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Inhibition of fungal growth by plant chitinases and β-1,3-glucanases

A morphological study

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Summary. Plant chitinases and β-1,3-glucanases have been demonstrated to inhibit fungal growth in model experiments, both on agar plates or in liquid media. Here, *Trichoderma longibrachiatum* was taken as a model to study the morphological changes caused by chitinase and glucanase treatments, using cytochemical techniques in combination with fluorescence and electron microscopy. Chitinase, alone or in the presence of glucanase, arrested growth of the hypha: it affected the extreme tip of the fungus producing a thinning of the wall, a balloon-like swelling and a rupture of the plasma membrane. Chitin and glucans were present in the wall, as shown by lectin- and enzyme-binding experiments, but they had a different susceptibility to chitinase and β-1,3-glucanase. Chitin was present at the apex and in the inner parts of the lateral walls; it was more susceptible to chitinase at the tip than in the subapical part. Glucans mostly occurred on the outer layer where they were degraded by glucanase. The latter did not affect the inner hyphal skeleton.
It is suggested that the growth inhibition of *Trichoderma* by hydrolytic enzymes is the consequence of a thinning of the cell wall in the hyphal apex, leading to an imbalance of turgor pressure and wall tension which causes the tip to swell and to burst.

Keywords: Fungal growth; Chitinase; β-1,3-Glucanase; Antifungal hydrolases; Pea; *Trichoderma*.

Abbreviations: WGA-FITC wheat germ agglutinin labelled with fluorescein isothiocyanate; ConA-FITC concanavalin A labelled with fluorescein isothiocyanate; PEG polyethylene glycol; SEM scanning electron microscopy; TEM transmission electron microscopy.

Introduction

Plant chitinases and β-1,3-glucanases have been called antifungal hydrolases (Boller 1988) since they inhibit fungal growth in model experiments, both on agar plates (Schlumbaum et al. 1986, Mauch et al. 1988 b) or in liquid media (Ludwig and Boller 1990). Rapidly growing saprophytes, including various species of *Trichoderma*, are inhibited by plant chitinases alone (Schlumbaum et al. 1986; Roberts and Selitrennikoff 1988; Broekaert et al. 1988, 1989). More slowly growing fungi, including many plant pathogens, are insensitive to either chitinase or β-1,3-glucanase alone, but most of them are strongly inhibited by combinations of the two enzymes (Mauch et al. 1988 b, Ludwig and Boller 1990). Combinations of the two enzymes inhibit most fungi with chitin-glucan cell walls although some symbiotic fungi with particularly slow growth appear not to be affected (Arlorio et al. 1992). Observations in the light microscope showed that growth inhibition by the enzymes was accompanied by swellings and lysis of the hyphal tips, indicating that the antifungal activity of the enzymes was caused by a weakening of the cell walls of the hyphal tips (Mauch et al. 1988 b, Broekaert et al. 1989, Arlorio et al. 1992). Here, we present a morphological study to examine the inhibitory effect of chitinase and β-1,3-glucanase in more detail, using combinations of cytochemical in situ techniques based on light, fluorescence and electron microscopy. *Trichoderma longibrachiatum*, a fungus highly sensitive to plant chitinases and β-1,3-glucanases, was used as an experimental model. The aims of the study were: (1) to describe the process of growth inhibition at the structural and ultrastructural level, (2) to study the presence and the accessibility of the substrates of chitinase and β-1,3-glucanase in the fungal cell walls, and (3) to identify the target zones of these enzymes.

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Material and methods

Biological material

Trichoderma longibrachiatum Rifai (strain SA 23; Botanisches Institut, Universität Basel) was cultivated on 3% malt extract agar.

Purified enzymes

The two antifungal hydrolases, chitinase (EC 3.2.1.14.) and β-1,3-glucanase (EC 3.2.1.39.) were purified from infected pea pods (*Pisum sativum* L. cv. "Dot" var. Alaska) and analyzed by SDS PAGE as described (Mauch et al. 1988 a, Ludwig and Boller 1990).

Bioassay of fungal growth inhibition

Growth inhibition on solid medium was studied with the technique of Schlumbaum et al. (1986). Briefly, *T. longibrachiatum* was grown on malt extract agar, and solutions of purified enzymes were applied in the range of 15–30 µl (75 ng/µl for chitinase and 93 ng/µl for β-1,3-glucanase) in wells punched into the agar in front of the growing fungus. Controls were performed by adding H_2O instead of the enzyme solutions in punched wells.

Light microscopy and affinity techniques

The effect of the enzymes was studied in hyphae growing on agar. Samples adjacent to the wells were taken at intervals from 0 to 6 h after application of the enzyme solutions. The samples were mounted on a glass slide and observed under a Zeiss light microscope equipped with Nomarski optics and epi-illumination. To avoid stresses due to the transfer, parallel experiments were performed with fungal cultures spreading on glass slide covered by a thin agar layer. Enzymes were directly applied to the growing hyphae and their effects were followed under the microscope. For fluorescence microscopy, unfixed hyphae were treated with 100 µg/ml fluorescein-labelled wheat germ agglutinin (WGA-FITC) or fluorescein-labelled concanavalin A (ConA-FITC) in 0.1 M HEPES buffer at pH 7.8 for 20 min and washed. Samples were mounted and observed in the epifluorescence mode using a BP 450–490 excitation filter and a BP 520–560 barrier filter. Some hyphal tips were treated with 0.6% NaClO to extract cell wall glucans before lectin staining. Controls with inhibitory sugars (N′,N″,N‴-triacetylchitotriose) were carried out as detailed in Bonfante et al. (1987). Samples of hyphae were also treated for 20 min with Calcofluor (0.01%), an apoplastic dye revealing the cell wall organisation. These samples were mounted and observed in the epifluorescence mode using a BP 395–446 excitation filter and a BP > 470 nm barrier filter.

