Mycoparasitism of the Extramatrical Phase of *Glomus intraradices* by *Trichoderma harzianum*

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ABSTRACT

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In the present study, the interaction between the saprophytic fungus *Trichoderma harzianum* and the arbuscular mycorrhizal (AM) fungus *Glomus intraradices* was studied by transmission electron microscopy (TEM) to delineate precisely the relationship established between both partners. An axenic system, divided into four compartments, proved useful for studying the interaction between *T. harzianum* and the extramatrical phase of *G. intraradices*. This experimental model, based on root-organ culture to obtain typical mycorrhizal infections, was selected as a reliable means of obtaining mycorrhizal spores and mycelium in root-free compartments. TEM observations of samples from the interaction region showed that hyphae of *T. harzianum* proliferated abundantly at the spore surface and penetrated the thick host wall through local hydrolysis of the wall polymers. Hyphae of the antagonist also were seen in the subtending hyphae of the AM fungus, and they grew actively in the main host hyphae. This massive colonization was associated with marked cell damage, involving partial to complete disorganization of the cytoplasm, which led in most cases to loss of the protoplasm and apparent bursting of the main hyphae of *G. intraradices*, resulting in the release of the actively proliferating *Trichoderma* hyphae. At an advanced stage of the colonization process, the main hyphae of *G. intraradices* were perforated in many places. The use of wheat germ agglutinin/ovomucoid-gold complex for the localization of chitin monomers resulted in regular labeling of the host cell walls even when spores, subtending hyphae, and main hyphae of *G. intraradices* were colonized massively. Chitinolytic degradation was seen only in areas adjacent to the sites of *Trichoderma* penetration. According to our observations, the interaction between *T. harzianum* and *G. intraradices* involves the following events: (i) recognition and local penetration of the antagonist into mycorrhizal spores; (ii) active proliferation of antagonist cells in mycorrhizal hyphae; and (iii) release of the antagonist through moribund hyphal cells.

Additional keywords: biocontrol agent, chitinase, parasitism, transformed roots.

Arbuscular mycorrhizal (AM) fungi are obligate biotrophs that have wide host ranges and that promote plant growth by increasing the efficiency of nutrient uptake (9,53). Such beneficial effects are, however, difficult to generalize, because mycorrhizal effectiveness is modulated by different plant-fungal interactions (40). Understandably, much attention has been given to attempting to understand the impact of genotypic and environmental factors on the differential effect of AM fungi in stimulating plant growth (51,53). Another facet that is receiving increasing attention is the reported influence of microbial interactions in the rhizosphere of AM fungi (45,58). Although mycorrhizal symbioses have been long considered two-partner interactions, a growing body of evidence supports the concept that both plant and AM fungi are affected markedly by a wide range of soil microorganisms that may act as beneficial or detrimental factors in the functioning of AM symbiosis (29).

In recent years, the relationships between AM fungi and soilborne pathogens have been studied extensively (39). The rationale for such an interest is that AM fungi could be not only potential inducers of plant disease resistance in a large number of crops but they could become a biologically, environmentally, and commercially valuable alternative to existing pathogen control methods. However, research progress over the past decade has strengthened the idea that the ability of AM fungi to colonize plant roots and promote disease resistance is influenced greatly by interactions between AM fungi and nonpathogenic soil microorganisms (23, 32). Convincing evidence has been provided that spores and mycelia of AM fungi could be vulnerable to parasitism from other soil organisms (21,41,47,48,49,55,56). However, the exact mechanisms by which such organisms contribute to the alteration of the viability of AM fungi are not understood fully, although a number of hypotheses, including antibiosis, production of cell-wall hydrolytic enzymes, and direct mycoparasitism, have been raised (29). These explanations are still a matter of speculation, and the exact contribution of each mechanism in the overall process remains controversial.

Among the most commonly occurring saprophytic fungi in the rhizosphere, *Trichoderma* spp. have been the focus of considerable interest because of their recognized potential to act as biocontrol agents against a wide range of root pathogens (1,7,17,28, 54). In spite of the increasing amount of research devoted to the antimicrobial activity of *Trichoderma* spp. against plant pathogens (18), the possibility that these antagonistic fungi also may interfere with other components of the soil rhizosphere has not been studied to any extent. Thus, our knowledge of the exact mechan-

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isms underlying microbial interactions in the rhizosphere are still incomplete, although circumstantial evidence correlating the presence of *Trichoderma* spp. in soil and a decrease in the extent of root colonization by AM fungi has been documented (45,58). At present, the situation is not clearly defined, and contradictory results about the effects of *Trichoderma* spp. on AM fungi have been reported (13,14,15).

An axenic system, previously designed by Bécard and Fortin (4) with root-inducing (Ri) transferred DNA (T-DNA)–transformed roots as a plant partner, was modified recently (30) to accurately delineate the time course of early events occurring with the interaction between the extramatrical phase of *Glomus intraradices* Schenck & Smith and *T. harzianum* Rifai. The advantages of such an experimental system are the absence of confounding effects caused by undesirable contaminants in nature and the ability to monitor precisely the events generated by contact between both partners. In an attempt to gain further insights into the sequence of events taking place during interaction between *Trichoderma* spp. and AM fungi and to assess the potential role of enzymatic hydrolysis in the antagonistic process, we have used transmission electron microscopy (TEM) and lectin gold cytochemistry to study the cytology of parasitism in terms of both potential benefit and disadvantage to the antagonist and the host, respectively.

MATERIALS AND METHODS

Fungal sources and growth conditions. Axenic spores of *G. intraradices* (DAOM 197198, Biosystematic Research Center, Ottawa, Canada) were cultivated on isolated tomato roots (*Lycopersicon esculentum* Mill.) in square (9 × 9 cm) petri dishes containing a minimal (M) medium at 27°C (16). The composition of M medium is for 1 liter of distilled water: 731 mg of $MgSO_4 \cdot 7H_2O$, 80 mg of KNO_3, 65 mg of KCl, 4.8 mg of KH_2PO_4, 288 mg of $Ca(NO_3)_2 \cdot 4H_2O$, 10 g of sucrose, 8 mg of NaFe EDTA, 0.75 mg of KI, 6 mg of $MnCl_2 \cdot H_2O$, 2.65 mg of $ZnSO_4 \cdot 7H_2O$, 0.0024 mg of $Na_2MoO_4 \cdot 2H_2O$, 3 mg of glycine, 0.1 mg of thiamine hydrochloride, 0.1 mg of pyridoxine hydrochloride, 0.5 mg of nicotinic acids, 50 mg of myo-inositol, and 0.35% (wt/vol) Gellan gum (ICN Biochemical, Cleveland) (4).

