antagonist grew abundantly on the sclerotial surface (Fig. 1D) and formed a dense branched mycelium that appeared to establish contact with the outer host cells through a thin mucilage readily observed in most samples studied (Fig. 1D, arrowhead). Features of potential host penetration between the collapsed rind cells were seen frequently (Fig. 1D, arrows).

Light microscope observations. At the light microscope level, examination of cross-sections of nonparasitized sclerotia showed that these structures were composed of two main layers: the rind, composed of darkly pigmented cells, and the medulla, in which cells were multishaped and apparently covered by a very thick wall-like envelope (Fig. 2A). The outermost rind cells were usually empty, collapsed, and sometimes broken, whereas the inner cells had a regular shape and contained vesicular bodies that stained densely with toluidine blue (Fig. 2A). The medullary cells consisted of

interwoven hyphae filled with granules showing strong staining, each surrounded by a thick wall that was itself surrounded by an amorphous matrix that often formed a bridge between hyphal cells (Fig. 2B).

Observation of cross-sections of parasitized sclerotia revealed that hyphae of the antagonist multiplied abundantly on the sclerotial surface and displayed the ability to penetrate the rind. Hyphal cells of *T. harzianum* ramified extensively through much of the rind and radiated in the medullary region (Fig. 2C). Growth of the antagonist in the rind layer was mainly intracellular, and host-wall penetration was achieved by means of constricted hyphae (Fig. 2C, arrows). Colonization of the medulla appeared to proceed via an intercellular mode of growth, because hyphae of the antagonist were never seen inside host cells (Fig. 2D). These observations by light microscopy could not clearly demonstrate whether the massive colonization by the antagonist was associated with host cell-wall alterations.

Ultrastructural observations. A more detailed picture of the structural organization of sclerotia was obtained through TEM observations of ultrathin sections. Examination of nonparasitized

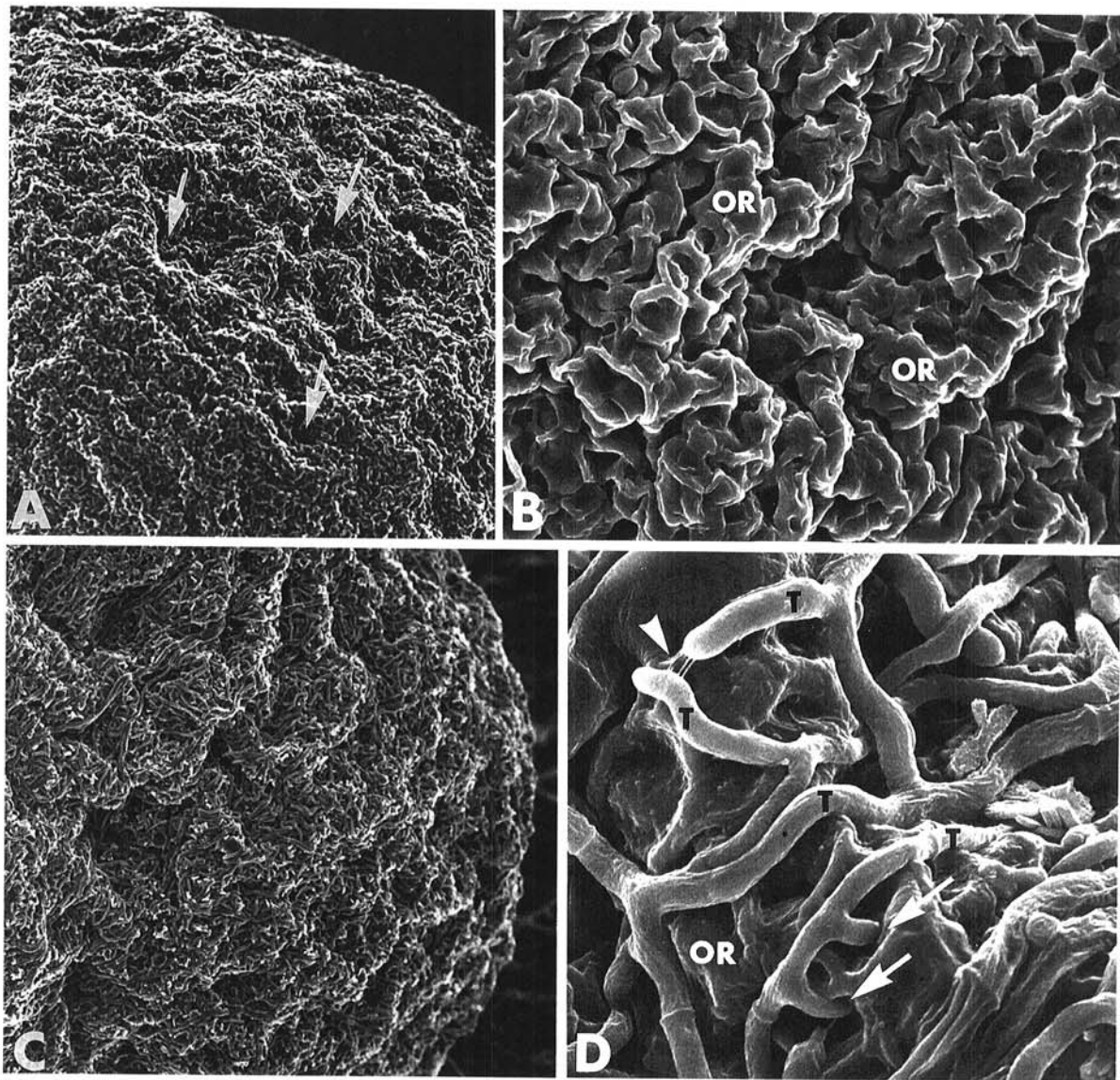


Fig. 1. Scanning electron micrographs of sclerotia of *Sclerotium rolfsii*. **A and B**, Nonparasitized sclerotium. **A**, The sclerotium appears as a spherical structure characterized by its rough surface on which marked depressions are visible (arrows). 200 \times . **B**, At a higher magnification, the sclerotial surface has a pitted, fractured appearance due to pronounced collapse and loss of turgor of the outer rind cells (OR). 1,000 \times . **C and D**, Sclerotium parasitized by hyphae of *Trichoderma harzianum* (T). **C**, Hyphae of the antagonist grow abundantly on the sclerotial surface. 200 \times . **D**, At a higher magnification, the branched mycelium of the antagonist (T) appears to establish contact with the outer rind cells (OR) through a thin mucilage (arrowhead). Features of potential host penetration between the collapsed rind cells are seen (arrows). 2,000 \times .

