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Cellular and Molecular Mechanisms Involved in the Interaction between *Trichoderma harzianum* and *Pythium ultimum*

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The interaction between *Trichoderma harzianum* and the soilborne plant pathogen *Pythium ultimum* was studied by electron microscopy and further investigated by gold cytochemistry. Early contact between the two fungi was accompanied by the abnormal deposition of a cellulose-enriched material at sites of potential antagonist penetration. The antagonist displayed the ability to penetrate this barrier, indicating that cellulolytic enzymes were produced. However, the presence of cellulose in the walls of severely damaged *Pythium* hyphae indicated that cellulolytic enzymes were not the only critical traits involved in the antagonistic process. The marked alteration of the β-1,3-glucan component of the *Pythium* cell wall suggested that β-1,3-glucanases played a key role in the process.

Pythium species are among the most aggressive soilborne pathogens, causing seed rot and seedling damping-off of many crops (14). Despite substantial economic losses associated with the conspicuous and destructive nature of *Pythium* spp., management of the diseases caused by these soilborne plant pathogens through fungicidal seed treatments has proved to be impractical, mainly because of increased concern about the fate of synthetic chemicals in the environment and the development of fungicide-resistant *Pythium* strains (14). In that context, the major task now facing scientists is to develop, using a combination of approaches, alternatives to chemicals for effective management of crop diseases caused by *Pythium* spp. One such alternative, which has been proposed for biological control of several plant pathogens, involves the introduction of selected microorganisms such as *Trichoderma* spp. to the soil (6, 14). However, while laboratory experiments (12) and biological control field trials (15) document the ability of some *Trichoderma* strains to reduce *Pythium* inoculum in soil, a clear answer to the process by which these fungal antagonists contribute to biological control of *Pythium* spp. has not yet emerged, although mechanisms of antagonism including mycoparasitism, antibiosis, and competition have been suggested (4, 12). If one considers that *Pythium* spp. belong to the oomycetes, which are exceptional in that their cell walls contain β-(1,3)-(1,6)-D-glucans and cellulose instead of chitin as major structural components (3), it can be speculated that the role played by glucanases in the antagonistic process is crucial. In this paper, the pattern of *Pythium ultimum* colonization by *Trichoderma harzianum*, the biological events underlying this interaction, and the relative importance of hydrolytic enzymes in the antagonistic process are reported.

Mycelial interactions between *T. harzianum* and *P. ultimum*. *P. ultimum* Trow (BARR 447) (Center for the Land and Resources Research, Ottawa, Ontario, Canada), was grown on potato dextrose agar (PDA) (Difco Laboratories, Detroit, Mich.) in a dark incubator at 26°C. The isolate of *T. harzianum* (T-203) was grown on PDA medium at 23 to 25°C. For dual-culture tests, PDA disks (5-mm diameter), collected from the growing margins of fresh fungal cultures, were placed 3 cm apart on the surface of the PDA medium and allowed to grow at 25°C under continuous light. The antagonist and its host grew towards each other, and overgrowth of *P. ultimum* mycelium by hyphae of *T. harzianum* was recorded by 4 to 5 days after inoculation. By 7 days after inoculation, macroscopic observations of the plates showed that the antagonist had multiplied and sporulated abundantly. Agar samples from the regions where the colonies merged were collected at 2, 3, 4, 6, and 7 days after inoculation and processed for scanning and transmission electron microscopy.

Scanning electron microscope studies. Mycelial samples, cut from the interaction region in dual-culture tests, 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 prior to examination with a Cambridge Stereoscan 5-150 scanning electron microscope (Cambridge Scientific Industries, Cambridge, Mass.) operating at 20 kV. As early as 2 days after the inoculation, hyphae of *T. harzianum*, easily recognized by their smaller diameter, coiled around the *Pythium* cells (Fig. 1a, arrows). The coils were usually very dense and tightly encircled the hyphae of *P. ultimum*, leading to strong compression of the host cells as illustrated by the wrinkled appearance of the cell surface (Fig. 1a). At a more advanced stage of parasitism, coiling of *T. harzianum* around *P. ultimum* hyphae increased and host cell collapse was easily discernible (data not shown).

