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Ultrastructure and Localization of Wall Polymers during Regeneration and Reversion of Protoplasts of *Schizophyllum commune*

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With 47 Figures

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Summary

Cell-wall regeneration and reversion of protoplasts of *Schizophyllum commune* were investigated using electron microscopic methods and X-ray diffraction.
After 3 hours of regeneration protoplasts have formed a loosely organized wall which does not react with Thiéry's stain for periodic acid sensitive carbohydrates. This wall largely consists of chitin microfibrils which are adpressed to the plasmalemma and which are covered by loose aggregates of alkali-soluble S-glucan (α-1,3-glucan). Both components are microcrystalline, at least partly. Walls formed in the presence of polyoxin D only consist of thick loose fibers of S-glucan.
From 3 hours onward the inner chitin microfibrils of the wall of the primary cells become embedded in alkali-insoluble material that stains heavily with the Thiéry reagent and probably is similar to the R-glucan of the mature wall (*i.e.*, β-1,3–β-1,6-glucan). The outer chitin microfibrils remain free of this matrix and are covered by S-glucan only.
Bud-like structures that arise have the same wall architecture as the primary cells, *i.e.*, only the inner chitin microfibrils are embedded in R-glucan and the S-glucan forms a fluffy coat. The walls of hyphal tubes that arise are distinct, however, in that all chitin microfibrils are embedded in R-glucan and the S-glucan forms a compact coat.
Cytoplasmic vesicles are sparse in primary cells except at the sites of emergence of bud-like structures and hyphae. They continue to be present in the apex of growing hyphae.

1. Introduction

The cell wall determines cell shape in fungi. Therefore, studies on the structure and biosynthesis of fungal cell walls are required to better understand the molecular basis of fungal morphogenesis.
Cell-wall regeneration by isolated protoplasts is a convenient system for studying cell-wall biosynthesis. Since cell-wall regeneration is a relatively slow process, taking some hours in fungi, it is possible to follow successive

stages in the biosynthesis of the new wall. In addition, such studies may lead to important conclusions about the role of specific wall components in determining the three-dimensional geometry of a cell.

The numerous publications on cell-wall regeneration by protoplasts of filamentous fungi mainly concern light microscopic observations of the various regeneration forms as influenced by the composition of the culture media (*cf.*, STRUNK 1970, VILLANUEVA and GARCÍA-ACHA 1971, WESSELS *et al.* 1976). In these systems the induction of normal morphogenesis (*i.e.*, the reversion to the original hyphal form) takes place in regenerated protoplasts (primary cells) but more often at the end of chain-cells, which arise by repeated budding of primary cells.

There are only a few publications concerning the fine-structural aspects of cell-wall formation by mould protoplasts (STRUNK 1969, GABRIEL 1970, CARBONELL *et al.* 1973, SIETSMA *et al.* 1975). These investigations show that the walls of regenerating protoplasts have a loose texture and contain both fibrillar and amorphous components. Only one study gives some information pertaining to the chemical identity of these wall polymers (CARBONELL *et al.* 1973).

A chemical analysis of cell-wall regeneration and reversion of protoplasts of the basidiomycete *Schizophyllum commune* (DE VRIES and WESSELS 1975) has shown that the regenerating protoplasts manufacture three main wall polymers, *viz.* S-glucan (α-1,3-glucan), R-glucan (β-1,3-β-1,6-glucan) and chitin. Here we report on the ultrastructural aspects of cell-wall regeneration and reversion of *S. commune* protoplasts. Close attention was paid to the ultrastructure and localization of the wall polymers and to changes in the cell wall and cytoplasm during initiation of bud-like structures and hyphae.

2. Materials and Methods

2.1. Organism

The monokaryotic strain 699 of *Schizophyllum commune* Fr. was used throughout.

2.2. Isolation of Protoplasts

Mycelium was grown for 2 days in shaken cultures with minimal medium (WESSELS 1965) at 25 °C. Protoplasts were liberated from this mycelium by using a wall-lytic enzyme system from *Trichoderma viride* (= *T. harzianum*) in the presence of 0.5 M $MgSO_4$ and 0.05 M sodium maleate (pH 5.8; osmolality: 0.68 Osm) according to the procedure of DE VRIES and WESSELS (1972). The enzymic digestion took place in shallow layers (depth about 2 mm) in Petri dishes to ensure aerobic conditions. After incubation for 11–18 hours the digestion mixture was centrifuged (20 minutes; 1400 g) in order to remove hyphal remnants and sedimenting protoplasts. The top-layer, containing highly vacuolated floating protoplasts, was collected.

2.3. Regeneration of Protoplasts

Floating protoplasts were prepared for regeneration studies according to the procedure of DE VRIES and WESSELS (1975). After removal of wall-lytic enzymes the protoplasts were

suspended in liquid regeneration medium, which was identical to the minimal culture medium except that glucose was present at 1% (w/v) and 0.5 M $MgSO_4$ was included (pH 5.8). In some experiments polyoxin D (generous gift of the Kaken Chemical Company, Tokyo, Japan) was added to the regeneration medium at a final concentration of 50 or 100 µg/ml. Regeneration of protoplasts (about 10^6/ml) took place under aerobic conditions in shallow layers (2 mm) at 25 °C (standing cultures).