sclerotia revealed that the rind layer was four to five cells thick in places. The outermost rind cells were characterized by their irregular shape and broken appearance. Most of these cells were empty and collapsed, whereas others contained aggregated remnants of cytoplasm (Fig. 3B). Such cells were always surrounded by very

thick, electron-dense cell walls. Rind cells beneath the outermost, empty cells differed in terms of protoplast density. Cells adjacent to the peripheral cell layer usually had a dense cytoplasm in which small, polymorphic vacuoles and electron-opaque inclusions were the only discernible structures (Fig. 3B). The vacuolar content was

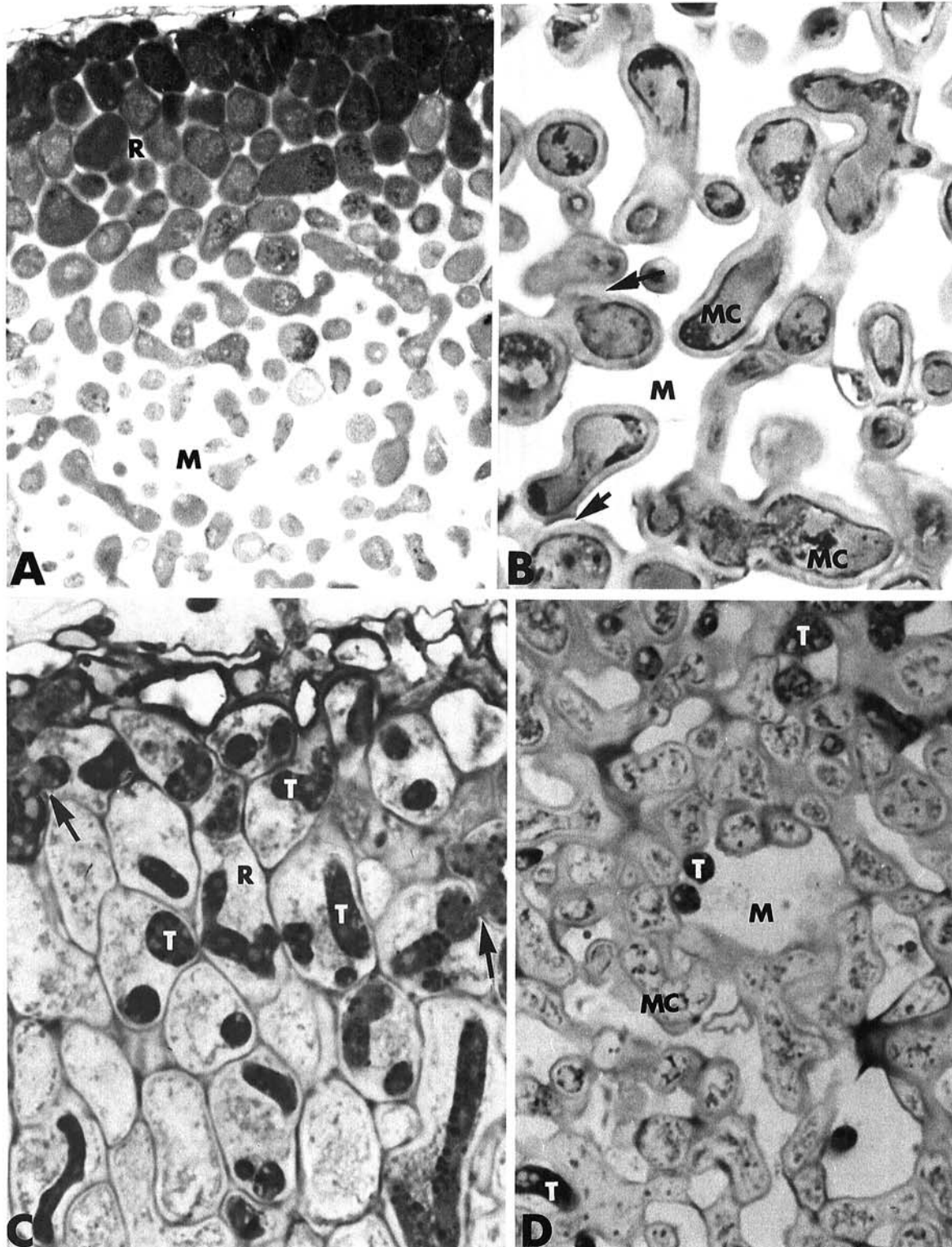


Fig. 2. Light microscope photographs of sclerotia of *Sclerotium rolfsii*. **A and B,** Nonparasitized sclerotium. **A,** The sclerotium is composed of two main layers: the rind (R), composed of darkly pigmented cells, and the medulla (M), in which the multishaped cells are loosely organized. 1,000 \times . **B,** Cells in the medulla (M) are filled with intensely stained granules and are surrounded by a thick wall that is surrounded by an amorphous matrix that forms a bridge between the medullary cells (MC) (arrows). 2,500 \times . **C and D,** Sclerotium parasitized by hyphae of *Trichoderma harzianum*. **C,** Hyphae of the antagonist (T) multiply abundantly throughout much of the rind (R) by an intracellular mode of growth. Host wall penetration is achieved by constricted hyphae (arrows). 2,000 \times . **D,** Colonization of the medulla (M) by the antagonist (T) proceeds via an intercellular mode of growth. 2,000 \times .

composed mostly of a fine granular ground matrix. Density of the cytoplasm decreased in inner rind cells that also contained numerous vacuoles and darkly stained inclusions (Fig. 3A). A significant decrease in the electron density of the cell walls also was observed. This change in the wall appearance revealed the presence

of an electron-opaque middle lamella at the junction between contiguous cells (Fig. 3A). Medullary cells were easily recognizable by their loosely arranged organization (Fig. 3C). Such polymorphic cells were surrounded by a thick electron-transparent wall connected in places to a fibrillar matrix that formed a bridge