To reveal enzyme substrates, enzymes linked to colloidal gold were used (Bendayan 1985), employing silver enhancement to detect the colloidal gold particles. Chitinase/colloidal gold and β-1,3-glucanase/colloidal gold were obtained as described by Bonfante et al. (1990) with the following modification: 100 µl of enzymatic solution (1 mg/ml) were mixed with 5 ml of colloidal gold (pH 9) and with 500 µl of 1% PEG (polyethylene glycol) in water, centrifuged at 25,000 rpm at 4 °C for 45 min and taken up in 0.05 M PBS buffer, pH 6, containing 1% PEG.

Cultures of *T. longibrachiatum* spreading on glass slides were washed in PBS buffer, pH 7.2 for 10 min, treated with the enzyme-gold complex for 40 min at room temperature and washed again in buffer and in water. The reaction given by the gold deposition was enhanced by a silver treatment according to Peretto et al. (1990) and observed under Nomarski optics. Control experiments were carried out by adding 1 mg/ml chitin (poly-N-acetyl glucosamine) to the chitinase-gold complex at least 1 h before use or by using the colloidal gold solution alone.

Scanning electron microscopy (SEM) and transmission electron microscopy (TEM)

Treated and control hyphae of *T. longibrachiatum* were fixed in 2% glutaraldehyde (GA) in 0.1 M cacodylate buffer at pH 7.4 for 2 h, pouring the fixative into the plates, in order to flood the cultures. After 1 h hyphal tips were cut, treated with fresh GA, postfixed in 1% osmium tetroxide in the same buffer for 2 h and dehydrated as described (Bonfante et al. 1987). A part of the samples was mounted on stubs, subjected to critical point drying, coated with gold and examined by SEM. A second part of the samples was embedded in Araldite and processed for the TEM (Roland and Vian 1991).

Ultrathin sections (0.05–0.07 µm) were cut using a diamond knife, handled with plastic rings and stained with the PATAg method (Roland and Vian 1991) in order to visualize polysaccharides. The sections were put on 200 mesh copper grids and observed with a CM 10 electron microscope (Philips).

For lectin labelling at the ultrastructural level, thin sections handled with plastic rings were pretreated with a saturated solution of sodium metaperiodate for 20 min and thoroughly washed in distilled water for 20 min, treated with WGA-colloidal gold complex, washed again and put on 200 mesh copper grids. They were then counterstained with uranyl acetate and lead citrate. Control sections were performed by adding N-acetylglucosamine to the lectin-gold complex 1 h before using it on the thin sections.

Analysis of cell wall glycans

To evaluate the presence of cell wall β-1,3 glucans, 8.3 g of *T. longibrachiatum* mycelium were shaken with 5 ml of NaClO (0.6%) at 21 °C for 20 min. The sugars released after NaClO treatment were estimated according to the phenol/sulphuric acid procedure of Dubois et al. (1956) using D-glucose as standard.

Chemicals

WGA-FITC and ConA-FITC were purchased from Vector Laboratories, Burlingame, CA; WGA-gold complex from Polysciences, Warrington, PA; N-acetylglucosamine and p-dimethylaminobenzaldehyde from Sigma, St. Louis, MO; Chitin from United States Biochemical Corporation, Cleveland, OH; Tetrachloroauric acid from Merck Laboratories, Darmstadt, Federal Republic of Germany; and a silver enhancement kit from Janssen, Olen, Belgium.

Results

Effect of antifungal enzymes on the morphology of hyphal tips

Combinations of chitinase and β-1,3-glucanase from pea pods have been shown to inhibit growth of various fungi (Mauch et al. 1988 b). We examined the effect of these enzymes on growth of *Trichoderma longibrachiatum*. The chitinase employed was a mixture of the forms CH 1 (33 kDa) and CH 2 (36 kDa), while β-1,3-glucanase was a single enzyme of 34 kDa (Fig. 1). In accordance with previous reports on other *Trichoderma* spe-

cies (Schlumbaum et al. 1986; Mauch et al. 1988 b; Broekaert et al. 1988, 1989), growth of *T. longibrachiatum* was inhibited by chitinase alone or by a combination of glucanase and chitinase, while glucanase alone was ineffective (Fig. 2).

In order to study the effects of the hydrolases on the morphology of *T. longibrachiatum*, hyphae were observed by light and fluorescence microscopy as well as by SEM and TEM. Hyphae of *Trichoderma longibrachiatum* grew by apical growth with a typical tapered tip which was continuous with the cylindrical sub-apical zones (Fig. 3 a and b). The most apical 1–2 µm had a homogenous dense cytoplasm (Fig. 3 b), while the adjacent subapical zone contained organelles, some of which were identified as mitochondria by their vermiform shape, refractile vesicles and nuclei. The first septum was usually found 50–60 µm from the tip (Fig. 3 c). There, hyphae became increasingly vacuolated with rounded vacuoles occupying the most of the cell volume (Fig. 3 c).