2.4. Isolation of Cell Walls

After 3 and 9 hours of normal regeneration and after 7 hours of regeneration in the presence of polyoxin D, cells were washed on the centrifuge (5 minutes; 3000 g; 4 °C) with water followed by 0.05 M KH_2PO_4-NaOH buffer (pH 7.0). Washed cells were disrupted with a sonifier (Branson, B 12) at 60 Watts for 4 minutes (normal regeneration) or 1 minute (regeneration in the presence of polyoxin D). Clean cell walls were prepared according to the procedure of WESSELS and MARCHANT (1974).

2.5. Fractionation of Cell Walls

S-glucan was extracted from the cell walls with 1 N KOH (20 minutes; 60 °C) and reprecipitated from the alkaline extract by addition of acetic acid to pH 5.5.
R-glucan was extracted from the alkali-resistant wall residues by treatment with 0.5 N HCl (1 hour; 100 °C) or with exo-β-1,3-glucanase from Basidiomycete QM 806 (REESE and MANDELS 1966). This enzyme was used at a concentration of 1 mg/ml in 0.05 M sodium acetate buffer (pH 5.6) at 35 °C for 24 hours. Toluene was added to prevent bacterial growth. S-glucan from normal hyphal walls was isolated as described by WESSELS *et al.* (1972) and "native" S-glucan was obtained by exhaustive digestion of hyphal walls with *T. viride* enzymes as described by DE VRIES and WESSELS (1973).

2.6. Electron Microscopic Techniques

2.6.1. Sectioning

Cells were fixed by mixing with 10 volumes of a solution containing 2% (w/v) glutaraldehyde, 0.2 M $MgSO_4$ and 0.1 M sodium cacodylate (pH 5.8; osmolality 0.68 Osm). After fixation for 30 minutes (isolated protoplasts) or 2 hours (regenerating protoplasts) the material was washed in a buffer containing 0.35 M $MgSO_4$ and 0.1 M sodium cacodylate (pH 7.2) and postfixed in 1% (w/v) OsO_4 in the same buffer for 30 minutes at room temperature (isolated protoplasts) or for 10 hours at 4 °C (regenerating protoplasts). After washing with buffer and water and block-staining in 0.5% (w/v) aqueous uranyl acetate (1 hour) the material was dehydrated through a graded series of ethanol solutions and embedded in Epon 812. Sections were stained with uranyl acetate and lead citrate (REYNOLDS 1963).
For the detection of periodic acid sensitive carbohydrates, the periodic acid-thiocarbohydrazide-silver proteinate technique of THIÉRY (1967) was used. The cells were fixed and embedded as described above but with the block-staining with uranyl acetate omitted. Thin sections were mounted on coated gold grids and treated as follows: (*i*) 1% (w/v) periodic acid (PA) for 30 minutes; (*ii*) 0.2% (w/v) thiocarbohydrazide (TCH) in 20% (v/v) acetic acid for 60 minutes and (*iii*) 1% (w/v) silver proteinate (SP) for 30 minutes. Two controls were run; one in which pure water was substituted for the periodic acid solution and another in which 20% (v/v) acetic acid was substituted for the thiocarbohydrazide solution.

2.6.2. Freeze-Etching

For freeze-etching cells were fixed with glutaraldehyde for 45 minutes at room temperature and washed on the centrifuge (5 minutes; 800 g) with distilled water (five times). This

thorough washing is essential to ensure that no eutectic layer is formed on the surface of the etched specimen. Concentrated suspensions of the cells were frozen in liquid Freon 22 and stored in liquid nitrogen for periods up to 60 minutes. Platinum/carbon replicas were made according to the method of MOOR and MÜHLETHALER (1963) using a Balzers BA 360 M freeze-etch unit. Replicas were cleaned overnight in a saturated solution of $K_2Cr_2O_7$ in 70% (v/v) H_2SO_4 and subsequently in 20% (w/v) NaOH for 2 hours. After rinsing in distilled water they were mounted on coated grids.

2.6.3. Shadowing and Negative Staining

Suspensions of isolated walls and wall fractions in water were placed on coated grids and dried at room temperature. These specimens were shadowed with platinum, platinum/carbon or platinum/iridium (80/20) at 30°. Specimens were negatively stained with 0.2% (w/v) aqueous uranyl acetate.
Specimens were examined in a Philips EM 300 electron microscope. In photographs of freeze-etched and shadowed specimens shadows are white.

2.7. X-Ray Diffraction

Powder diagrams of freeze-dried preparations of isolated walls and wall fractions were taken in a flat film camera with Ni-filtered CuKα radiation.