The strain of *T. harzianum* (T-203) used in this study was isolated by Elad et al. (25). *T. harzianum* was cultured on Difco potato-dextrose agar medium (Difco Laboratories, Detroit) at 25°C.

Transplant procedure. A clone of transformed pea roots (*Pisum sativum* L., *frisson*, P64, provided by G. Duc, Dijon, France) was maintained on modified White's medium (2) containing for 1 liter of distilled water: 731 mg of $MgSO_4 \cdot 7H_2O$, 453 mg of $Na_2SO_4 \cdot 10H_2O$, 80 mg of KNO_3, 65 mg of KCl, 19.0 mg of $NaH_2PO_4 \cdot H_2O$, 288 mg of $Ca(NO_3)_2 \cdot 4H_2O$, 30 g of sucrose, 8 mg of NaFe EDTA, 0.75 mg of KI, 6 mg of $MnCl_2 \cdot H_2O$, 2.65 mg of $ZnSO_4 \cdot 7H_2O$, 0.0024 mg of $Na_2MoO_4 \cdot 2H_2O$, 3 mg of glycine, 0.1 mg of thiamine hydrochloride, 0.1 mg of pyridoxine hydrochloride, 0.5 mg of nicotinic acids, 50 mg of myo-inositol, and 0.35% (wt/vol) Gellan gum (4). These transformed pea roots were transferred into four-compartment petri dishes containing M medium and grown as illustrated in Figure 1.

Mycorrhizal establishment and development of extramatrical mycelium of *G. intraradices*. Mycorrhizal colonization was achieved by depositing spores (15 to 20) into two opposite compartments along the transformed pea roots. After spore germination and root colonization, hyphae of *G. intraradices* were able to grow toward the two root-free compartments. After 1 month, hyphae of *G. intraradices* developed abundantly in these compartments and produced spores. This was considered the extramatrical phase of *G. intraradices*.

Inoculation of extramatrical mycelium of *G. intraradices* by *T. harzianum*. Hyphal interactions between *G. intraradices* and *T. harzianum* were studied according to the following procedure. Mycelial disks (5 mm diameter) of *T. harzianum*, cut from 4-day-old actively growing colonies, were placed in the root-free compartments containing the extramatrical phase of *G. intraradices*. One week later, after macroscopic observations, fungal samples from the interaction region between both partners were collected and processed for electron microscopy.

TEM. Compartments containing *G. intraradices* and *T. harzianum* were flooded with a mixture of 3% (vol/vol) glutaraldehyde and 3% (wt/vol) paraformaldehyde in 0.2 M sodium cacodylate buffer, pH 7.2, for 2 h at room temperature. Mycelial samples (2 mm^3) were excised carefully at potential sites of interaction between the two microorganisms and were immersed immediately in the same fixative and maintained overnight at 4°C. Samples were postfixed with 1% (wt/vol) osmium tetroxide in 0.1 M sodium cacodylate buffer for 2 h at 4°C, dehydrated in a graded ethanol series, and embedded in Epon 812 (JBEM Chemical Company, Pointe-Claire, Québec). Ultrathin sections were collected on Formvar-coated nickel grids and were contrasted either with uranyl acetate and lead citrate for direct examination with a JEOL 1200 EX electron microscope (Tokyo) at 80 kV or further processed for cytochemical labeling.

Cytochemistry. Colloidal gold with an average particle diameter of 15 nm was prepared according to Frens (31). 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 (6). Because of its low molecular weight, this lectin could not be directly complexed to colloidal gold, instead it was used in a two-step procedure with ovomucoid as a second-step reagent. This glycoprotein was complexed to colloidal gold at pH 5.4.

Sections first were incubated on a drop of phosphate-buffered saline (PBS), pH 7.2, for 5 min, then transferred on a drop of WGA (25 µg/ml of PBS, pH 7.2) for 30 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.

Specificity of the labeling was assessed by the following control tests: (i) incubation with WGA, to which an excess of *N*-*N*′-*N*″-triacetylchitotriose (1 mg/ml in PBS) was previously added; (ii) incubation with WGA, followed by unlabeled ovomucoid and ovomucoid-gold complex; and (iii) incubation with stabilized or unstabilized gold suspension.

RESULTS

Ultrastructural features of the interaction region between *G. intraradices* and *T. harzianum*. Examination of ultrathin sections from mycelial samples collected in the interaction region of 7-day-old dual cultures showed that hyphae of *T. harzianum*, easily

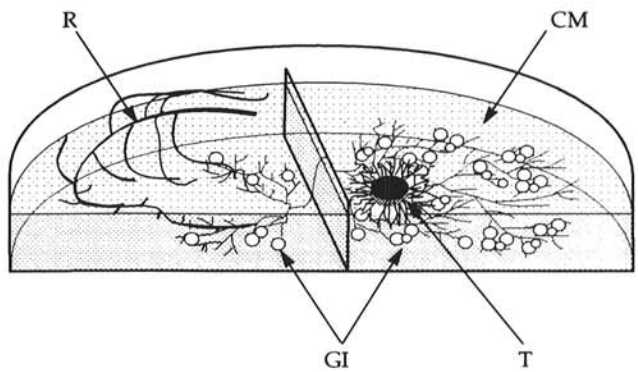

Fig. 1. Schematic representation of the axenic four-compartment system. Root-inducing transferred-DNA–transformed pea roots (R) and spores of *Glomus intraradices* (GI) were deposited in two opposite compartments. After germination and root colonization, hyphae of *G. intraradices* grew toward the root-free compartments, which were subsequently inoculated with mycelial disks of actively growing colonies of *Trichoderma harzianum* (T). CM = culture medium.

recognized by the strong electron density of their protoplasm, were closely appressed against spores of the mycorrhizal fungus (Fig. 2A). Spore invasion occurred through localized host wall penetration (Fig. 2A). Channels of penetration were always much narrower than the average hyphal diameter and usually were associated with little wall displacement in the growth direction (Fig. 2A, arrow). *Trichoderma* ingress in the spore cavity coincided with extensive cell alterations, leading to dissolution of the cytoplasm, which was often reduced to a few aggregated remnants (Fig. 2A and B). At this stage after inoculation, host cell walls were apparently not altered, although some local splitting could be observed at a distance from the point of fungal penetration (Fig. 2A, arrowhead).