Cellular events involved in the *Trichoderma-Pythium* interaction. Mycelial samples from the interaction region were preembedded in 2% (wt/vol) aqueous Bacto-agar, immersed in 3% (vol/vol) glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 2 h at room temperature, and postfixed with 1% (wt/vol) osmium tetroxide in the same buffer for 1 h at 4°C prior to being dehydrated in a graded ethanol series and embedded in Epon 812. For the localization of cellulosic β-1,4-glucans, an exoglucanase (β-1,4-D-glucan cellobiohydrolase [EC 3.2.1.21]), purified from a cellulase produced by the fungus *T. harzianum*, was complexed to colloidal gold at pH 9.0 (2). For the localization of β-1,3-glucans, a purified tobacco β-1,3-glucanase (the PR protein, PR-N) (7) was complexed to colloidal gold at pH 5.5 (1). Ultrathin sections were first

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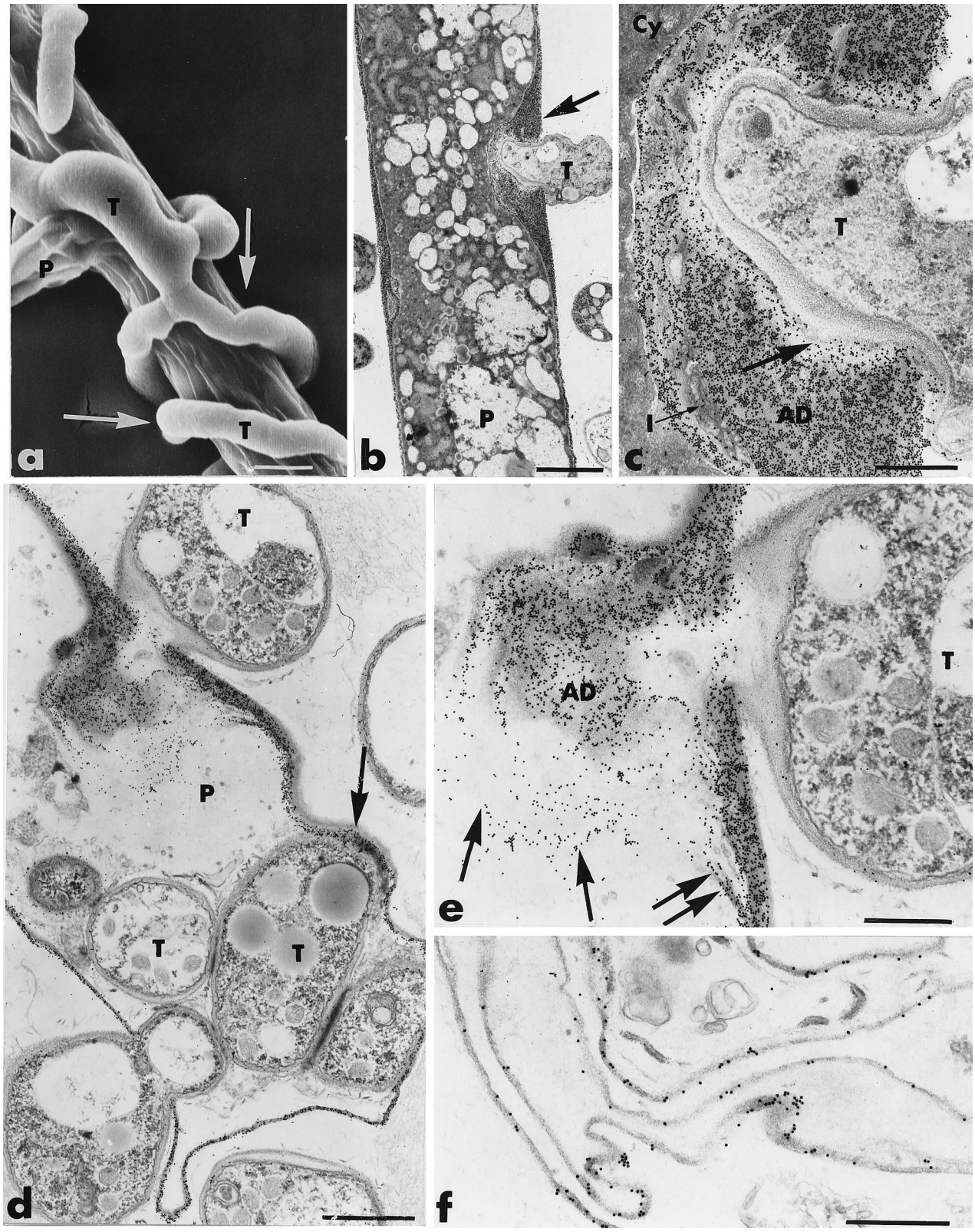
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FIG. 1. Scanning and transmission electron micrographs of *T. harzianum* (T) hyphae interacting with cells of *P. ultimum* (P) in dual cultures. (a) Scanning electron micrograph of the interaction, 3 days after inoculation. *Trichoderma* hyphae form dense coils (arrows) and tightly encircle hyphae of *P. ultimum*. A strong compression of the host cell is shown by the wrinkled appearance of the cell surface. Bar, 3 μm. (b to f). Transmission electron micrographs of the interaction, showing labeling of cellulosic β-1,4-glucans with the exoglucanase-gold complex. (b and c) Interaction 2 days after inoculation. A hypha of *P. ultimum* is encircled by hyphae of *T. harzianum*. Structural changes characterized by an increase in the number of vacuoles and a local retraction of the plasma membrane (arrow in panel b) are visible. An amorphous deposit (AD) accumulates between the retracted plasma membrane and the cell wall. The newly deposited material is intensely labeled except in areas corresponding to inclusions (I). A decrease in labeling intensity is seen along the channel of *Trichoderma* penetration (arrow in panel c). Bars, 2 μm (b) and 0.5 μm (c). (d and e) Interaction 6 days after inoculation. *Pythium* hyphae appear as empty pleiomorphic shells. Cells of the antagonist invade the area originally occupied by the host cytoplasm and exert a mechanical pressure on the host cell wall (arrow in panel d). Loosening and even dissolution of the wall-like deposits (AD) are seen in areas of *Trichoderma* penetration, as illustrated by the release of gold particles at a distance from the deposits (arrows in panel e). Shredding of the innermost pathogen wall layers is visible (double arrows in panel e). Bars, 1 μm (d) and 0.5 μm (e). (f) Interaction 7 days after inoculation. *Pythium* cells are reduced to some wall remnants which are slightly labeled with the exoglucanase-gold complex. Bar, 0.25 μm.