3. Results

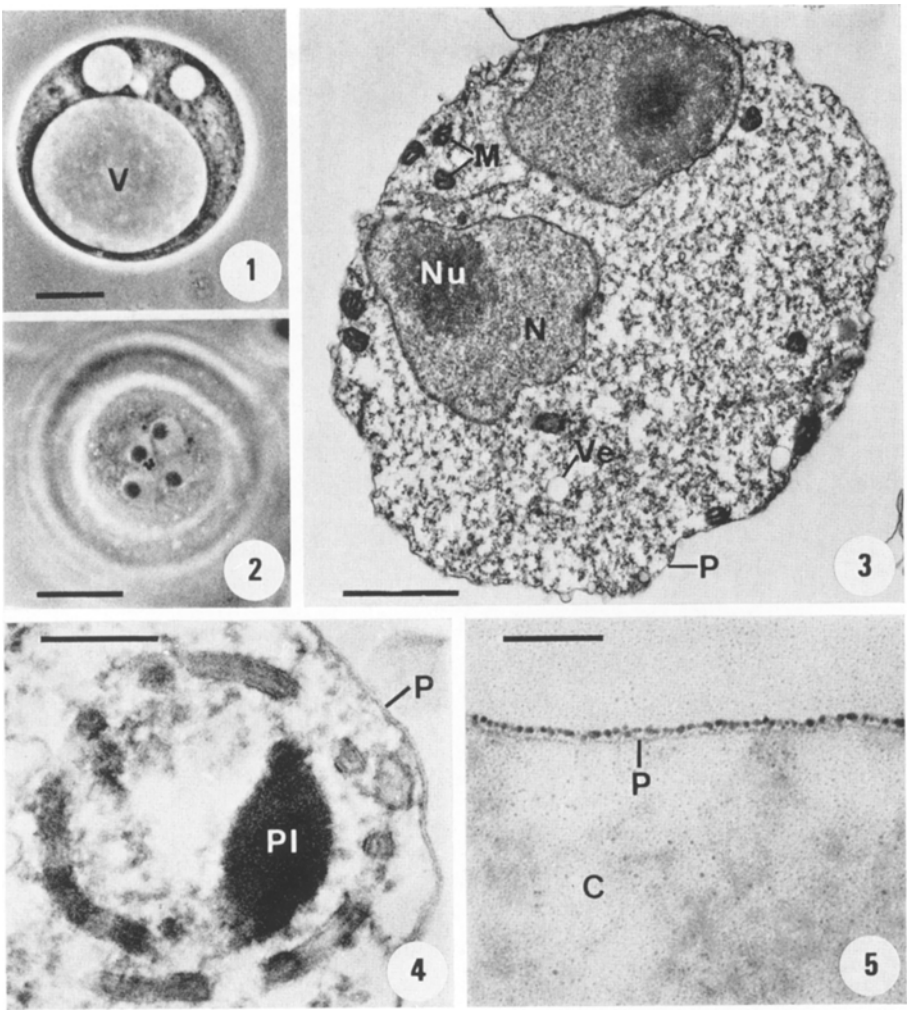
3.1. Examination of Thin Sections

3.1.1. Isolated Protoplasts

Isolated protoplasts were characterized by large vacuoles and often contained many nuclei (Figs. 1–3), which arose by synchronous nuclear divisions (VAN DER VALK and WESSELS 1973). Protoplasts were bounded by a clearly defined trilamellar plasmalemma (Fig. 4), which stained strongly with the periodic acid-thiocarbohydrazide-silver proteinate reagent (THIÉRY 1967), especially at the outside (Fig. 5). The specificity of this staining reaction was demonstrated by the absence of staining in controls from which periodic acid or thiocarbohydrazide had been omitted. The cytoplasm of isolated protoplasts often was dilute (Figs. 3 and 4). Mitochondria were common but cytoplasmic vesicles and glycogen aggregates were sparse (Fig. 3). Septal pore caps with or without septal pore plugs were regularly found (Fig. 4). These perforated caps apparently detached from the septum during protoplast formation and appeared to float freely in the cytoplasm. Complex plasmalemma invaginations, as seen in regenerating protoplasts (see below) were not observed prior to regeneration. The protoplast surface appeared free of wall remnants (Fig. 3).

3.1.2. Regenerating Protoplasts

During wall regeneration the cytoplasmic density often was much higher than prior to regeneration (Figs. 6 and 8). This appeared to be mainly due to



Figs. 1 and 2. Phase-contrast micrographs of isolated protoplasts. Note the large vacuole (*V*) (Fig. 1) and the nuclei with prominent nucleoli (Fig. 2). Scale lines = 10 μm

Figs. 3–5. Sections of isolated protoplasts stained with uranyl acetate and lead citrate (Figs. 3 and 4) or stained as specified. *C* = cytoplasm; *M* = mitochondrion; *N* = nucleus; *Nu* = nucleolus; *P* = plasmalemma; *Pl* = septal pore plug; *Ve* = vesicle

Fig. 3. Nucleate protoplast. No wall remnants are visible at the plasmalemma. Note the dilute cytoplasm and the scarcity of cytoplasmic vesicles. Scale line = 1 μm