A close examination of some spores revealed that hyphae of *T. harzianum* were able to colonize the subtending hyphae that support the spores (Fig. 2B). *Trichoderma* colonization was not restricted to spores and subtending hyphae; it also occurred in the main hyphae of *G. intraradices* (Fig. 2C). Fungal growth in the mycorrhizal hyphae was associated with marked cell damage, involving marked disorganization of the cytoplasm (Fig. 2C), which led eventually to complete loss of the protoplasm (Fig. 3A and B). Cells of the antagonist ramified so extensively in main host hyphae that it was often difficult to delineate free space in the area originally occupied by the host cytoplasm (Fig. 3A). Such massive colonization frequently resulted in mechanical pressure against the main host hyphal cell walls, ultimately leading to apparent bursting of host hyphae and release of actively growing *Trichoderma* hyphae (Fig. 3A). At an advanced stage of the colonization process, main hyphae of *G. intraradices* were perfor-

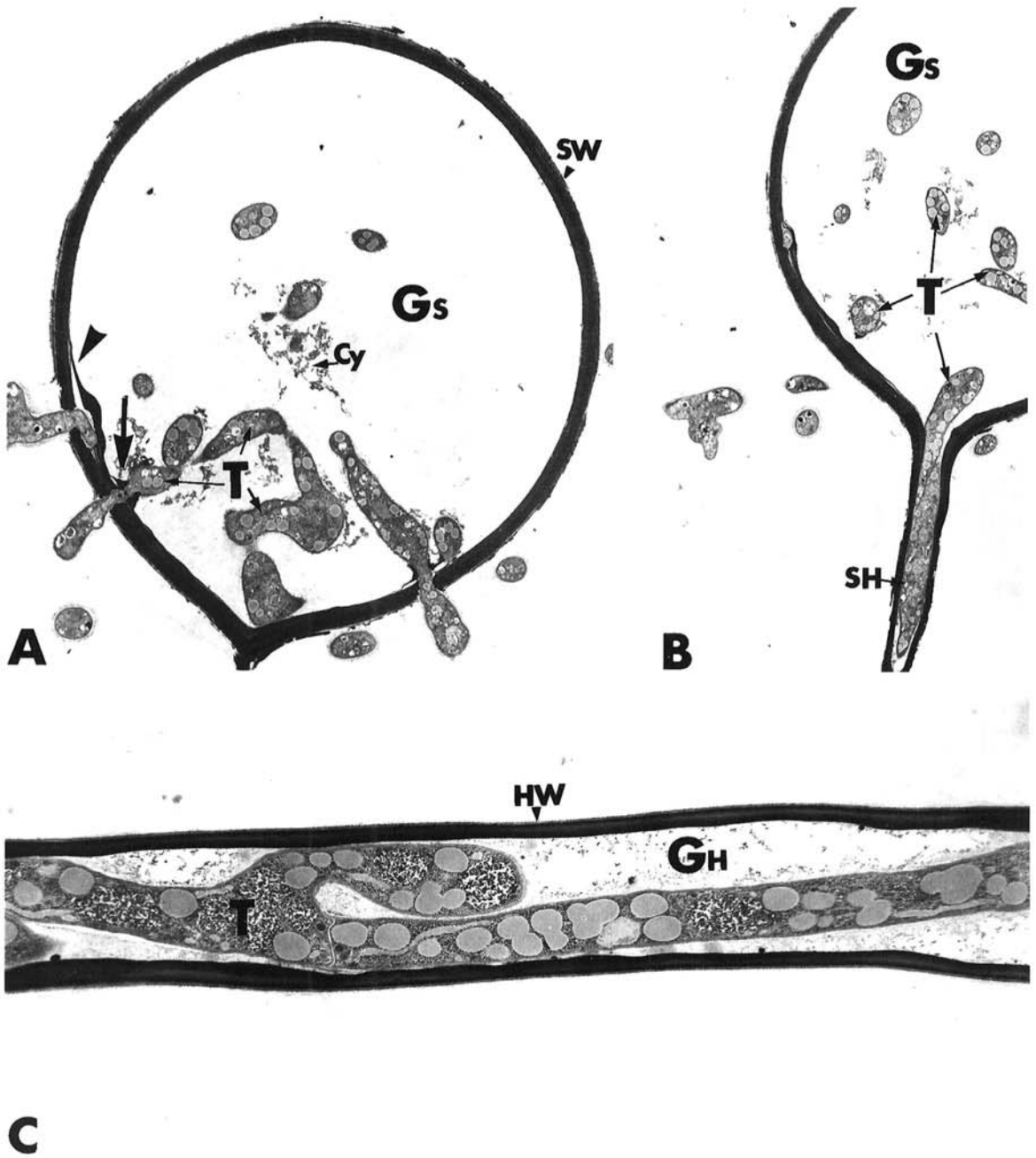

Fig. 2. Transmission electron micrographs of spores of *Glomus intraradices* (Gs) parasitized by *Trichoderma harzianum* (T) 7 days after inoculation. **A,** Spore invasion by constricted hyphae of the antagonist occurred through localized host wall penetration and often was accompanied by little wall displacement in the growth direction (arrow). The host cytoplasm (Cy) was reduced to a few aggregated remnants. The spore cell wall (SW) apparently was not altered, although some local splitting can be seen at a distance from the point of fungal penetration (arrowhead). 1,900×. **B,** A hypha of *T. harzianum* (T) that colonizes the host subtending hypha (SH) supporting the arbuscular mycorrhizal *G. intraradices* spore (Gs). 1,500×. **C,** *Trichoderma* (T) colonization in a main hypha of *G. intraradices* (GH) in which cytoplasm disorganization is visible while the cell wall (HW) is apparently intact. 5,000×.

ated in many places, and host wall displacement in the direction of *Trichoderma* growth was easily discernible (Fig. 3B, arrows). To determine whether the apparent mechanical pressure exerted by the antagonist was accompanied by a hydrolytic action of cell wall-degrading enzymes, cytochemical tests were carried out to study the distribution of chitin, which is one of the most important wall-bound compounds in AM fungi (34).