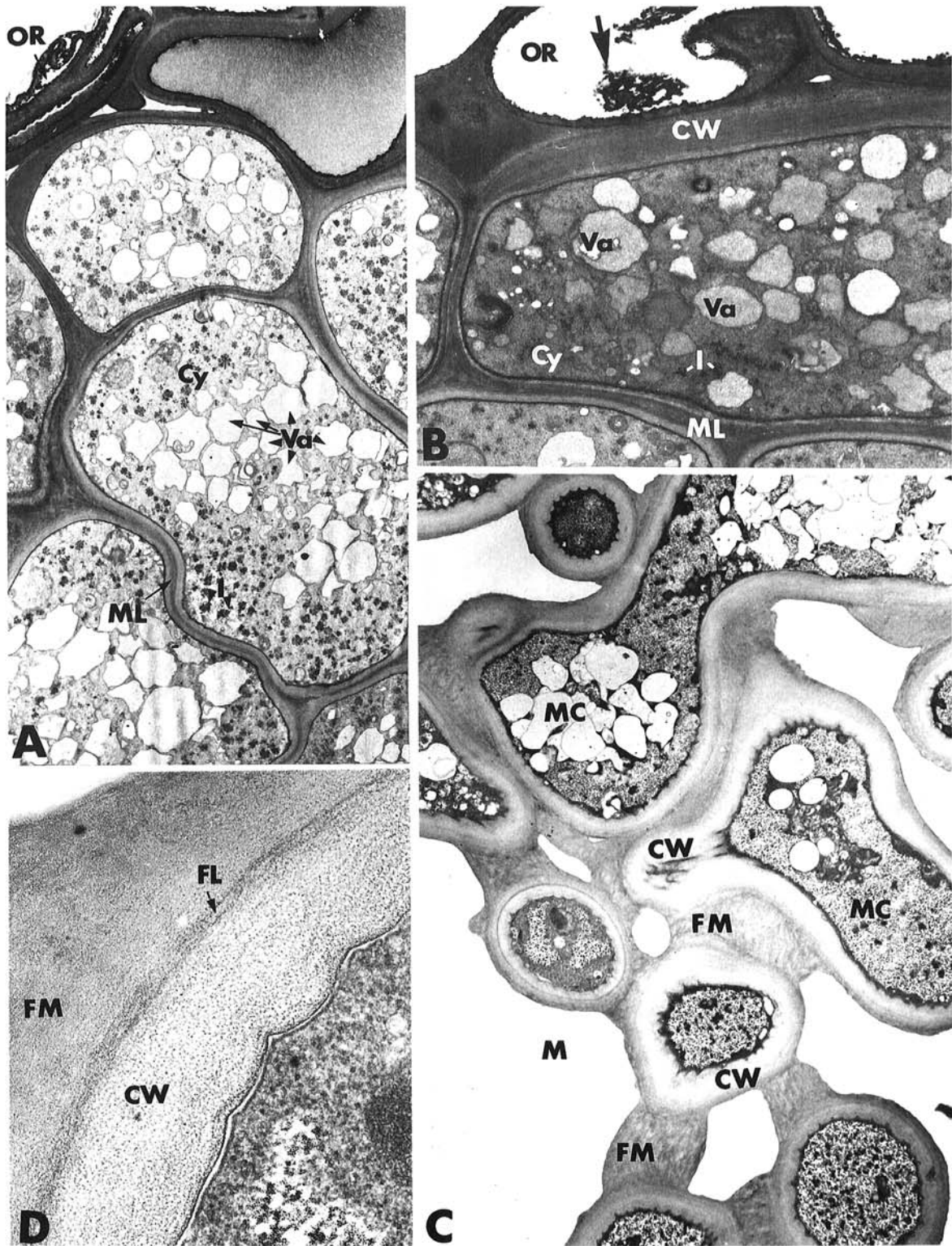


Fig. 3. Transmission electron micrographs of nonparasitized sclerotia of *Sclerotium rolfsii*. **A and B**, Rind. **A**, The outer rind cells (OR) are characterized by their irregular shape and broken appearance. Inner rind cells contain a regular cytoplasm (Cy) in which small, polymorphic vacuoles (Va) and electron-opaque inclusions (I) are visible. The walls of contiguous cells are separated by an electron-opaque middle lamella (ML). 5,000X. **B**, Some outer rind cells (OR) contain aggregated remnants of cytoplasm (arrow). Such cells are surrounded by a very thick, electron-dense cell wall (CW). Rind cells beneath the outer cells contain a dense cytoplasm (Cy) in which small vacuoles (Va) and electron-opaque inclusions (I) are seen. 8,000X. **C and D**, Medulla (M). **C**, Medullary cells (MC) are characterized by their loosely arranged organization. These polymorphic cells are surrounded by a thick, electron-transparent wall (CW) connected in places to a fibrillar matrix (FM), which forms a bridge between neighboring cells (arrows). 5,000X. **D**, The interface between the wall (CW) and the fibrillar matrix (FM) shows that both structures are separated by a thin, electron-opaque fibrillar layer (FL). 50,000X.

between neighboring cells (Fig. 3C). A close examination of the interface between the wall and the fibrillar matrix showed that both structures were separated by a thin, electron-opaque fibrillar layer (Fig. 3D).

Examination of numerous sections from parasitized sclerotia showed that hyphae of *T. harzianum* had ramified abundantly through much of the rind cells and reached the medulla by centripetal growth (Fig. 4). Fungal growth mainly was intracellular in the rind layer, and cell invasion occurred through localized host-wall penetration (Fig. 4A and B). Channels of penetration were

much narrower than the hyphal diameter and usually were associated with little wall displacement in the growth direction (Fig. 4A and C, arrows). *Trichoderma* ingress toward the medulla coincided with extensive cell alterations, such as retraction and aggregation of the cytoplasm and vacuole breakdown (Fig. 4C and D). In the invaded outer rind cells, host cell walls apparently were not altered, as judged by their structural integrity (Fig. 4D). In contrast, cell breakdown due to host cell-wall disruption was observed more frequently in inner rind cells adjacent to medullary cells (Fig. 5A and B). In some cases, the wall at the junction be-