floated for 5 min on a drop of 0.01 M sodium phosphate-buffered saline containing 0.02% polyethylene glycol 20,000 at the pH corresponding to that for the optimal activity of the protein tested (6.0 for the two enzymes). Sections were transferred to a drop of each gold-complexes enzyme, kept in a moist chamber for 30 to 60 min, washed with phosphate-buffered saline (pH 7.4), rinsed with distilled water, and stained with uranyl acetate and lead citrate for direct examination with a 1200 EX electron microscope (JEOL, Tokyo, Japan) operating at 80 kV. An average of five samples from each sampling time was examined, with five sections per sample. The specificity of the labelings was assessed by at least two control tests: (i) addition of the corresponding substrate to each protein-gold complex for a competition experiment (β-1,4-glucans from barley [1 mg ml^{-1}] for the β-1,4-exoglucanase–gold complex and laminarin [1 mg ml^{-1}] for the β-1,3-glucanase–gold complex and (ii) incubation of the sections with the enzyme-gold complexes under nonoptimal conditions for biological activity. The density of labeling obtained with the enzyme-gold complexes over cell walls of *P. ultimum* was determined by counting the number of gold particles per square micrometer. Area determinations were carried out by the point counting method established by Weibel (13), using negatives of electron micrographs projected on a lattice. The amount of labeling over specified wall areas (Sa) was estimated by counting the number of gold particles (Ni) on a photographic enlargement. The density of labeling (Ns) was calculated as Ns = Ni/Sa, where Ns represents the number of gold particles per unit of surface area.