Cytochemical localization of *N*-acetylglucosamine residues in parasitized spores of *G. intraradices*. Incubation of ultrathin sections of parasitized spores with the WGA/ovomucoid-gold complex for the localization of chitin monomers resulted in regular labeling of cell walls, except in areas of *Trichoderma* penetration. Cell surface interactions between both partners initially were characterized by localized disruption of the outermost spore wall layers (Fig. 4A). Although intense chitinolytic degradation at a large distance from the site of *Trichoderma* penetration was not observed, the presence of small electron-lucent wall areas, apparently free of gold labeling, in the immediate vicinity of the antagonist indicated the production of chitinolytic enzymes that diffused in advance of the fungus (Fig. 4A, arrow).

Penetration of the thick spore wall by *Trichoderma* hyphae was achieved either through wall splitting (Fig. 4B and C) or direct wall breakdown (Fig. 4D). In spite of this, the overall structural integrity of the electron-dense spore cell wall was maintained, and gold particles were distributed evenly, except in areas of close contact with the antagonist (Fig. 4B, arrows). This phenomenon was exemplified under higher magnification, because chitinolytic degradation at a large distance from the site of *Trichoderma* penetration was not observed (Fig. 4C). The only typical features associated with hyphal ingress were the presence of unlabeled, electron-lucent areas along the channel of penetration (Fig. 4C, arrows). A similar labeling pattern of the host cell wall was noticed when hyphal ingress proceeded through direct wall penetration (Fig. 4D). Although typical disruption of both the outer and inner spore wall layers was observed along the channel of penetration, this degradation event remained localized and apparently did not affect the neighboring wall areas, as judged by the regular distribution of gold labeling (Fig. 4D). All control tests, including previous adsorption of WGA with *N*-*N*′-*N*″-triacetylchitotriose, resulted in a near absence of labeling over the cell walls of both fungi (data not shown).

Cytochemical localization of *N*-acetylglucosamine residues in parasitized hyphae of *G. intraradices*. As previously mentioned, *T. harzianum* frequently was found in the subtending host hyphae (Fig. 5A). Surprisingly, host wall alterations associated with growth of the antagonist were never observed in subtending hyphae, even when close contact was established between the two partners (Fig. 5A, arrow). In all cases and even in massively invaded subtending hyphae, there was no indication of cell wall disorganization in either the host or the antagonist, as shown by the regular distribution of labeling (Fig. 5A).

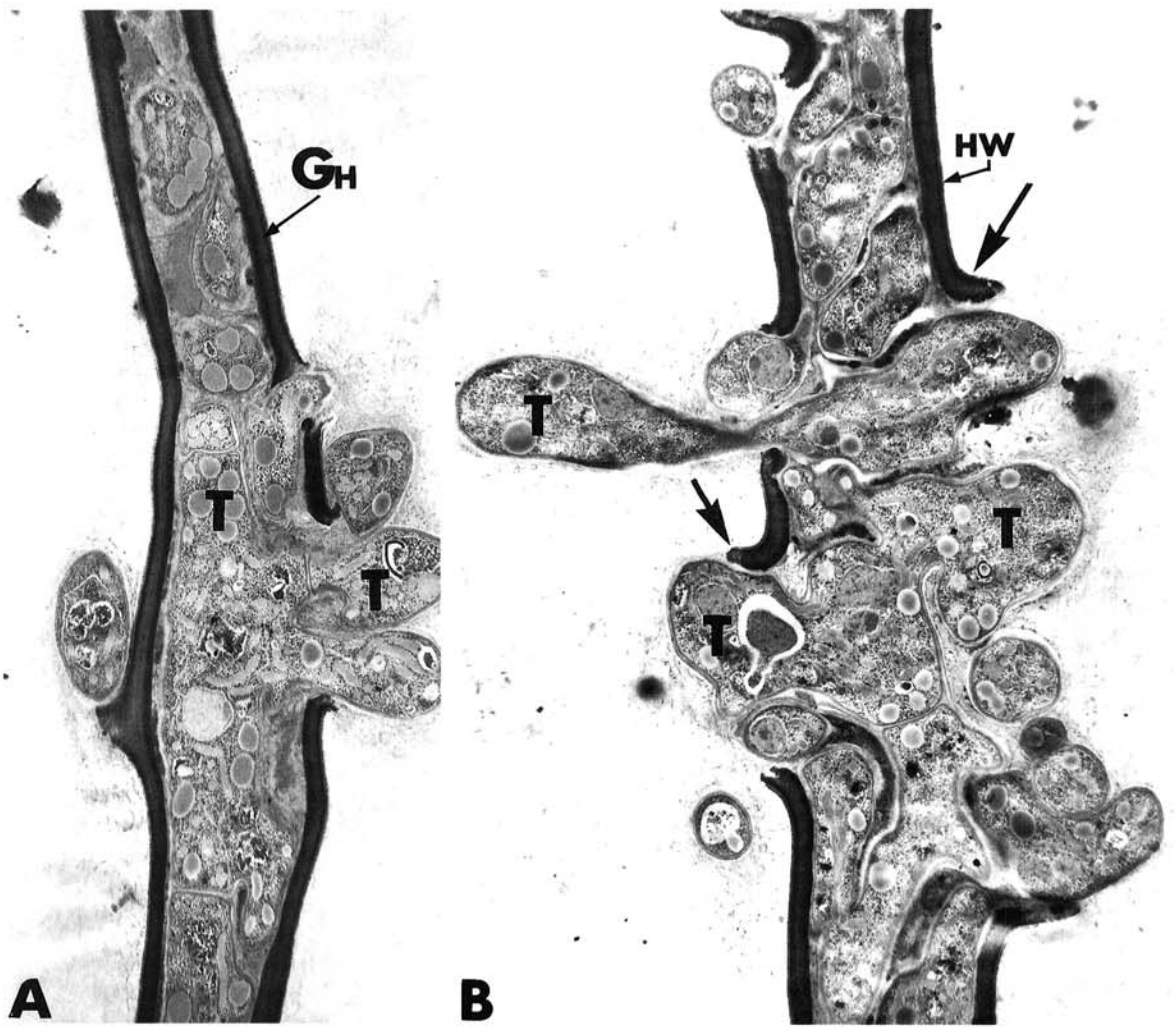

Fig. 3. Transmission electron micrographs of hyphae of *Glomus intraradices* (GH) parasitized by *Trichoderma harzianum* (T) 7 days after inoculation. **A,** Hyphae of the antagonist ramified extensively in a *Glomus* main hypha and entirely colonized the area that originally was occupied by the host cytoplasm. Such intense colonization apparently resulted in strong mechanical pressure against the host hyphal cell walls, leading to apparent bursting of the main host hypha and release of actively proliferating *Trichoderma* cells. 5,000×. **B,** In a massively invaded hypha of *G. intraradices*, the hyphal wall (HW) was perforated in many places, and host wall displacement in the direction of *Trichoderma* growth (T) is discernible (arrows). 5,000×.