Fig. 4. Septal pore cap which is free in the cytoplasm. Note the layered structure of the perforated cap and the enclosed septal pore plug. Scale line = 0.2 μm

Fig. 5. Plasmalemma stained with the Thiéry reagent. Note that the stain is confined to the outer half of the membrane. Scale line = 0.1 μm

accumulation of ribosomes. Mitochondria (Fig. 6) and rough endoplasmic reticulum (Fig. 8) were common. Cytoplasmic vesicles were sparse in regenerating protoplasts as in isolated protoplasts (Fig. 8). Vacuoles mostly were irregularly shaped (Fig. 17) and appeared to decrease in size during regeneration. Glycogen masses (Figs. 6 and 9), which stained heavily with the PA-TCH-SP reagent (Figs. 17 and 20–22), were abundant in the cytoplasm of most cells and often had a peripheral position (Figs. 6, 9, and 17). Notable was the association of glycogen with lipid granules (Figs. 6, 8, and 21). As in isolated protoplasts, the specificity of the staining reaction with the Thiéry reagent was demonstrated by the absence of staining in controls from which periodic acid or thiocarbohydrazide had been omitted. As prior to regeneration, the plasmalemma of the regenerating protoplasts showed a strong affinity to the Thiéry reagent (Figs. 12–16). Observations at high magnification sometimes showed a bilateral staining of the plasmalemma (Fig. 14). The plasmalemma of many regenerating protoplasts exhibited invaginations, which varied in complexity and also showed a high affinity to the Thiéry stain (Figs. 14—16). Other cellular membranes showed a low affinity to this stain.

After 3 hours of regeneration the walls of most cells were loosely organized (Figs. 6–8). These walls often varied considerably in thickness and appeared to consist mainly of loose aggregates of fibers with irregular outlines (Fig. 7). Such fibers were also found in the surrounding medium suggesting that the fibers easily detached from the cell surface, especially during wall isolation (see below).

After 9 hours of regeneration the walls of most primary cells consisted of two layers, *viz.* a fluffy, loosely organized outer layer as referred to above and

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Figs. 6–11. Sections of regenerating protoplasts stained with uranyl acetate and lead citrate. *CL* = compact layer; *ER* = endoplasmic reticulum; *FC* = fluffy coat; *G* = glycogen; *L* = lipid; *M* = mitochondrion; *N* = nucleus; *V* = vacuole; *Ve* = vesicle

Fig. 6. Nucleate cell after 3 hours of regeneration, showing dense cytoplasm, a thin cell wall, and peripherally located glycogen. Scale line = 1 μm

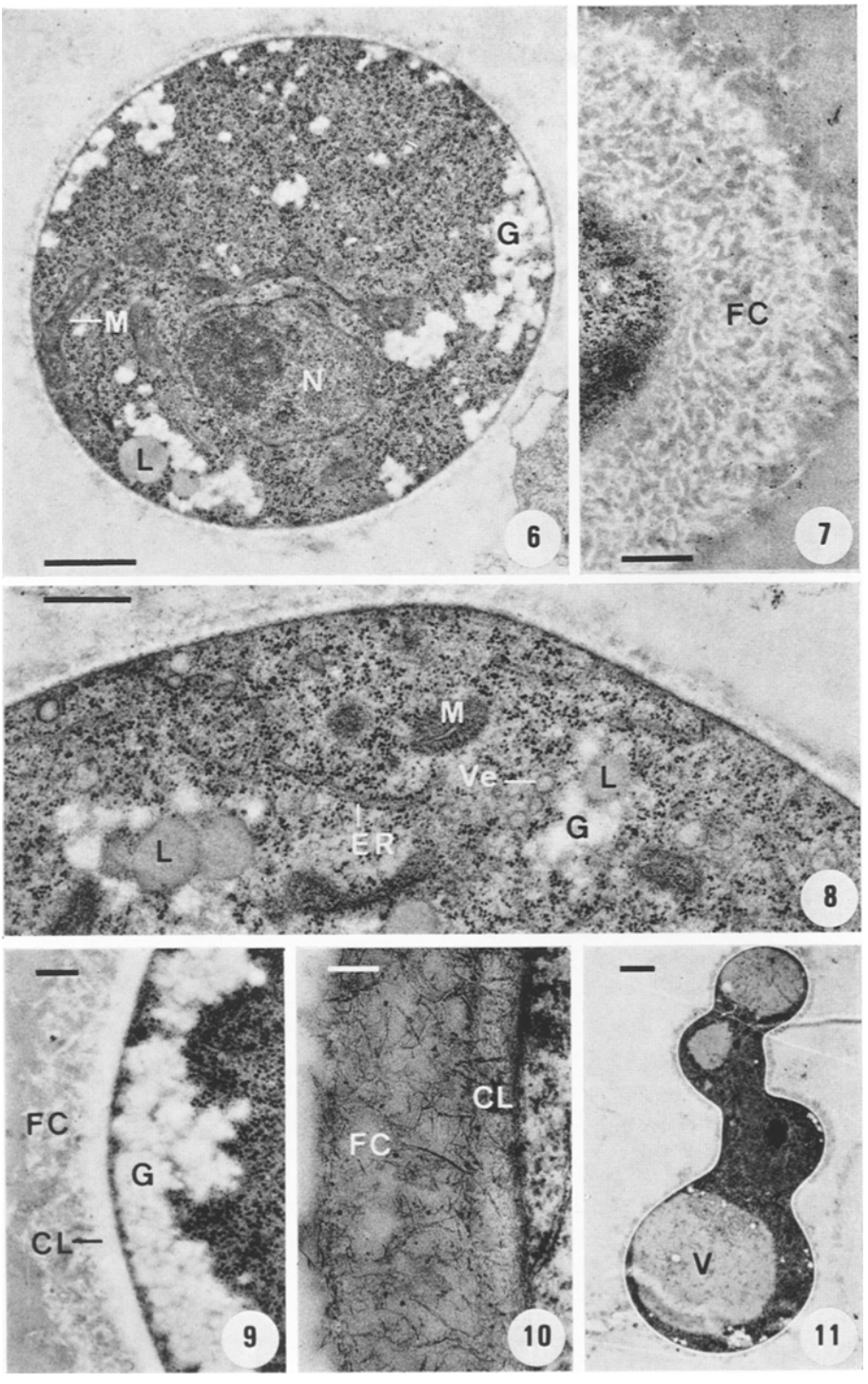
Fig. 7. Cell wall after 3 hours of regeneration. The thick wall is loosely organized and consists of aggregates of irregular fibers. Scale line = 0.5 μm