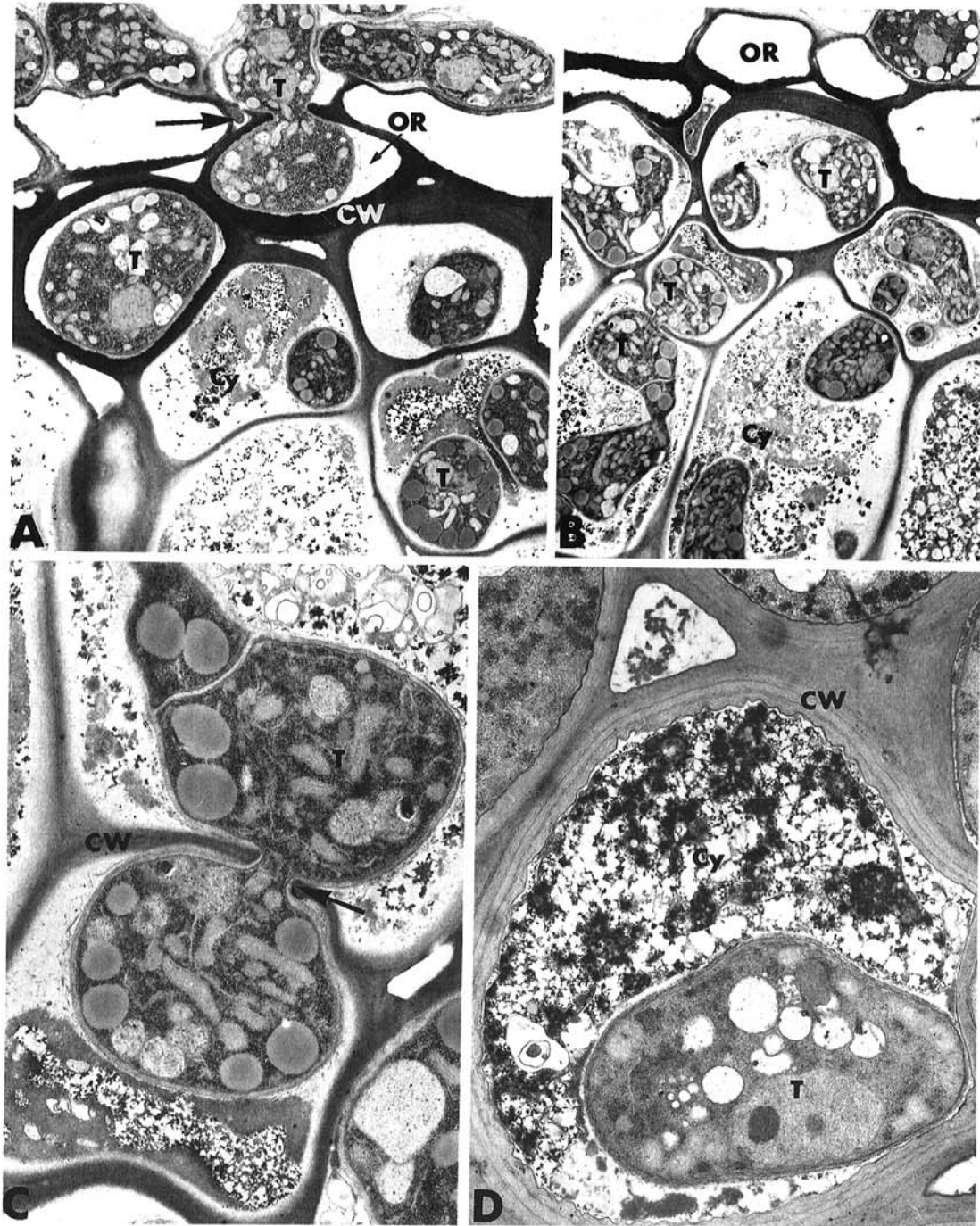


Fig. 4. Transmission electron micrographs of sclerotia of *Sclerotium rolfii* parasitized by *Trichoderma harzianum* (T). **A and B,** Outer rind cells. **A,** Invasion of the outer rind cells (OR) through direct penetration of the electron-dense cell walls (CW) (arrow). 5,000 \times . **B,** *Trichoderma* (T) growth is mainly intracellular and coincides with extensive cell alterations, such as retraction and aggregation of the cytoplasm (Cy) and vacuole breakdown. 4,000 \times . **C and D,** Inner rind cells. **C,** Cell invasion by *Trichoderma* (T) hyphae occurs through localized host-wall (CW) penetration. The channel of penetration is much narrower than the hyphal diameter (arrow). 15,000 \times . **D,** The host cell wall is not apparently altered, as judged by its structural integrity. 10,000 \times .

tween two adjacent cells was no longer discernible, and leakage of cytoplasmic remnants from one cell to another was seen (Fig. 5B). Ingress of *T. harzianum* hyphae in the medulla was characterized mainly by a change in the mode of growth from intra- to intercellular (Fig. 5C). Except in the area surrounding the rind (Fig. 5C,

arrow), *Trichoderma* hyphae usually did not penetrate the medullary cells, although these latter showed pronounced alterations, such as cytoplasm disorganization and aggregation. Growth of the antagonist apparently proceeded through partial degradation of the fibrillar matrix connecting medullary cells (Fig. 5D, arrowheads).