After treatment with β-1,3-glucanase, no morphological modifications were evident (Fig. 3 d) compared to a control (Fig. 3 a and b). However, 1–2 h after a treatment with chitinase alone or with a combination of chitinase and β-1,3-glucanase (Fig. 3 e–l), many hyphal tips showed a marked swelling, reaching 20–30 µm in diameter. A close examination in the light microscope showed that some swollen tips had clearly defined outlines (Fig. 3 e–f); a septum was often seen between the subtending hypha and the swollen tip (Fig. 3 f–h). The swollen tips were brightly fluorescent after calcofluor staining or after WGA-FITC treatment (Fig. 3 j). Other tips showed fuzzy outlines (Fig. 3 i and k), apparently because the ballooned tip had burst. In this stage, the tips showed no longer any fluorescence after calcofluor or WGA-FITC staining (Fig. 3 l).

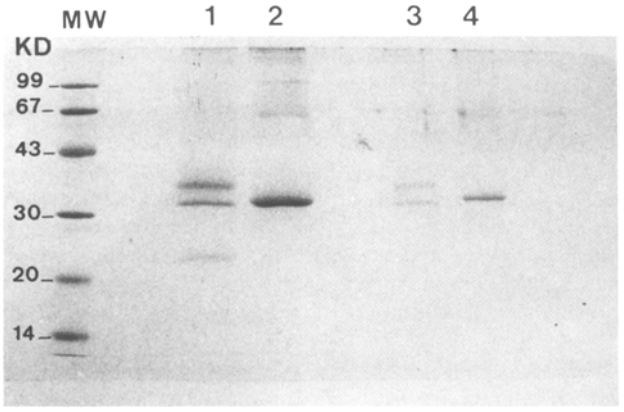

Fig. 1. Analysis of purified pea chitinase and β-1,3-glucanase by SDS PAGE. *1* and *3* 180 and 37 µg of the purified chitinase applied, respectively. *2* and *4* 232 and 46 µg of the purified glucanase applied

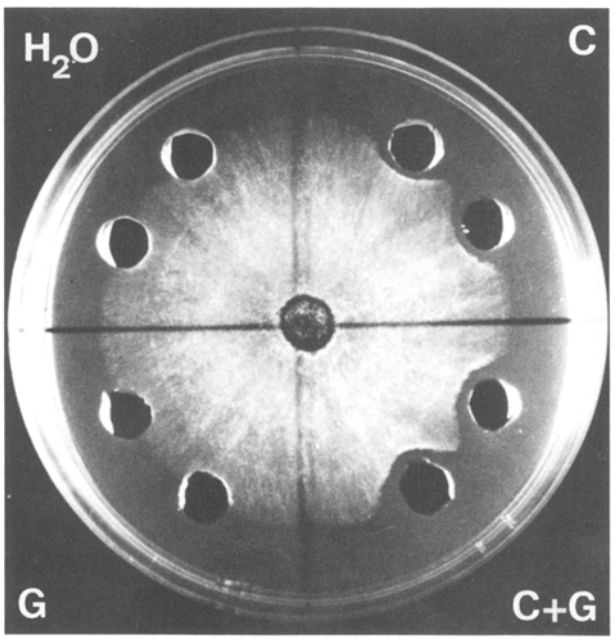

Fig. 2. Effect of H_2O, chitinases (*C*), β-1,3-glucanase (*G*), and chitinase and glucanase (*C* + *G*) on radial growth of *T. longibrachiatum*

In situ localization of substrates for chitinase and β-1,3-glucanase in the hyphae

After treatment with WGA-FITC, a chitin-specific lectin, a weak but regular fluorescence was observed along the hyphal walls (Fig. 4 a). A similar but more intense pattern of staining was observed after labelling with ConA-FITC (Fig. 4 c). In both experiments, preincubation of the lectins with low molecular weight ligands (N′,N″,N‴-triacetylchitotriose and α-methylmannoside, respectively) prevented appearance of the fluorescence (results not shown). Hyphae were incubated with 0.6% NaClO, a treatment which removes glucans. Analysis of the NaClO extract indeed revealed the removal of 23.3 µg glucose equivalents per g mycelium (data not shown). After this treatment, the hyphal walls became strongly fluorescent after WGA-FITC labelling (Fig. 4 b) but showed only a very weak fluorescence limited to the cytoplasmic part, and none at all over their cell walls after ConA-FITC labelling (Fig. 4 d).

When examined at ultrastructural level, the fungal cell wall appeared very thin (50–100 nm), and after PATAg reaction for the polysaccharide localization there was a diffuse distribution of silver granules (Fig. 5 a). A more reactive outer layer was evident (Fig. 5 a and b). Glycogen particles were labelled, too. WGA-gold complex labelled the wall in a regular pattern (Fig. 5 c).