Fig. 8. Part of a cell after 3 hours of regeneration, showing rough endoplasmic reticulum, cytoplasmic vesicles and lipid/glycogen complexes. Note the numerous ribosomes and the thin cell wall. Scale line = 0.5 μm

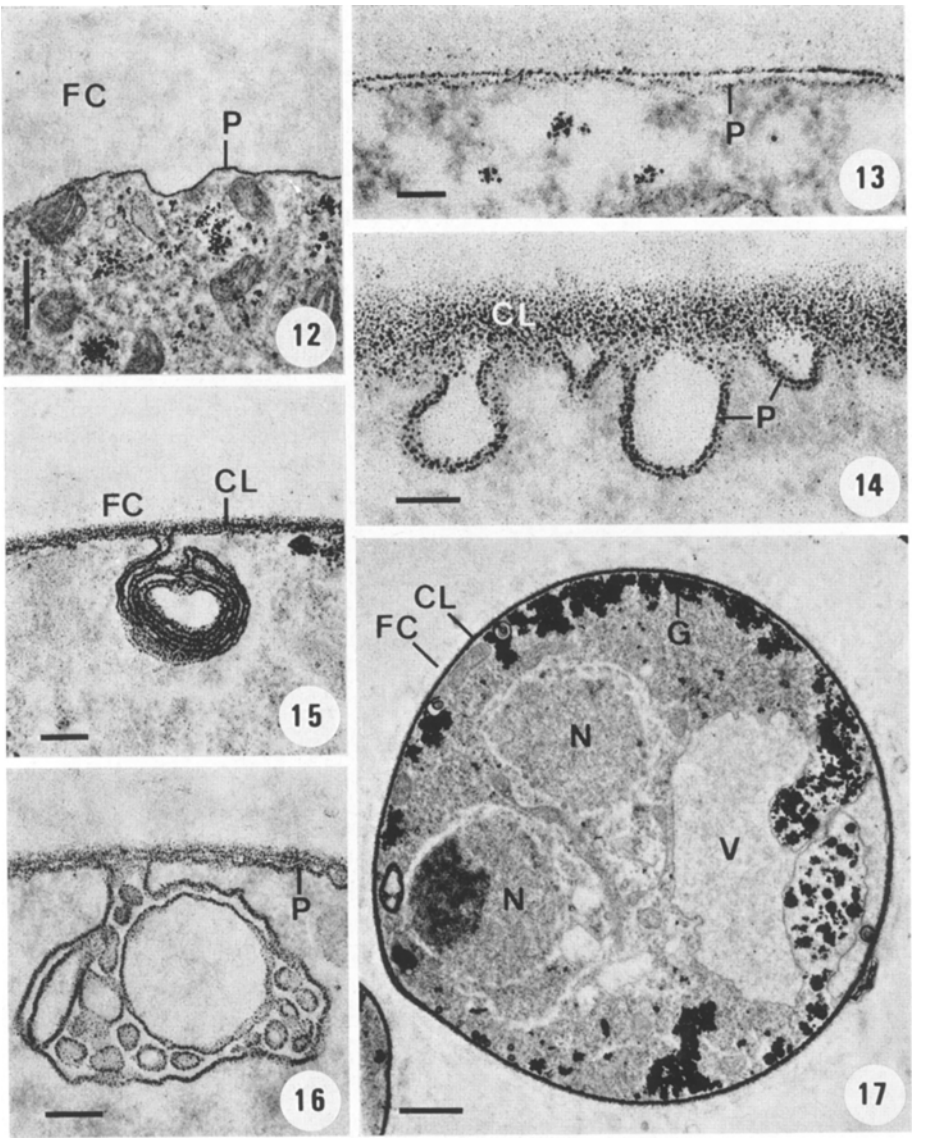
Fig. 9. Cell wall after 9 hours of regeneration. The wall consists of two layers, *viz.* a fluffy coat and a compact inner layer. Scale line = 0.2 μm