Incubation of sections from nonparasitized (control) *Glomus* hyphae with the WGA/ovomucoid-gold complex revealed specific deposition of gold particles over the wall. However, preferential accumulation of gold labeling was detected over the outermost wall layers (Fig. 5B). In parasitized main hyphae, growth of the antagonist was not accompanied by extensive structural alterations of the host walls, especially during the early stages of colonization (Fig. 5C). Although the labeling pattern often was difficult to visualize due to the strong electron density of the walls, close examination showed that labeling was evenly distributed over the

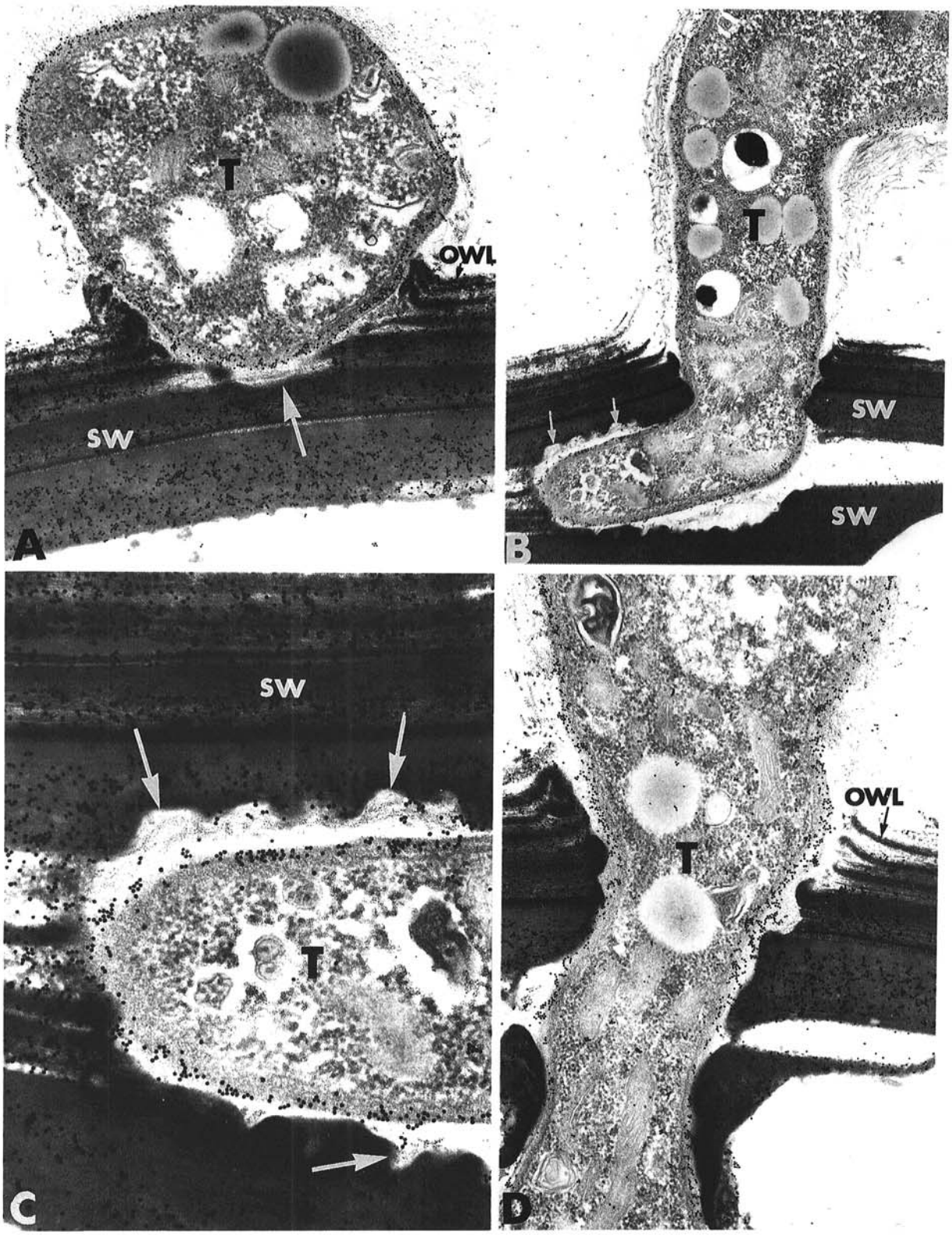

Fig. 4. Transmission electron micrographs of spores of *Glomus intraradices* parasitized by *Trichoderma harzianum* (T) 7 days after inoculation. *N*-acetylglucosamine residues (chitin) were labeled with wheat germ agglutinin/ovomucoid-gold complex. **A,** Regular labeling of the mycorrhizal spore wall (SW) was observed, except in the area of *T. harzianum* (T) penetration. A localized disruption of the outermost spore wall layers (OWL) was visible. Small electron-lucent wall areas, apparently free of gold labeling, were seen in the immediate vicinity of the antagonist (arrow). 25,000×. **B through D,** Penetration of the thick spore wall by the antagonist (T) was achieved either through wall splitting (**B and C**) or direct wall breakdown (**D**). *Trichoderma* ingress was accompanied by the formation of unlabeled, electron-lucent areas along the channel of penetration (**B and C,** arrows). **B,** 13,000×. **C,** 50,000×. **D,** 25,000×.

walls and occurred over a fine band of retracted fibrillar material that was still in apparent connection with the wall in places (Fig. 5C, arrows). In massively invaded *Glomus* main hyphae, wall alterations ranging from slight deformations (Fig. 5D, arrow) to complete disruption (Fig. 5D, double arrows) frequently were observed.