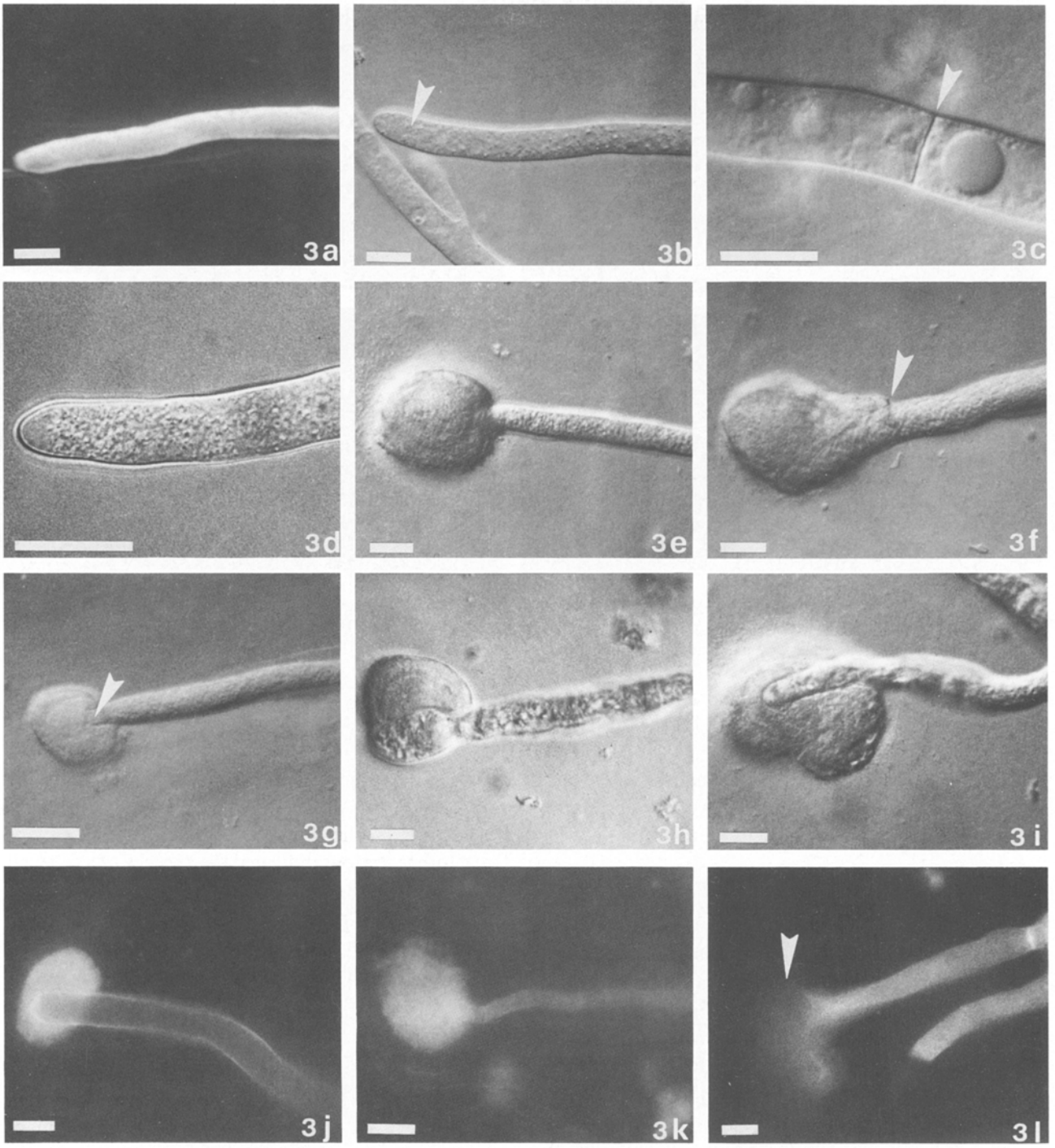

Fig. 3. Morphology of hyphae growing in the absence (**a–c**) and in the presence of plant enzymes (**d–l**). **a** and **b** SEM and LM of a growing hypha. ▶ Subapical region. **c** Beginning of the vacuolated region near the first septum (▶). **d** A growing tip appears unaffected by the glucanase treatment. **e–i** Balloon-like swelling of the hyphal apex after chitinase and glucanase treatment. **j** and **k** WGA-FITC and calcofluor treatments cause a fluorescence over the apical swelling. **l** No labelling is observed over the burst swelling after the Calcofluor treatment. Bars: 10 μm

In order to examine whether glucans and chitin are accessible to the enzymes in vivo, living hyphae of *T. longibrachiatum* were treated with gold complexes of chitinase and glucanase, and the gold particles were localized at the level of light microscopy by silver enhancement. The glucanase-gold complex was irregu-