Examination of ultrathin sections from the interaction region of 2-day-old dual cultures showed that cells of *P. ultimum*, encircled by hyphae of *T. harzianum*, exhibited changes, mainly characterized by an increased vacuolation and a local retraction of the plasma membrane at sites of potential antagonist penetration (Fig. 1b, arrow). Observations at a higher magnification revealed that, in most cases, retraction of the plasma membrane was accompanied by the deposition of an amorphous matrix in which small electron-dense structures were embedded (Fig. 1c). These hemispherical deposits were heavily labeled by the β-1,4-exoglucanase–gold complex (Fig. 1c). Attempts of the antagonist to penetrate this newly formed material frequently succeeded and resulted in a decrease of labeling intensity along the channel of penetration (data not shown). The mechanisms that control the process of cellulose deposition at such sites are unclear, although at least two explanations may be advanced. First, the accumulating material may originate from the host cell wall through the swelling and stretching of preexisting polymers. This obviously would require the action of cellulases produced by the antagonist and would imply a subsequent decrease in wall-bound cellulosic compounds in *Pythium* cells. However, at that time after inoculation, we did not observe marked alterations of *Pythium* cell walls or significant differences in the intensity of cellulose labeling compared to that for control hyphae grown in single cultures (Table 1). Second, the deposited material may be laid down as newly synthesized molecules. This possibility raises the question of how the fungal cell can regulate the deposition of wall-like material in unusual cell areas. Membrane-bound proteins such as enzymes involved in the synthesis of structural compounds are known to be linked to lipids which play a major role in regulating the permeability of the plasma membrane (9, 10). Alteration in the lipid composition of the plasma membrane of *Pythium* cells, possibly resulting from the action of toxic compounds produced by *T. harzianum*, may therefore induce a deregulation of membrane-bound enzymes, resulting in areas of abnormal wall-like deposition. The exact biological function of the cellulose-rich deposits is not understood, but one may speculate that the massive accumulation of structural compounds at unusual sites reflects a defense strategy elaborated by *Pythium* cells for preventing penetration of the antagonist.

By 3 to 4 days after inoculation, *Pythium* cells appeared to be highly damaged as judged by the complete retraction and/or the rupture of the plasma membrane and by the pronounced disorganization of the cytoplasm. Cell walls appeared thicker than normal and were evenly labeled by the gold-complexed exoglucanase except in the areas of *Trichoderma* penetration (data not shown). By 6 days after inoculation, *Pythium* hyphae appeared essentially as abnormally shaped, empty pleiomorphic shells (Fig. 1d). At that advanced state of parasitism, cells of the antagonist ramified extensively in the host hyphae and invaded the area which originally was occupied by the host cytoplasm. Such a massive colonization frequently resulted in a strong mechanical pressure (Fig. 1d, arrow) against the host hyphal cell walls. In spite of this severe host cell degradation, exoglucanase-binding sites still occurred over the host cell walls, even in areas adjacent to the sites of fungal penetration (Fig. 1e). However, quantification of labeling indicated a significant decrease in the labeling intensity of these cell wall

TABLE 1. Density of labeling obtained with the gold-complexes exoglucanase and β-1,3-glucanase over cell walls of *P. ultimum* during the interaction with *T. harzianum*

Time after inoculation in dual cultures (days)	Mean no. of gold particles per μm^2 ± SD[a] with:	
	Exoglucanase-gold	β-1,3-Glucanase-gold
Control (single culture)	94.6 ± 14.8	51.3 ± 12.2
2	87.8 ± 10.3	22.6 ± 10.1
3	78.2 ± 9.5	10.3 ± 6.4
4	60.5 ± 12.2	8.2 ± 2.0
6	31.8 ± 5.3	1.0 ± 0.5
7	18.5 ± 6.4	0

[a] Densities were determined by counting the number of gold particles over specified areas of cell walls on 20 micrographs.