Fig. 10. Cell wall (9 hours of regeneration) after heavy staining with uranyl acetate and lead citrate. Note the thin fibrils in both wall layers. Scale line = 0.2 μm

Fig. 11. Chain-cell (9 hours of regeneration) with a two-layered cell wall. Note the absence of septa between the compartments of the chain-cell. Scale line = 1 μm



Figs. 6–11



Figs. 12–17. Sections of regenerating protoplasts stained with the Thiéry reagent. *CL* = compact layer; *FC* = fluffy coat; *G* = glycogen; *N* = nucleus; *P* = plasmalemma; *V* = vacuole

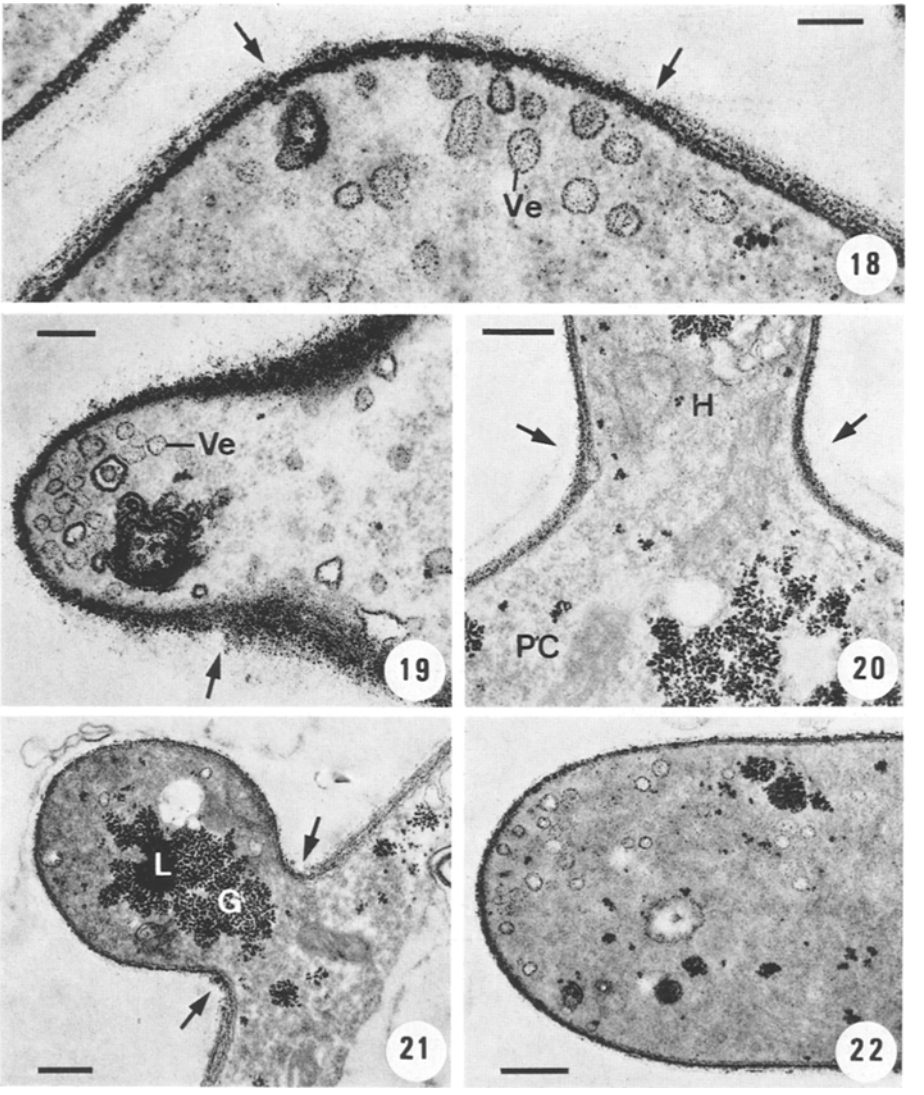
Fig. 12. Part of a cell after 3 hours of regeneration. The wall consists of an unstained fluffy coat. Note the stained plasmalemma. Scale line = 0.5 μm

Fig. 13. As Fig. 15 but showing a wall that consists of an unstained fluffy coat and a very thin stained inner wall layer. Note the staining of the plasmalemma. Scale line = 0.1 μm