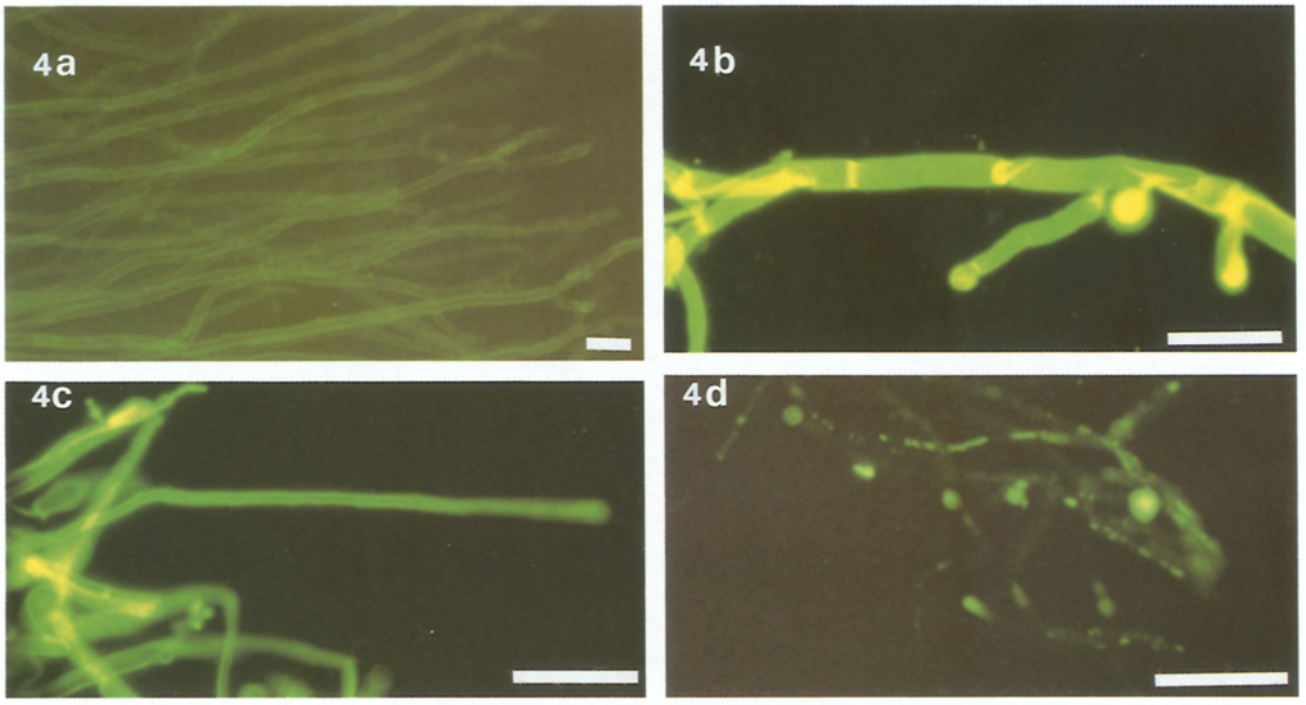

Fig. 4. Staining of hyphae growing in the absence of plant enzymes with fluorescence-labelled lectins. **a** WGA-FITC: a regular labelling is present over the walls. **b** The labelling is increased after the NaClO treatment. **c** ConA-FITC produces an intense labelling over all the hyphae. **d** After NaClO treatment, no labelling is observed over the cell walls. Bars: 25 μm

larly distributed over the entire hyphal wall (Fig. 6 a), while the chitinase-gold complex was present primarily at the tips of the growing fungus (Fig. 6 b).

Ultrastructural features of the fungal cell wall after enzyme treatments

After a treatment with β-1,3-glucanase, the hyphal cell walls showed minor but distinct morphological changes, appearing slightly swollen (Fig. 7 a). The outer transparent layer disappeared, and the reactivity of the wall to the PATAg test was strongly reduced (Fig. 7 b). In contrast, the cytoplasmic glycogen was still strongly labelled (Fig. 7 c).

After treatments with chitinase alone or with combinations of chitinase and β-1,3-glucanase, the hyphal wall was much more swollen, appearing loose with detaching fibrils (Fig. 7 c). Labelling with WGA-gold was intense (Fig. 7 d), with a cloud of gold granules surrounding the hyphae.

Upon formation of the balloon-like tip and the septum below it, different features were observed: the swollen tips had a strongly altered cytoplasm, containing a mass of disorganized membranous structures (Fig. 8 a–c). Some of the sections across the balloon-like tips showed the presence of an uneven and fluffy wall (Fig. 8 a) which was labelled by WGA-gold (Fig. 8 b). In contrast, other sections indicated the absence of a cell wall, with the plasma membrane still intact but exposed to the medium or ruptured, releasing cytoplasmic remnants into the medium (Fig. 8 c).

The wall of the subapical part of the hypha was labelled by the WGA-gold complex (Fig. 8 b). Some subapical hyphae appeared to have maintained their structural integrity, as seen by the normal appearance of mitochondria and other organelles (Fig. 8 a and b). In other cases, the cytoplasm was altered (Fig. 8 c) and detached from the wall, as if a strong plasmolysis event had occurred, and showed considerable degeneration (Fig. 8 d).

Discussion

The biochemistry and molecular biology of plant chitinases and β-1,3-glucanases have been studied extensively as paradigms of active resistance mechanisms against fungi, based on the idea that these enzymes act against fungi with chitin-glucan cell walls (for reviews,