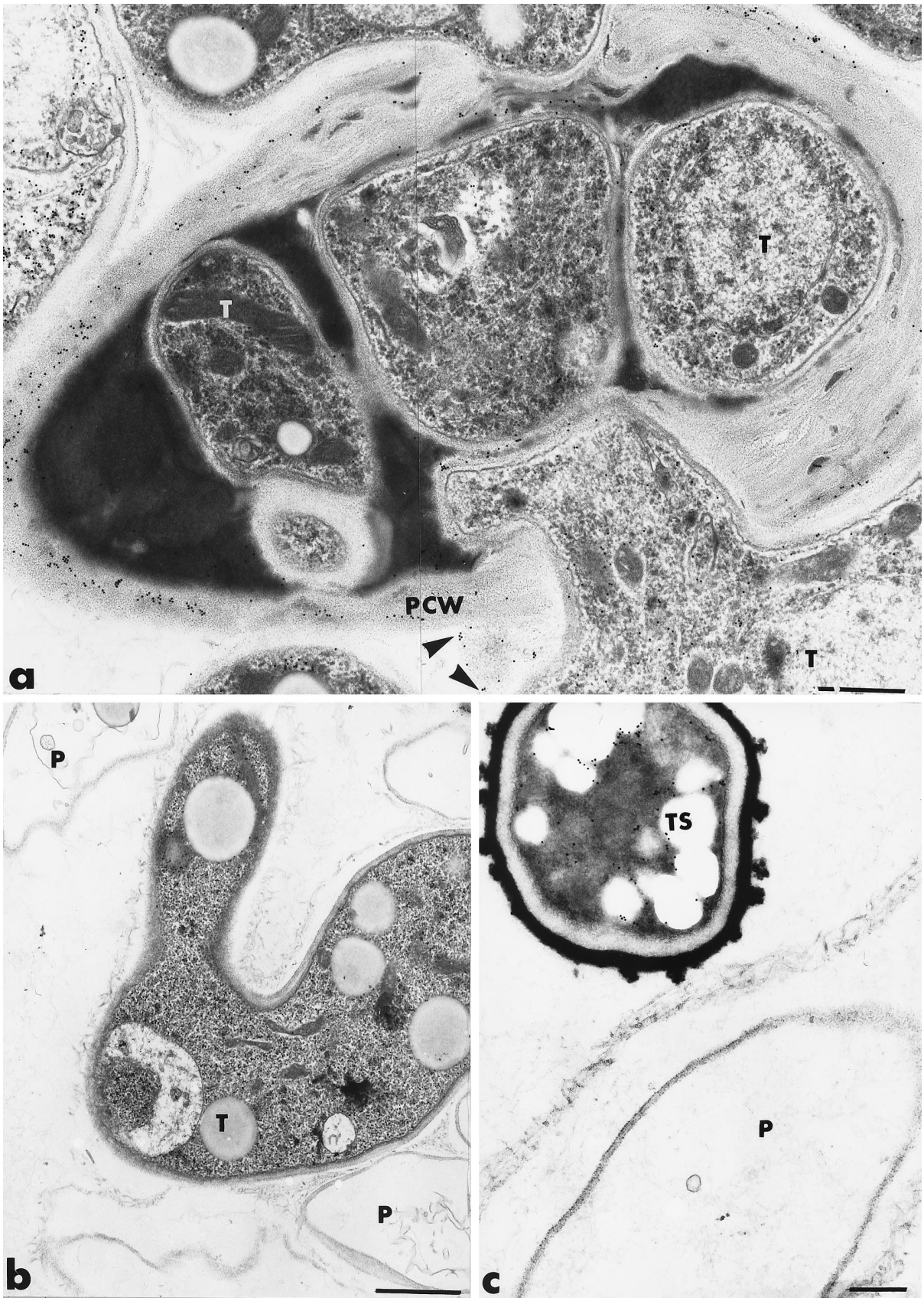

FIG. 2. Transmission electron micrographs of *T. harzianum* (T) hyphae interacting with cells of *P. ultimum* (P) in dual cultures, showing labeling of β-1,3-glucans with the tobacco β-1,3-glucanase. (a) Interaction 3 days after inoculation. In a massively colonized *Pythium* cell, the cytoplasm is highly aggregated and labeling is restricted to the outer wall layers. No labeling over the wall-like deposits is seen. At the site of *Trichoderma* penetration, the release of labeled fibrillar fragments is visible (arrowheads). Bar, 0.5 μm. (b and c) Interaction 6 to 7 days after inoculation. β-1,3-Glucans are no longer present in the walls of altered *Pythium* hyphae. Spores of *Trichoderma* (TS) are seen in the agar medium. Bars, 0.5 μm (b) and 0.25 μm (c).

layers compared to that found in *Pythium* cells collected 2 days after inoculation in dual cultures (Table 1). Loosening and even dissolution of the deposits initially formed between the invaginated plasma membrane and the host cell walls were often seen in areas of *Trichoderma* penetration, as exemplified by the release of numerous gold particles at a distance from the deposits (Fig. 1e, arrows). This obviously indicates that large amounts of cellulolytic enzymes are produced. A close examination revealed that some features of degradation, mainly characterized by the shredding of the innermost wall layers, occurred in the host wall areas neighboring fungal penetration (Fig. 1e, double arrows). However, the finding that cellulose labeling still occurred over the walls of damaged *Pythium* suggested that cellulolytic enzymes were not the only critical traits involved in the antagonistic process. Although it is clear that such enzymes play a key 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 main wall-bound compounds are utilized as a food source, providing the energy required to multiply and sporulate abundantly. This was corroborated by labeling quantification. Although a decrease in wall labeling intensity, probably attributable to penetration by an increased number of *Trichoderma* hyphae, was recorded during the course of the mycoparasitic process, it was only at an advanced stage of the interaction (6 to 7 days) that substantial cellulose digestion could be detected. Indeed, by 7 days after inoculation, *Pythium* cells were completely degraded, and, in most cases, only some slightly labeled wall remnants were indicative of the former presence of a fungal cell (Fig. 1f; Table 1).