Close examination of the process leading to the release of *Trichoderma* hyphae revealed that cell wall breakdown was preceded by a series of chronological events involving not only apparent mechanical pressure exerted by the actively growing *Trichoderma* mycelia but also enzymatic hydrolysis of some wall portions at

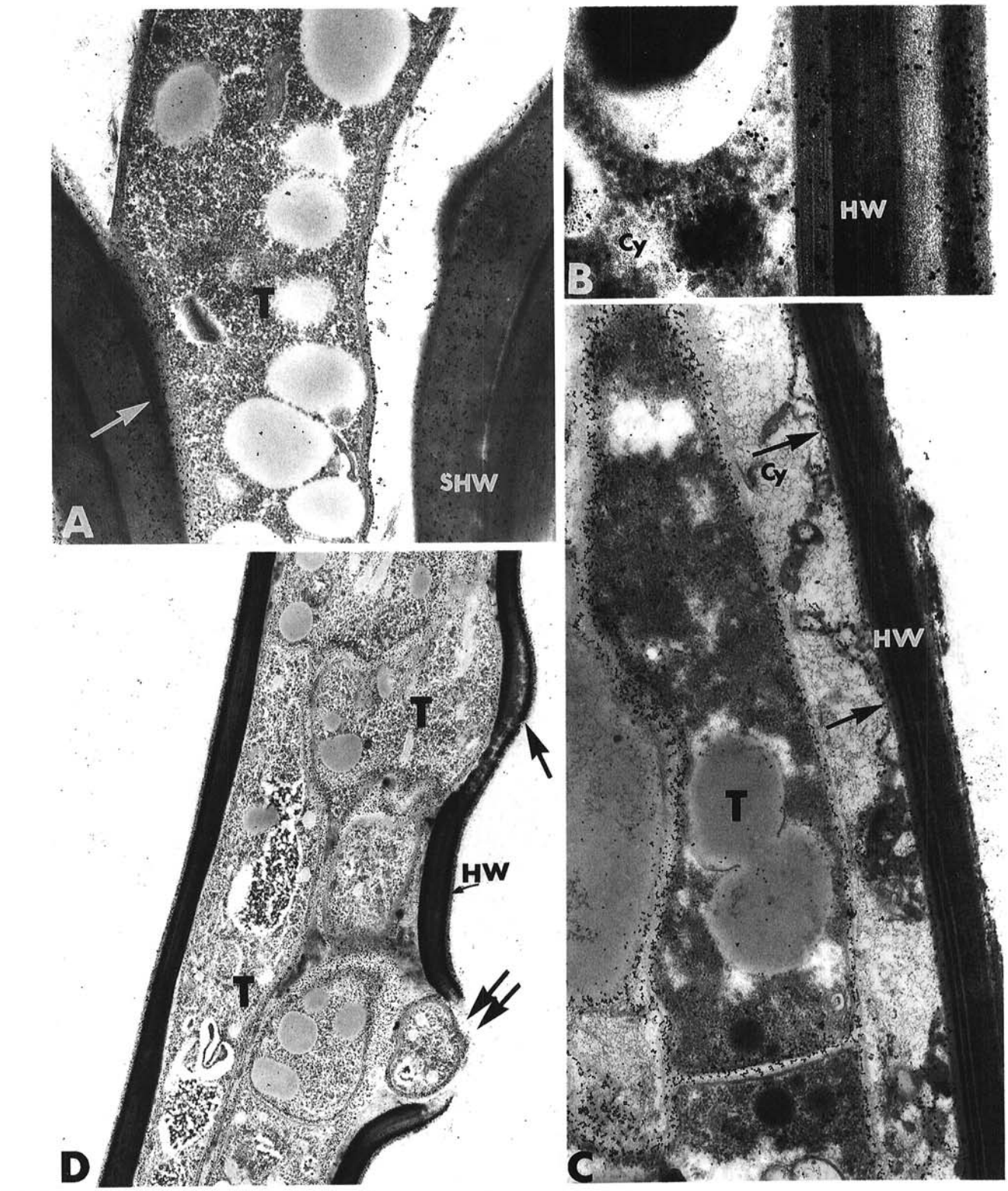

Fig. 5. Transmission electron micrographs of hyphae of *Glomus intraradices* parasitized by *Trichoderma harzianum* (T) 7 days after inoculation. *N*-acetylglucosamine residues (chitin) were labeled with wheat germ agglutinin/ovomucoid-gold complex. **A,** A hypha of *T. harzianum* (T) growing in a subtending hypha of *G. intraradices*. The subtending hyphal wall (SHW) is regularly labeled (arrow). 20,000×. **B,** Specific deposition of gold particles detected over the hyphal wall (HW) of a nonparasitized *G. intraradices* hypha with, however, a preferential accumulation of gold particles over the outermost wall layer. Cy = cytoplasm. 50,000×. **C,** The hyphal wall (HW) of *G. intraradices* was not apparently altered by *T. harzianum*. Gold labeling also occurred over a fine band of retracted fibrillar material (arrows). 20,000×. **D,** In a massively invaded *Glomus* main hypha, wall alterations ranging from slight deformation accompanied by a decrease in electron density (arrow) to complete disruption (double arrows) were observed. 8,500×.

strategic sites (Fig. 6). The first typical manifestations of host wall disorganization were characterized mainly by a slight decrease in the electron density of the wall regions at sites of potential *Trichoderma* release (Fig. 6A). This early process was followed by localized disruptions of altered regions (Fig. 6B, arrow) that gradually extended to larger wall portions (Fig. 6C), finally creating openings by which hyphae of *T. harzianum* could escape easily (Fig. 6D). These degradation events always coincided with the release of slightly labeled, fibrillar fragments that accumulated in the vicinity of the invaded *Glomus* main hyphae (Fig. 6C and D).