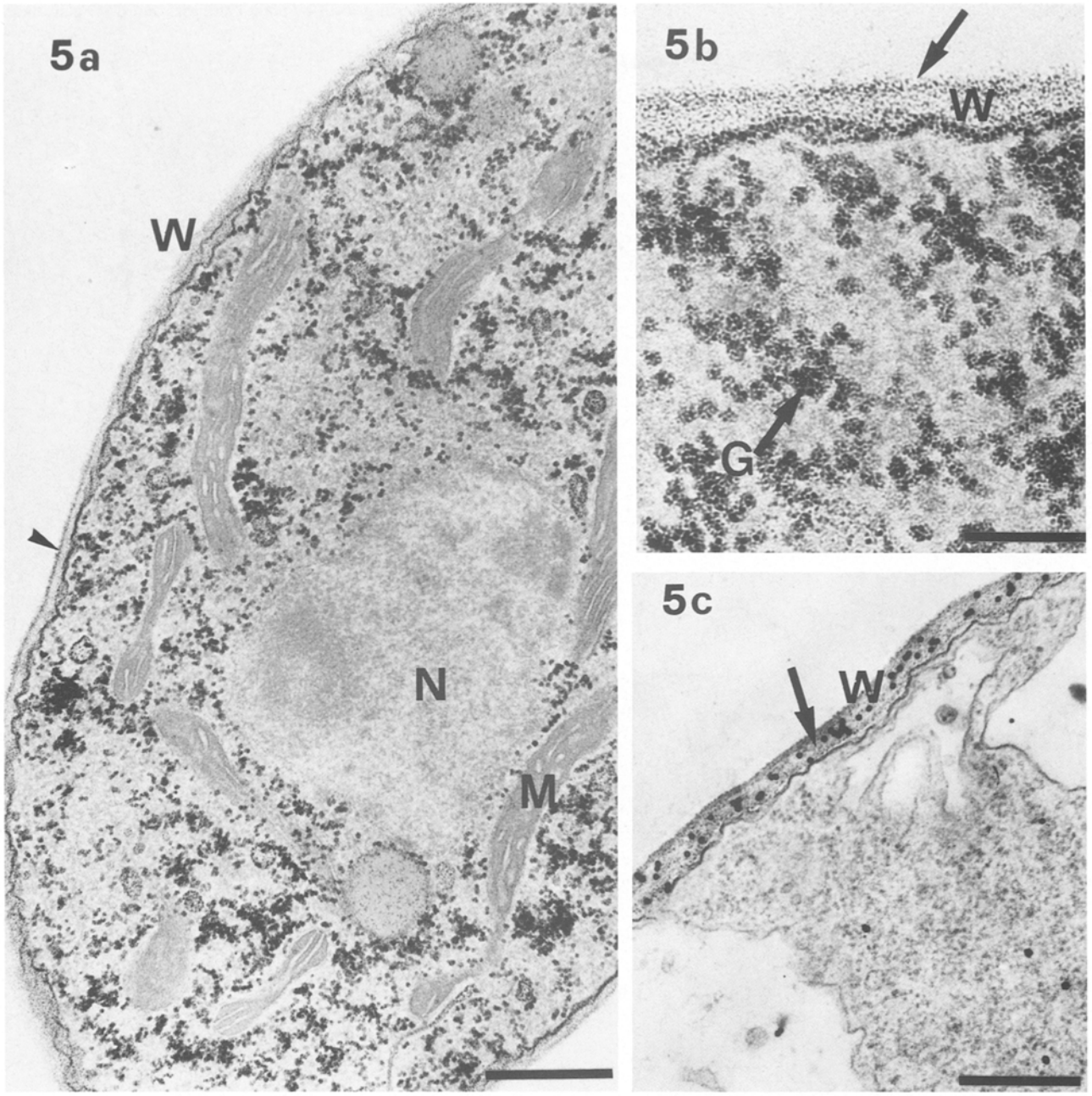

Fig. 5. Ultrastructural cytochemical characterization of the cell walls of hyphae growing in the absence of plant enzymes. **a** The wall (*W*) is thin with an outer more reactive layer (►). *N* Nucleus, *M* mitochondrion. **b** Magnification of the wall (*W*), showing the reactivity to the PATAg test. *G* Glycogen. **c** A regular distribution of gold granules is present over the inner part of the wall (*W*) after treatment with WGA-gold. Bars: 0.5, 0.12, 0.5 µm, respectively

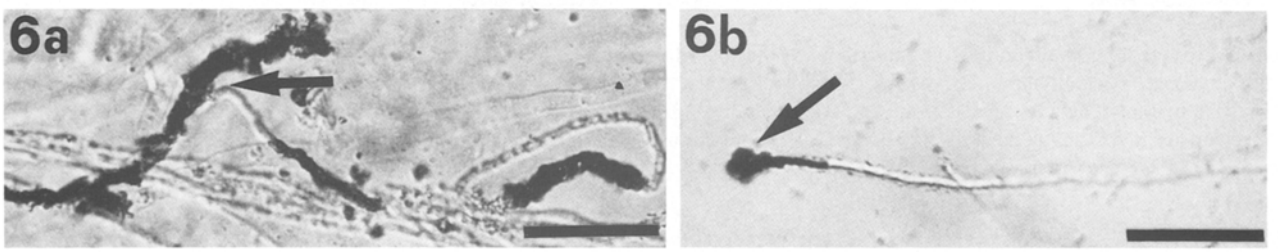

Fig. 6. Labelling of cell walls of hyphae growing in the absence of plant enzymes with gold complexes of plant β-1,3-glucanases and chitinases. **a** Glucanase-gold complex irregularly binds to the fungal walls. **b** Chitinase-gold complex only binds to the tip. Bars: 25 µm