Following incubation of sections from the interaction region with the gold-complexed β-1,3-glucanase, a significant decrease in the labeling of *Pythium* cell walls was observed as early as 2 days after inoculation (Table 1). By 3 to 4 days after inoculation, labeling was reduced to a fine band of gold particles over the outermost host wall layers (Fig. 2a). At sites of antagonist penetration, the release of labeled fibrillar fragments was recorded (Fig. 2a, arrowheads). At an advanced stage of the antagonism (6 to 7 days after inoculation), labeling of *Pythium* cell walls with the β-1,3-glucanase disappeared (Fig. 2b). At that time, the antagonist sporulated abundantly (Fig. 2c).

The observed alteration of the β-1,3-glucan component of the host cell wall is consistent with the known capacity of *Trichoderma* spp. to produce hydrolytic enzymes, such as β-1,3-glucanases (8, 12). Alteration of β-1,3-glucans occurred not only in wall areas adjacent to *Trichoderma* cells but also at a distance from the sites of antagonist entry, so β-1,3-glucanases may have freely diffused extracellularly, likely facilitating *Trichoderma* ingress through loosened wall matrices. These observations raise the question as to how much weakening of the cell wall through the action of *Trichoderma* β-1,3-glucanases may facilitate the diffusion of toxic compounds towards membrane receptors by increasing the wall permeability, as recently pointed out by Di Pietro et al. (5) for the biocontrol process mediated by *Trichoderma virens*. A similar scheme of events was described by Schirmböck et al. (11), who confirmed that the cooperation between enzymes and antibiotics also was an important feature of the antagonistic process in the *T. harzianum-Botrytis cinerea* interaction.

Based on the present observations, the following scheme of sequential events can be proposed: (i) recognition and attachment of *T. harzianum* to *Pythium* hyphae, (ii) production of β-1,3-glucanases to weaken the host cell wall and slight production of cellulases to allow local penetration of the antagonist, (iii) production of antibiotic substances to deregulate the host cell metabolism, and (iv) host cell invasion and active production of cellulases, leading to pathogen cell breakdown.

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