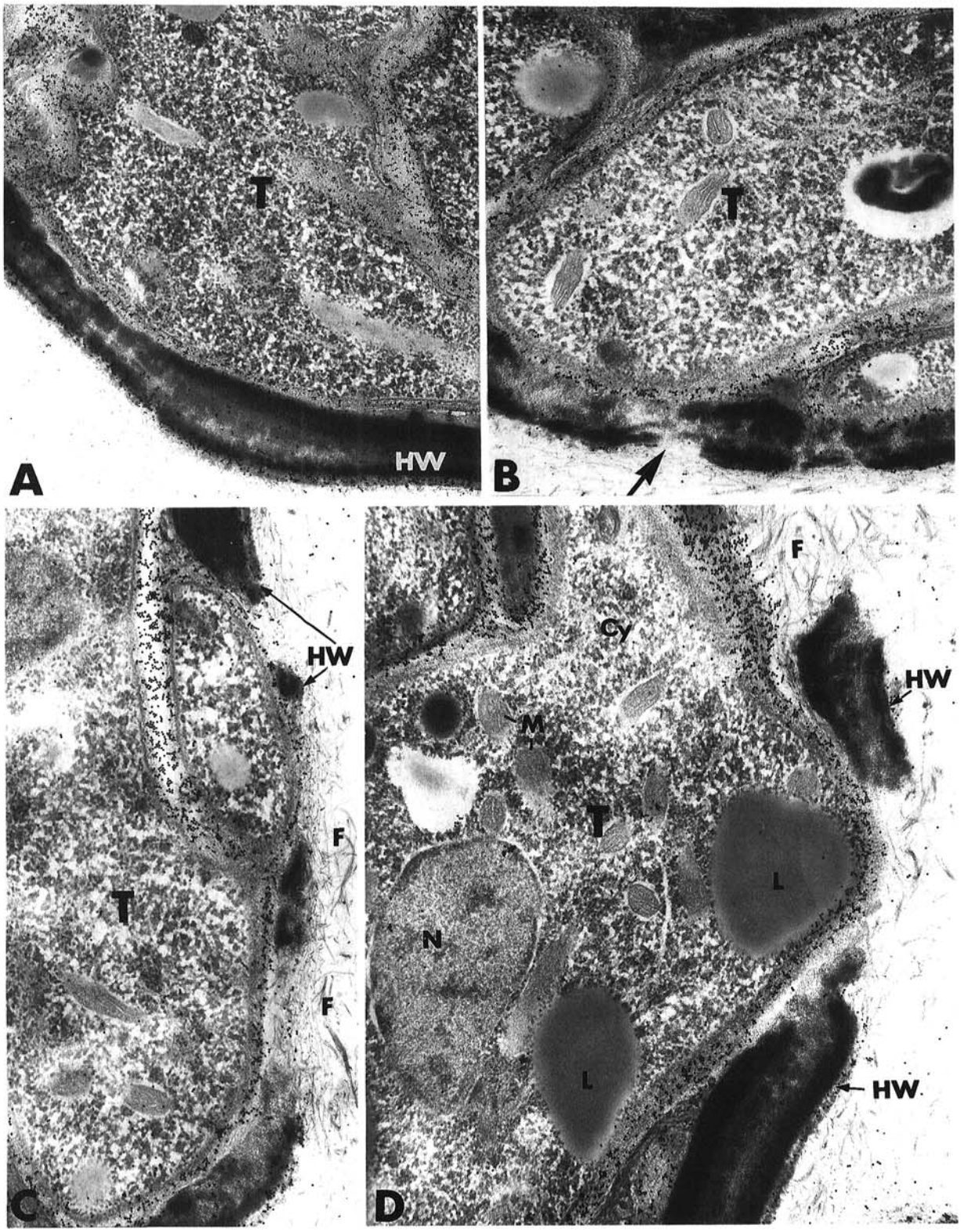

Fig. 6. Transmission electron micrographs of hyphae of *Glomus intraradices* parasitized by *Trichoderma harzianum* (T) 7 days after inoculation. *N*-acetylglucosamine residues (chitin) were labeled with wheat germ agglutinin/ovomucoid-gold complex. **A,** A slight decrease in the electron density of the wall region at a site of potential *Trichoderma* release was visible. HW = host wall. 20,000×. **B,** Localized host wall disruptions were observed in areas where the antagonist (T) was closely appressed against the host cell wall (arrow). 25,000×. **C and D,** Extensive host wall (HW) alterations, coinciding with the release of slightly labeled, fibrillar fragments (F), apparently led to the formation of openings through which cells of *T. harzianum* could escape easily (**D**). **D,** *Trichoderma* hyphae showed intense metabolic activity, as exemplified by their ribosome-rich cytoplasm (Cy) in which mitochondria (M), lipid bodies (L), and nuclei (N) were seen. **C and D,** 25,000×.

Trichoderma hyphae showed intense metabolic activity, exemplified by their ribosome-rich cytoplasm in which a large number of organelles, such as mitochondria and nuclei, were seen (Fig. 6D).

DISCUSSION

In the current study, the experimental model with Ri T-DNA–transformed pea roots (2) proved to be a distinct improvement over existing methods for delineating the mechanisms underlying the interaction between *T. harzianum* and the extramatrical mycelium of *G. intraradices*. This simple and easily reproducible axenic system, modified by Fortin (30), for precisely monitoring the formation of extramatrical mycelium is remarkably well adapted to maintain the growth of isolated plant roots and fungi with the use of M medium (4). Such a system provides a powerful tool for studying the sequence of events occurring not only during the establishment of symbiotic associations but also after colonization with pathogenic (8) and nonpathogenic (i.e., *T. harzianum*) fungi.

Our results demonstrate that the mycorrhizal fungus *G. intraradices* is highly vulnerable to attack by the saprophytic fungus *T. harzianum*, which is probably the most studied of the *Trichoderma* species identified for biological control and which is arguably the most effective in suppressing disease (7,18,20,26,27). Although such impressive results are difficult to generalize, because they may be linked to the particular aggressiveness of the *Trichoderma* strain (T-203) used in this study, our observations confirm that competitive interactions between AM fungi and some soil microorganisms may reduce considerably mycorrhizal inoculum, inducing marked disorders on mycorrhizally dependent plants (42). These results are in line with earlier reports on the potential of some *Trichoderma* spp. to cause adverse effects on AM fungi (45,58) and provide the first in vitro ultrastructural evidence that both spores and hyphae of a mycorrhizal fungus may suffer from severe damage when competition with aggressive soil fungi is established.