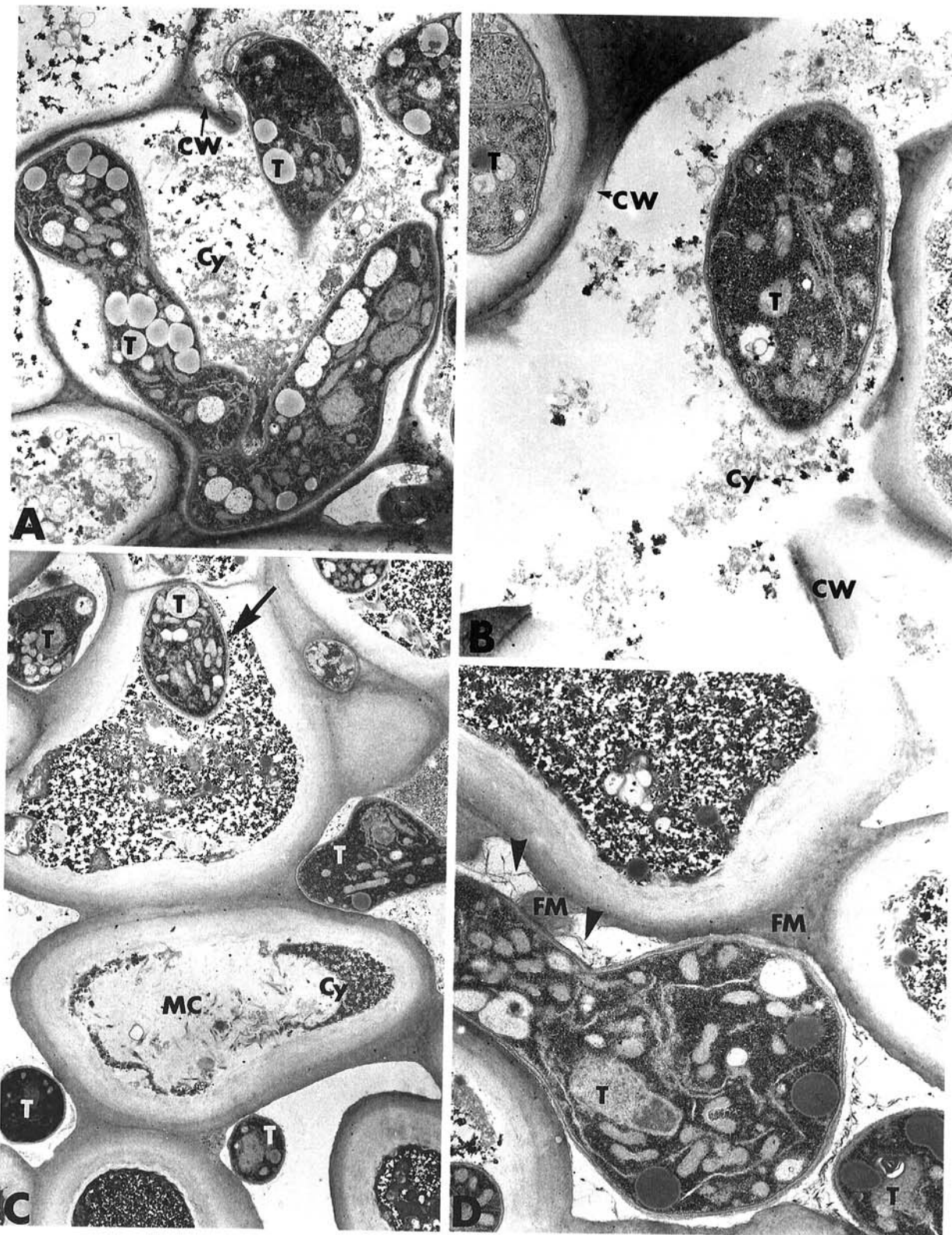


Fig. 5. Transmission electron micrographs of sclerotia of *Sclerotium rolsfii* parasitized by *Trichoderma harzianum* (T). **A and B**, Inner rind cells adjacent to medullary cells. **A**, Cell breakdown due to local host cell-wall (CW) disruption is observed. The cytoplasm (Cy) is disintegrated and reduced to a few granules. 8,000X. **B**, Marked host cell-wall disruption leads to cytoplasm leakage. 10,000X. **C and D**, Medullary cells. **C**, *Trichoderma* (T) hyphae do not penetrate the medullary cells directly (MC), although the latter show pronounced alterations, such as cytoplasm (Cy) disorganization and aggregation. The cell wall (CW) apparently is not altered. 5,000X. **D**, Growth of the antagonist (T) apparently proceeds through partial degradation of the fibrillar matrix (FM) connecting medullary cells (arrowheads). 10,000X.

Cytochemical observations. Incubation of ultrathin sections of parasitized sclerotia with the WGA/ovomucoid-gold complex for localization of chitin monomers resulted in regular labeling of both host and antagonist cell walls (Fig. 6), even when sclerotia were massively colonized. As mentioned above, invasion of the rind by

hyphae of *T. harzianum* proceeded via the formation of narrow channels of penetration (Fig. 6A). In spite of this, the structural integrity of the electron-dense outermost rind cell wall was maintained, and gold particles were evenly distributed, even in areas in close contact with the antagonist (Fig. 6A). This phenomenon was