Fig. 14. Wall after 9 hours of regeneration. The wall contains a thick heavily stained inner wall layer. Note the bilateral staining of the plasmalemma. Scale line = 0.1 μm

Figs. 15–16. Parts of regenerating protoplasts (9 hours of culture) showing heavily stained plasmalemmasomes with lamellar (Fig. 15) and vesicular (Fig. 16) elements. Scale lines = 0.2 μm

Fig. 17. Nucleate protoplast after 9 hours of regeneration, showing heavily stained peripherally located glycogen masses and an irregularly shaped vacuole. Note that the wall consists of an unstained fluffy coat and a stained inner wall layer. Scale line = 1 μm



Figs. 18–22. Sections of regenerating protoplasts (9 hours culture) stained with the Thiéry reagent and showing bud and hyphal tube initials. The site of emergence is characterized by a discontinuity in the outer part of the stained inner layer of the cell wall (arrows). *G* = glycogen; *H* = hyphal tube; *L* = lipid; *PC* = primary cell; *Ve* = vesicle

Fig. 18. Cytoplasmic vesicles, showing affinity to the Thiéry reagent, occur in a region near the modified wall. Scale line = 0.2 μm

Fig. 19. Advanced stage in the emergence of a hypha-like structure. Note the cluster of cytoplasmic vesicles in the apex of the emerging hypha. Scale line = 0.3 μm

Fig. 20. The wall of the emerged hypha lacks the fluffy coat. This coat apparently ruptured at the site of emergence whereas the stained inner layer dit not. Scale line = 0.5 μm

Fig. 21. Emergence of a bud-like structure. The wall of the bud initial is continuous with the inner portion of the wall of the primary cell. Scale line = 0.5 μm

Fig. 22. Hyphal tip of reverted protoplast. Note the presence of cytoplasmic vesicles in the apex. Scale line = 0.5 μm

a densely packed layer at the inside (Fig. 9). There was no sharp boundary between the two layers. The wall showed a low affinity to staining with uranyl acetate/lead citrate (Figs. 6–9). However, prolonged staining revealed thin fibers in both wall layers (Fig. 10). Staining with the Thiéry reagent differentiated between the two wall layers: the outer fluffy layer remained unstained whereas the inner layer stained heavily. This inner stained layer was very thin (Fig. 13) or absent (Fig. 12) after 3 hours of culture but was well developed in most cells after 9 hours (Figs. 14–17). After 9 hours of regeneration only a small proportion of the cells lacked the PA-TCH-SP stained inner wall layer and were surrounded only by a fluffy coat of unstainable material.

3.1.3. Emergence of Buds and Hyphae

After 9 hours of regeneration many cells were already engaged in the formation of bud-like structures (buds) or hyphal tubes. Buds resembled primary cells in having a fluffy outer wall layer (Fig. 11). Hyphae of reverted protoplasts, however, lacked the fluffy coat and instead had a compact wall (Figs. 20 and 22). Buds and hyphae were only initiated in primary cells possessing the PA-TCH-SP stained inner wall layer.

The PA-TCH-SP stained portion of the wall of most emerging buds and hyphae had an irregular outline, was rather thin and continuous with only the inner portion of the stained inner wall layer of the primary cell (Figs. 18–21).

Contrary to the sparse and irregular distribution of cytoplasmic vesicles in

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Figs. 23–26. Surfaces of freeze-etched protoplasts before and during wall regeneration. *IFF* = inner fracture face; *OS* = outer surface

Fig. 23. Prior to regeneration no fibrils are present on the outer surface of the plasmalemma. Numerous intramembranous particles are visible at the inner fracture face of the plasmalemma. Scale line = 0.2 μm

Fig. 24. After 30 minutes of regeneration a thin mat of microfibrils is adpressed to the plasmalemma. Arrow indicates a bundle of microfibrils. Scale line = 0.2 μm