According to our cytological observations, the process of *Glomus* colonization by *T. harzianum* strain T-203 involves a sequence of events: (i) recognition and local penetration of the antagonist into mycorrhizal spores; (ii) active growth of antagonistic cells in mycorrhizal hyphae; and (iii) release of the antagonist through moribund hyphal cells. One of the most typical and surprising features of this antagonistic process is the remarkable affinity of *T. harzianum* hyphae for the spores of the mycorrhizal fungus. The exact mechanisms by which the antagonist effectively recognizes the spore surface of *G. intraradices* are still unknown, but it is likely that they are derived from a complex strategy involving several successive steps. A similar phenomenon has often been reported in a number of antagonistic interactions and described as chemotropic growth of the antagonist in response to stimuli or a gradient of chemicals produced by the host (18,19). In the legume-*Rhizobium* interaction, a root exudate factor accounts for the attraction of the bacteria, which, in turn, synthesize a bacterial factor that causes an altered growth pattern of the plant root (24). Whether such a phenomenon occurs as an early step in the interaction between *T. harzianum* and *G. intraradices* warrants further investigation. The observation that the antagonist invaded the mycorrhizal fungus suggests that molecular signaling occurred between the antagonist and the AM fungus, leading to an exchange of information preceding further recognition and penetration.

In a number of biological systems, cell surface molecules play an important role in cell-to-cell interactions or cell surface recognition phenomena (38,52). Several lines of evidence have provided support for the concept that early recognition events, mediated by molecules with sugar-binding affinity, are important determinants in the mycoparasitic relationship between *Trichoderma* hyphae and host mycelia (3,7). Using a biomimetic system, Inbar and Chet (37) recently reported that nylon fibers coated with either Concanavalin A or *Sclerotium rolfsii* 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. rolfsii* hyphae.

In light of these findings, one may assume that the interaction observed between hyphae of the antagonist and spores of *G. intraradices* is mediated by a specific cell surface recognition process that, in turn, triggers a program of events, including host wall penetration. Preferential interactions between *T. harzianum* hypha and *G. intraradices* spores may be explained by changes in wall chemical composition after spore germination and, consequently, in either the loss or the modification of target recognition molecules. Structural and chemical modifications of the cell wall occur during the life cycle of Glomalean fungi (10,11,12), and it is likely that such changes are related to the particular relationship established between these fungi and their partners.

Successful penetration of the host spore wall by the antagonist frequently was achieved, indicating that at least small amounts of cell wall hydrolytic enzymes, such as chitinases, were produced by the antagonist to locally weaken or loosen the spore cell wall. The production of extracellular lytic enzymes by *T. harzianum* is a well-documented phenomenon (43,44) involved in the process of antagonism against an array of pathogenic fungi (17,18). However, observations of the spore ultrastructure showed that generalized disorganization of the spore integrity and ultimately complete loss of the protoplasm occurred often in advance of *Trichoderma* penetration. This suggested that diffusible compounds with antifungal properties were likely produced by the antagonist. In all examined samples, spore penetration by the antagonist seldom was associated with marked host cell wall alterations. This was corroborated by the labeling pattern of *N*-acetylglucosamine, which showed that, except in the area of hyphal penetration, the chitin component of the host cell walls was structurally preserved.

Since the pioneering work of Weindling (57), the exact contribution of hydrolytic enzymes and antibiotics in the antagonistic process developed by *Trichoderma* spp. has been the focus of considerable interest and the subject of numerous debates (33,36). The observed reactions of the mycorrhizal spores neighboring *Trichoderma* hyphae are reminiscent of the disturbances detected in fungal cells exposed to antibiotics. In a recent study, Bélanger et al. (5) reported that antibiosis leading to host hypha death was an early event preceding the action of digestive enzymes in the interaction between a Québec isolate of *T. harzianum* and *Botrytis cinerea*. A similar pattern of reactions was described previously by Hajlaoui et al. (35) 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. In addition, in a recent experiment Schirböck et al. (50) demonstrated a synergistic interaction between hydrolytic enzymes and peptaibol antibiotics in the antagonistic action of *T. harzianum* against phytopathogenic fungi. In agreement with these studies, our results suggest that antibiosis causing cytoplasm disintegration precedes parasitism and subsequent internal colonization of host spores. One may presume that weakened or dead cells are invaded more easily by hyphae of the antagonist, which may then use the host cells as an essential food source for their growth and multiplication.

Trichoderma hyphae did proliferate abundantly in both the spores and the emerging hyphae of *G. intraradices*. Such active growth was accompanied by apparent mechanical pressure, leading to hyphal bursting of the host. However, in light of the labeling pattern of chitin, it seems reasonable to assume that the physical pressure exerted by the actively growing hyphae of the antagonist was not the only mechanism involved in host breakdown. The observation of digested wall areas at sites of potential release of the antagonist indicates that chitinases were produced and likely contributed to the rapid disruption of the hyphal wall in areas where mechanical pressure also occurred.

Based on the present observations, the antagonism of *G. intraradices* by *T. harzianum* appears to follow a specific schedule of events: (i) recognition between *Trichoderma* and mycorrhizal spores; (ii) possible production of antibiotic substances by the antagonist; (iii) production of cell wall-degrading enzymes, such as chitinases; (iv) spore penetration and active growth of the antagonist in mycorrhizal hyphae; and (v) excretion of hydrolytic enzymes and release of the antagonist. It remains to be shown whether such events occur in nature and to what extent they affect plant colonization by *G. intraradices*. It is important to note that the *Trichoderma* strain used in this study was selected for its superior biocontrol potential and that properties such as dual production of antibiotics and hydrolytic enzymes may not be shared by all *Trichoderma* isolates found in the rhizosphere.

In conclusion, this in vitro study provides evidence that the extramatrical phase of AM fungi is not protected from an attack by antagonistic fungi present in the mycorrhizosphere. Interestingly, McGovern et al. (46) reported that *T. harzianum* reduced significantly the colonization of tomato roots by *G. intraradices*, but a recent field study conducted by Datnoff et al. (22) disclosed that the combination of *T. harzianum* and *G. intraradices* was more effective than either fungus used singly in reducing Fusarium crown and root rot of tomato. Therefore, additional research is needed to evaluate the complex interactions between *T. harzianum* and *G. intraradices* that occur at the root-soil interface. The use of rRNA-targeted oligonucleotide probes would be an appropriate approach to determine by in situ hybridization or in situ polymerase chain reaction whether *Trichoderma* spp. infect AM fungi under natural conditions.

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