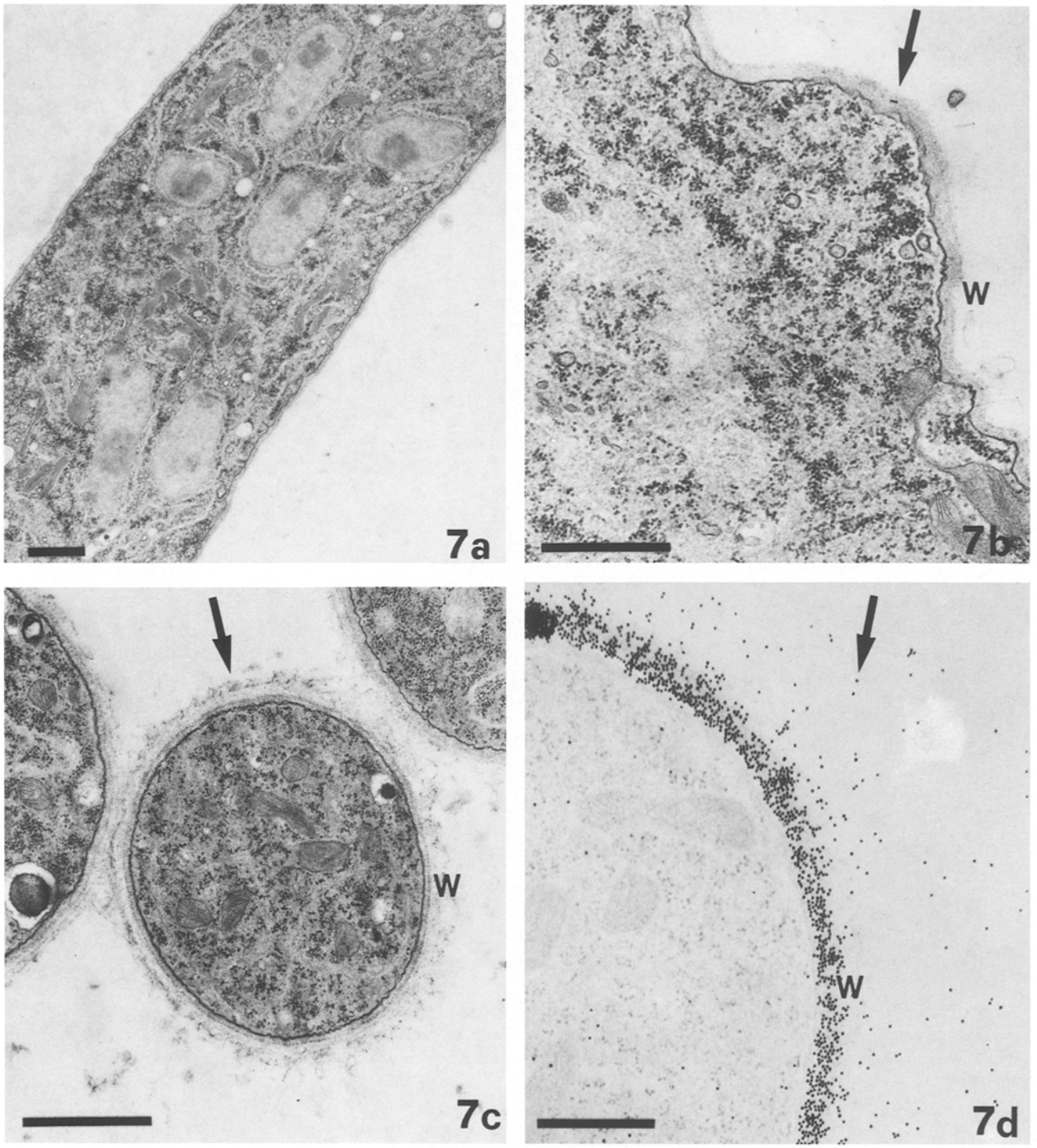

Fig. 7. Changes in cell wall morphology induced by treatments with plant β-1,3-glucanase and chitinase. **a** Subapical part of a hypha treated with glucanase. The wall does not show any reactivity after the PATAg reaction. **b** Magnification of the wall (*W*) showing a loose texture. Glycogen is still strongly reactive after the PATAg reaction. **c** Subapical part of a hypha treated with chitinase. The wall (*W*) is loose with detaching fibrils. **d** Detail of the wall (*W*) after WGA-gold treatment. Gold deposition is prominent over the wall as well as over the detaching material. Bars: a and c, 1 μm; b and d, 0.5 μm

see Boller 1987, 1988). Isolated plant chitinases and β-1,3-glucanases indeed inhibit fungal growth in vitro (Schlumbaum et al. 1986; Mauch et al. 1988 b; Roberts and Selitrennikoff 1988; Broekaert et al. 1988, 1989; Ludwig and Boller 1990). However, the cellular target of these enzymes and their mode of action have not