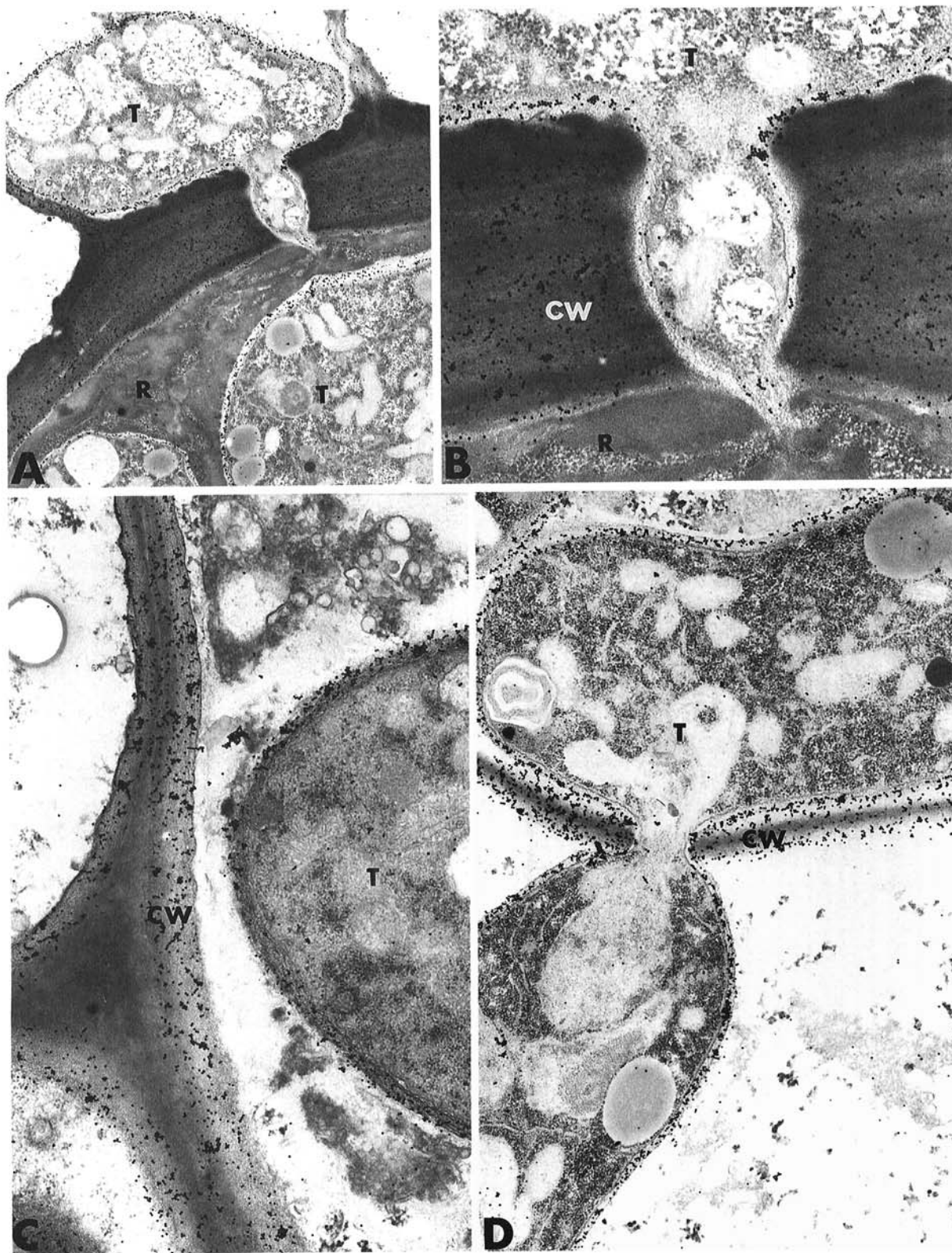


Fig. 6. Transmission electron micrographs of sclerotia of *Sclerotium rolfsii* parasitized by *Trichoderma harzianum* (T). Chitin is labeled with the wheat germ agglutinin/ovomucoid-gold complex. **A**, Invasion of the rind (R) by hyphae of *T. harzianum* (T) proceeds via the formation of narrow channels of penetration. 10,000 \times . **B**, Higher magnification shows that the structural integrity of the electron-dense outermost rind cell wall (CW) is preserved and gold particles are distributed evenly, even in areas of close contact with the antagonist (T). 30,000 \times . **C and D**, In inner rind cells massively invaded by hyphae of *T. harzianum* (T), labeling is distributed evenly over the host cell walls (CW). **C**, 24,000 \times ; **D**, 20,000 \times .

well exemplified under higher magnification, because chitolytic degradation at a distance from the site of *Trichoderma* penetration was not observed (Fig. 6B). A similar labeling pattern was observed in most intact inner rind cells, even when these were massively invaded (Fig. 6C and D). There was no indication of cell-wall disorganization in either the host or antagonist, as shown by the regular distribution of labeling (Fig. 6C), even in zones of penetration by constricted *Tricho-*

derma hyphae (Fig. 6D). In the medulla, gold labeling was regularly distributed over the thick host cell walls, even when the medullary cells showed obvious signs of disorganization (Fig. 7A and B). In contrast, the fibrillar matrix connecting the medullary cells to each other was unlabeled (Fig. 7B). All control tests, including previous adsorption of WGA with *N-N-N*-triacetylchitotriose, resulted in a near absence of labeling over fungal cell walls (not shown).

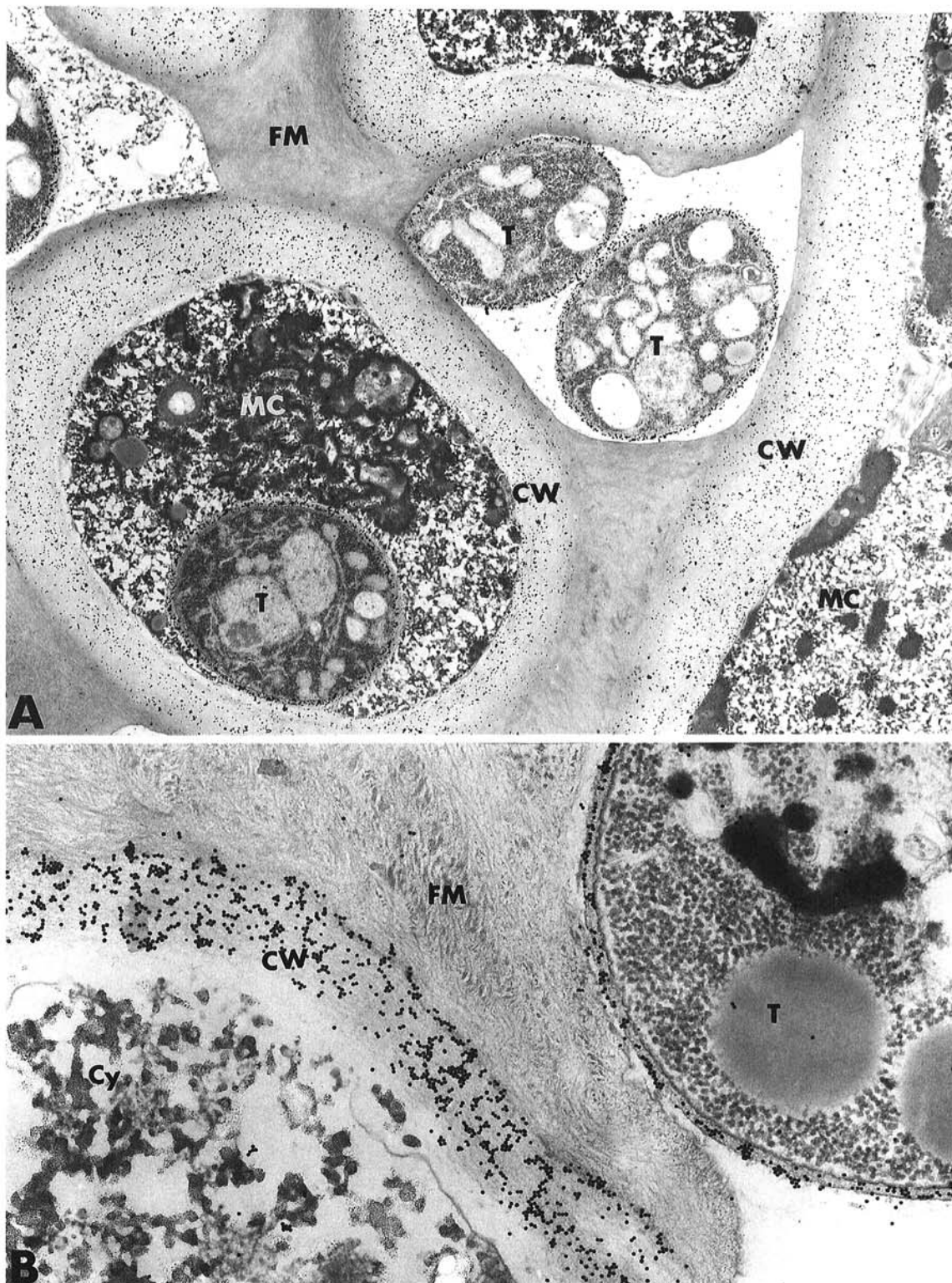


Fig. 7. Transmission electron micrographs of sclerotia of *Sclerotium rolfii* parasitized by *Trichoderma harzianum* (T). Chitin is labeled with the wheat germ agglutinin/ovomucoid-gold complex. **A and B**, In the medulla, gold labeling is distributed evenly over the thick host cell walls (CW), even when the medullary cells (MC) show obvious signs of cytoplasm (Cy) disorganization. In contrast, the fibrillar matrix (FM) connecting the medullary cells to each other is unlabeled. **A**, 10,000 \times ; **B**, 40,000 \times .

When ultrathin sections of parasitized sclerotia were incubated with gold-complexed β -1,3-glucanase for localization of β -1,3-glucans, a regular distribution of gold particles was observed over the walls of both outer and inner rind cells, even when these exhibited a disorganized cytoplasm that was reduced to a few rem-

nants of aggregated material (Fig. 8A). Except at sites of potential penetration by the antagonist (Fig. 8A, arrow), this labeling pattern was similar to that observed in nonparasitized sclerotia. In the medulla, the cell walls as well as the fibrillar matrix surrounding the medullary cells also were labeled (Fig. 8B).

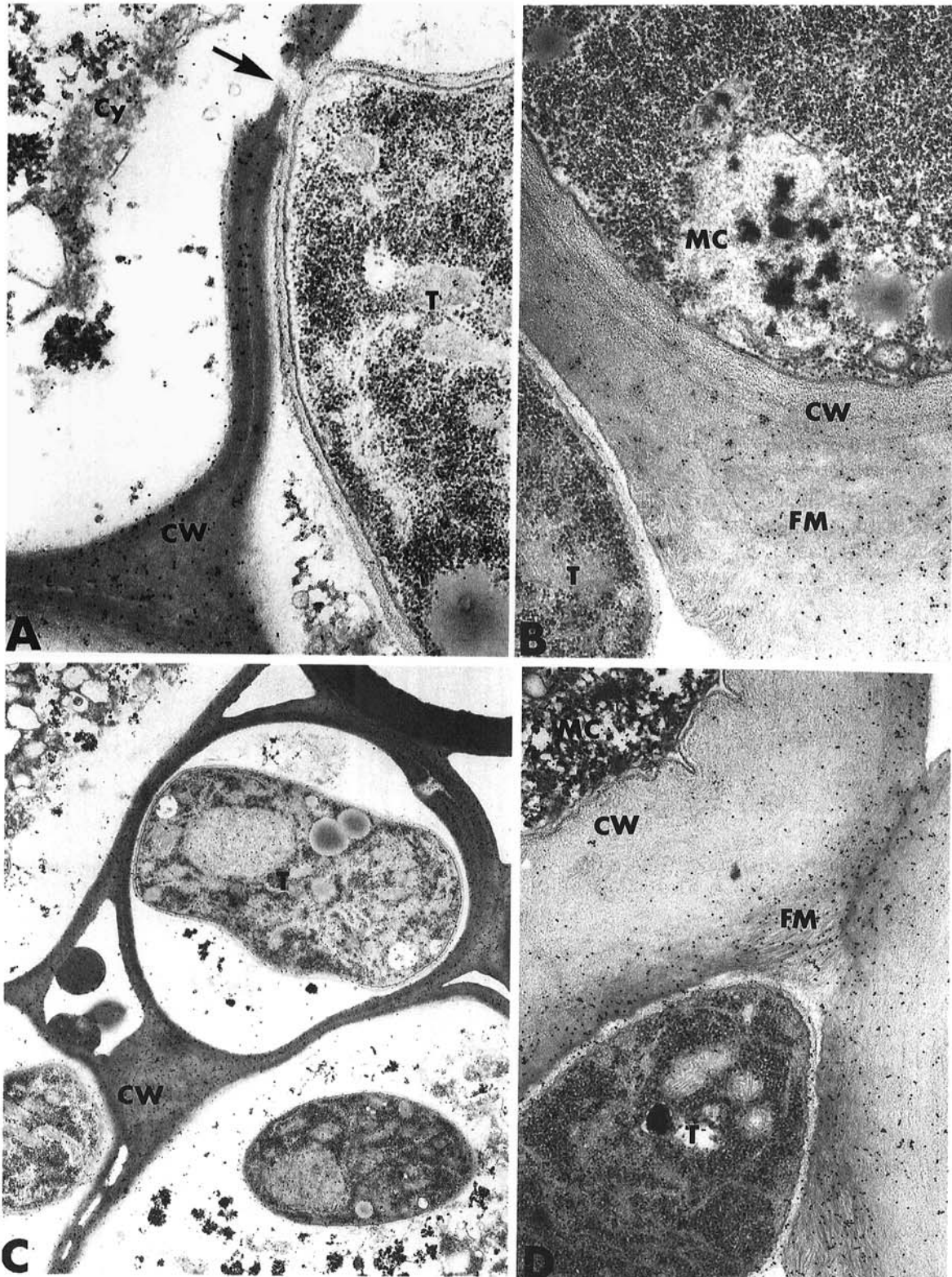


Fig. 8. Transmission electron micrographs of sclerotia of *Sclerotium rolfsii* parasitized by *Trichoderma harzianum* (T). **A and B**, β -1,3-glucans are labeled with gold-complexed tobacco β -1,3-glucanase. Gold particles are distributed evenly over the rind cell wall (CW) (**A**) and over the medullary cell wall (**B**). Local wall breaching is visible at the site of potential penetration by the antagonist (T) (arrow in **A**). The cytoplasm (Cy) in the rind cell (**A**) is markedly disorganized. **A**, 30,000 \times ; **B**, 30,000 \times . MC, medullary cell. FM, fibrillar material. **C and D**, Labeling of lipids with gold-complexed lipoprotein lipase. The cell wall (CW) is intensely labeled in both the rind (**C**) and the medulla (**D**), where fibrillar material (FM) is visible. **C**, 10,000 \times ; **D**, 25,000 \times .