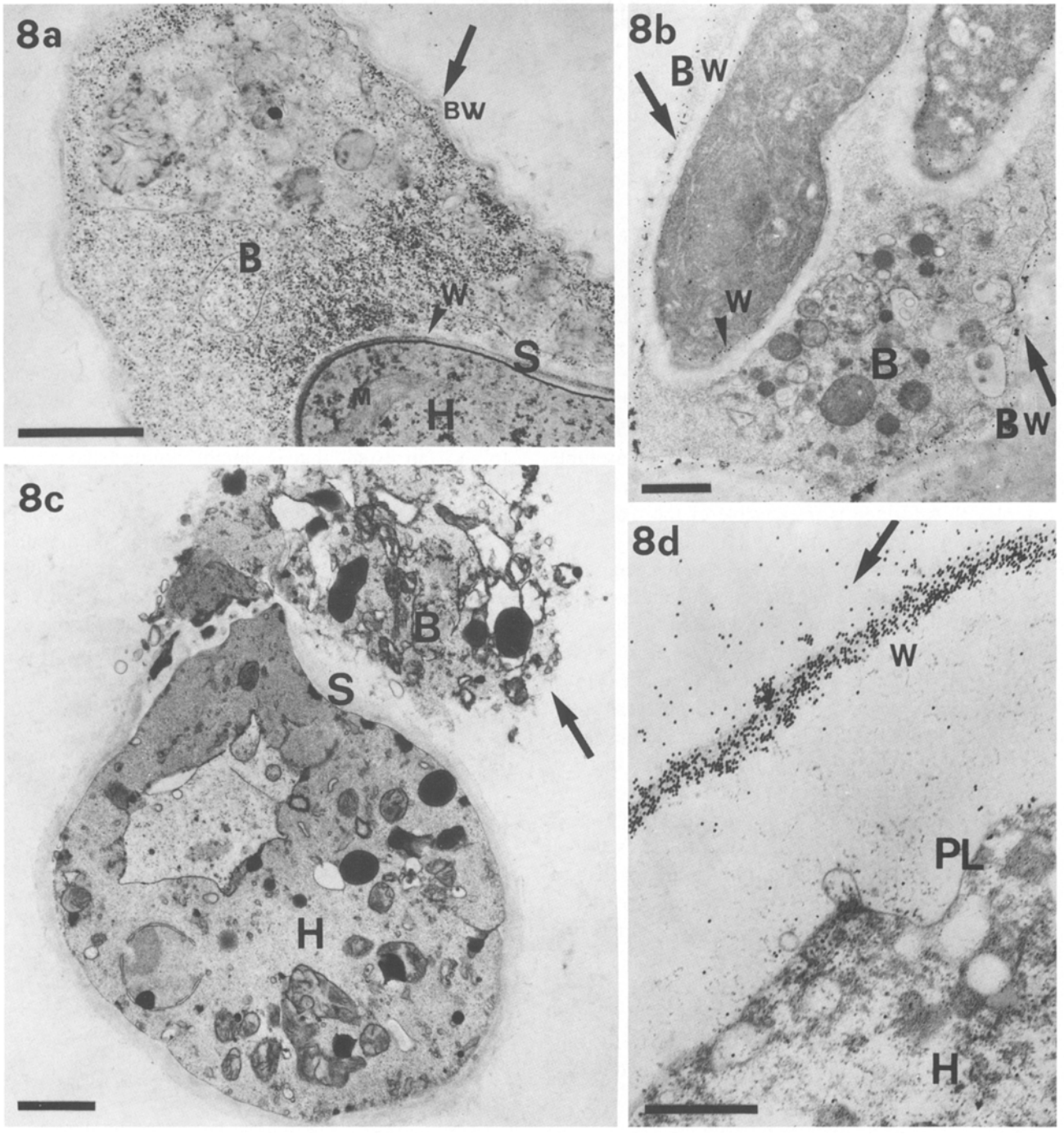

Fig. 8. Ultrastructural morphology after treatments with plant β-1,3-glucanase and chitinase. **a** The balloon-like swelling (*B*) has a thin wall (*Bw*) and a degenerated cytoplasm, while the subtending hypha (*H*), separated by the septum (*S*) has a well preserved cytoplasm. *M* Mitochondrion. **b** A similar section treated with WGA gold shows a regular distribution of gold granules over the wall of the balloon swelling (*Bw*) as well as of the subtending hypha (*W*). **c** Transverse section of the swelling (*B*) surrounded by the naked membrane. The subtending hypha (*H*) has a degenerated cytoplasm. **d** Detail of a subtending hypha (*H*) where cytoplasm is detached by the wall (*W*). This is still labelled by the WGA-gold treatment. *PL* Plasma membrane. Bars: a–c, 1 μm; d, 0.5 μm

been investigated. The morphological and cytochemical data presented here contribute new information with regard to both questions.

The fungal cell wall is the target of the hydrolases

Trichoderma longibrachiatum is a rapidly growing conidial fungus related to the ascomycetes. The cell walls

of filamentous ascomycetes consist mainly of chitin and β-glucans (Wessels 1991). Our studies show that chitin is in the inner part of the wall, while glucans seem to be localized both in the inner part together with the chitin and in the outer, more electron-dense part. A comparable layering of the cell wall has already been observed in some endomycorrhizal fungi (Bonfante et al. 1987, 1990).

These data should be seen in the context of a fungal cell wall model established on the basis of an analysis of *Schizophyllum commune* (Wessels 1991). According to this model, the extreme hyphal apex is characterized by alkali-insoluble glucosaminoglycans which are synthesized in a non-fibrillar texture and are very susceptible to chitinase and hot dilute mineral acid. Water soluble β-glucans are present at the tip, too, while subapically, water soluble glucans become alkali-insoluble and available for linkages to glucosaminoglycans. The complex thus formed is responsible of the rigidity of the fungal wall (Wessels 1991).

The experiments with *T. longibrachiatum* suggest a comparable organization of the cell wall: chitin present at the apex seems to be more susceptible to chitinase, while in the subapical part it seems to be stabilized inside a highly resistant glucan-chitin complex. Glucanase is able to degrade the outer electron dense layer, which is no more seen in ultrastructural pictures after the enzyme treatment. The disappearance of the PATAg reactivity through all the wall suggests that polysaccharides with vic-glycol groups are lost (Roland and Vian 1991). β-Glucans consisting of a mixture of β-1,3-glucans and β-1,6-glucans (which are reactive to the PATAg test) might be responsible of the effect. Notwithstanding this, glucanase does not affect the inner, rigid hyphal skeleton and the growth.

Even in the presence of glucanase, chitinase appears to have limited access to the inner parts of the lateral walls in the subapical and basal parts, where it only causes small modifications. It strongly affects only the extreme tip.

How does the plant chitinase inhibit the fungal growth?

Morphological observations show that chitinase produces a swelling of the wall texture, probably loosening the chitin strands, as suggested by the cloud of WGA-colloidal gold particles observed around the wall of the treated hyphae. This effect is particularly spectacular at the apex, where the wall becomes thinner and thinner and finally disappears. As a consequence, the naked tip swells in a balloon-like fashion until the plasma membrane is ruptured. These events present some features which are in common with those shown by hyphae treated with Calcofluor or Congo red. These dyes disturb the growth in yeasts, chitinous and cellulosic fungi (Fevre et al. 1991 and related references). In *Saprolegnia*, tips swell producing large bulges after 1–3 h from the application of Congo red. Even in this case, there is a block in the hyphal extension and a tip expansion (Fevre et al. 1991). However, after Congo red the wall is thick, since cellulose synthesis occurs while its crystallization into the microfibrils is prevented. This event produces a loss in cell wall rigidity.

Unlike this situation, in *Trichoderma* hydrolytic enzymes do not seem to affect the machinery for the cell wall synthesis. They directly weaken the wall of the tip to such an extent that it cannot counterbalance the turgor pressure, leading to the balloon-like swelling of the tip. For these reasons and for the formation of a septum between the ballooning hyphal tip and the subtending hypha, the effect on fungal growth is short-lived and the fungal re-growth is allowed.

According to Gooday and Gow (1991) apex is life for the fungus: any change in this compartment may produce important alterations in the growth process.

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