Incubation of ultrathin sections with gold-complexed lipoprotein lipase yielded a pattern of labeling similar to that obtained with gold-complexed β -1,3-glucanase (Fig. 8C). Gold particles were distributed evenly over the host cell walls in the rind layer (Fig. 8C). Although qualitatively less numerous, they also occurred over the cell walls and fibrillar material in the medulla (Fig. 8D). All control tests performed to assess the specificity of labeling for both gold-complexed β -1,3-glucanase and lipoprotein lipase resulted in a near absence of labeling (not shown).

DISCUSSION

Trichoderma species have received considerable attention as possible biocontrol agents of soilborne plant pathogens, and their recognized antagonistic potential often has been used as a means of in vitro screening for the best biocontrol candidates (13). *T. harzianum* is probably the most studied of the *Trichoderma* species identified for biological control and, arguably, is the most effective in disease suppression (9). In the last few years, several authors have debated the role of antifungal molecules in the antagonistic process (15). Although production of extracellular cell wall-degrading enzymes, such as β -1,3-glucanases and chitinases, has often been considered the main mechanism involved in biological control of fungal pathogens by *T. harzianum* (7,13,17,20), circumstantial evidence implicating antibiosis as a contributing mechanism in pathogen cell breakdown is increasing (23).

Complementary to earlier investigations of the interaction between *T. harzianum* and sclerotia of *S. rolfii* (17), the present study was initiated to gain further insight into the cellular and molecular events associated with sclerotial colonization by *T. harzianum*. In line with previous observations (17), our results confirm that *T. harzianum* strain T203 successfully penetrates and colonizes sclerotia of *S. rolfii*, causing marked cytological alterations. SEM observations of samples from the interaction region in dual-culture tests showed that hyphae of *T. harzianum* multiplied abundantly at the sclerotial surface, forming a dense mycelium that established close contact with the outer host cells through a thin mucilage.

Recent studies have shown convincingly that cell-surface molecules, such as lectins, produced by pathogenic fungi are closely involved in the attachment, recognition, and specificity of the interaction between *T. harzianum* and its hosts (2,6). Whether the fine mucilage, apparently connecting *Trichoderma* hyphae to the outer sclerotial cells, plays a role in the initial recognition between both partners in a way similar to the matrix found at the surface of *R. solani* hyphae (6), warrants further investigation. However, the finding that this mucilage occurred only at the surface of *Trichoderma*-parasitized sclerotia suggests that early recognition events, mediated by molecules with agglutinating properties, may be important determinants in the mycoparasitic relationship between hyphae of the antagonist and sclerotia of the pathogen. Support for this assumption is provided by the elegant experiment recently conducted by Chet and Inbar (13). Using a biomimetic system, the authors were able to show that nylon fibers coated with either Concanavalin A or *S. rolfii* lectin provided an attractive "host support" for *T. harzianum* hyphae that coiled around and produced hooks in a way similar to that observed with *S. rolfii* hyphae.

In an attempt to identify the target molecules at the surface of *T. harzianum*, a number of gold-complexed lectins, including Concanavalin A (mannose-glucose), *Ricinus communis* agglutinin (galactose), *Helix pomatia* agglutinin (*N*-acetylgalactosamine), and *Ulex europaeus* (fucose), were applied to sections from the interaction sites (N. Benhamou, unpublished data). None of the tested lectins showed significant binding to *Trichoderma* hyphae, as judged by the scarce gold labeling observed at the cell surface of the antagonist. Failure of Concanavalin A to effectively bind to receptors at the antagonist cell surface, as might be expected in light of the

biomimetic assays (13), may reflect the inaccessibility of the probe to its target molecules in resin-embedded specimens or sugar extraction during sample processing.

Ultrastructural observations of sections from parasitized sclerotia provided evidence that *Trichoderma* ingress toward the medullar region is associated with a series of degradation events, including retraction of the plasmalemma, generalized disorganization of the cytoplasm, and, ultimately, complete loss of the protoplasm in both melanized and nonmelanized host cells. These results confirm earlier observations of the ability of *T. harzianum* to induce pronounced cytoplasmic damage when invading sclerotial cells (17) and bring new insights into the exact mode of parasitism of the antagonist.

According to our observations, host cytoplasmic disorganization in the medullar region often occurred in advance of physical contact with *T. harzianum*, suggesting that diffusible compounds were responsible for the observed disturbances. In all examined samples, hyphal penetration of the antagonist into rind cells was seldom associated with marked host cell-wall alterations. This was corroborated by the pattern of labeling obtained with the WGA/ovomucoid-gold complex, which showed that, except in the area of hyphal penetration, the chitin component of the host cell walls was structurally preserved. Similarly, comparison of the chronology of cell reactions with labeling of β -1,3-glucans by the gold-complexed β -1,3-glucanase from tobacco provided evidence that the host cytoplasm had undergone complete disorganization at a time when β -1,3-glucans were still distributed evenly over the rind cell walls (Fig. 8A).

These observations indicated that production of cell wall-degrading enzymes by *T. harzianum* probably was not the first event involved in sclerotial decay as previously suggested by Elad et al. (17). This was confirmed by the finding that medullary cells, which were not colonized internally by *Trichoderma* hyphae, also were affected markedly, as judged by the significant aggregation of the cytoplasm. *T. harzianum* is known to produce a wide array of extracellular lytic enzymes that are involved in the process of antagonism against pathogenic mycelia and sclerotia (6,29). However, the observed sclerotial cell reactions after invasion by *T. harzianum* are reminiscent of the disturbances detected in fungal cells treated with some fungicides or exposed to antibiotics (14). Studying the effect of cyproconazole on the structural integrity of *S. rolfii* hyphae, Robertson and Fuller (32) and Fuller et al. (22) reported that marked cytological alterations were visible in hyphal tip cells, whereas labeling for chitin was nearly intact over the cell walls. A similar scheme of reactions was recently described by Hajlaoui et al. (24) who found that an antibiotic substance produced by the antagonist *Sporothrix flocculosa* induced major cytoplasmic disorders and loss of cell turgor in *Sphaerotheca pannosa* var. *rosae*, whereas the level of chitin integrity in the host cell walls was maintained. Although it is clear that enzymes produced by *T. harzianum* play a major role in breaching the host cell walls at sites of attempted penetration, it seems likely that further enzymatic production contributes more to the saprophytic phase of the antagonist when the cell content is utilized as a food source providing the energy required to multiply and sporulate abundantly.

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