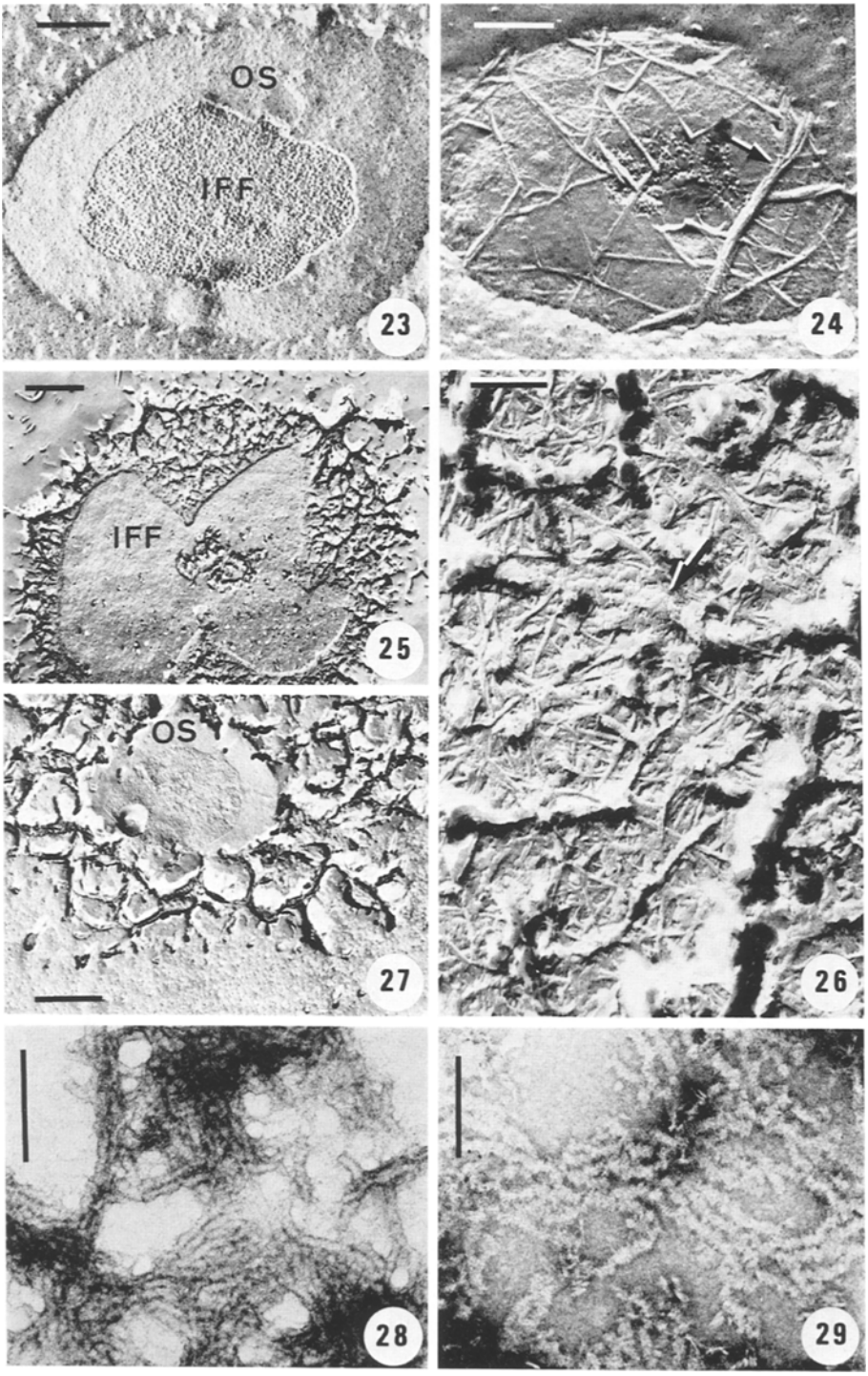
Fig. 25. Protoplast after 4 hours of regeneration. Reticulate wall material extends far into the medium. Scale line = 1 μm

Fig. 26. As Fig. 25 but at higher magnification. Reticulate wall material, often forming irregular fibers (arrow) covers the microfibrils. Scale line = 0.2 μm

Fig. 27. Regeneration in the presence of polyoxin D (100 μg/ml; 4 hours). The wall lacks the microfibrillar component and only consists of reticulate material. Scale line = 0.5 μm

Fig. 28. Negatively stained isolated wall fragment of regenerated protoplast cultured in the presence of polyoxin D (50 μg/ml; 7 hours), showing irregular fibers (fiber diameter about 20 nm). Scale line = 0.2 μm

Fig. 29. Negatively stained preparation of reprecipitated S-glucan from normal hyphal walls, showing aggregates of irregular fibers (fiber diameter 20–30 nm). Scale line = 0.2 μm



Figs. 23–29

primary cells, vesicles (diameter about 100 nm) were commonly found in the tips of emerged hyphae (Fig. 22) and in the initials of either hyphal apices or buds (Fig. 19). Even the very inception of these initials could be traced by the presence of such vesicles (Fig. 18). The origin of these vesicles, which showed affinity to the Thiéry reagent, is not clear.

3.2. Examination of Freeze-Etched and Negatively Stained Preparations

Prior to regeneration the outer surface of the plasmalemma of the protoplasts appeared completely free of wall remnants (Fig. 23). After 30 minutes of regeneration, however, the plasmalemma of most protoplasts was covered by a thin net of apparently randomly arranged microfibrils (Fig. 24). These microfibrils appeared tightly adpressed to the plasmalemma. Impressions of microfibrils in the plasmalemma were regularly observed both at the inner and outer fracture face of the plasmalemma.

After 4 hours of regeneration the density of the microfibrillar net was much higher than after 30 minutes. The microfibrils often formed bundles even when the density of the microfibrillar net was still low (Fig. 24).

Besides the microfibrillar nets the walls of regenerating protoplasts contained reticulate material, that covered the microfibrils (Fig. 26) and often extended far into the medium (Fig. 25). This material often formed thick irregular fibers (Fig. 26, arrow).

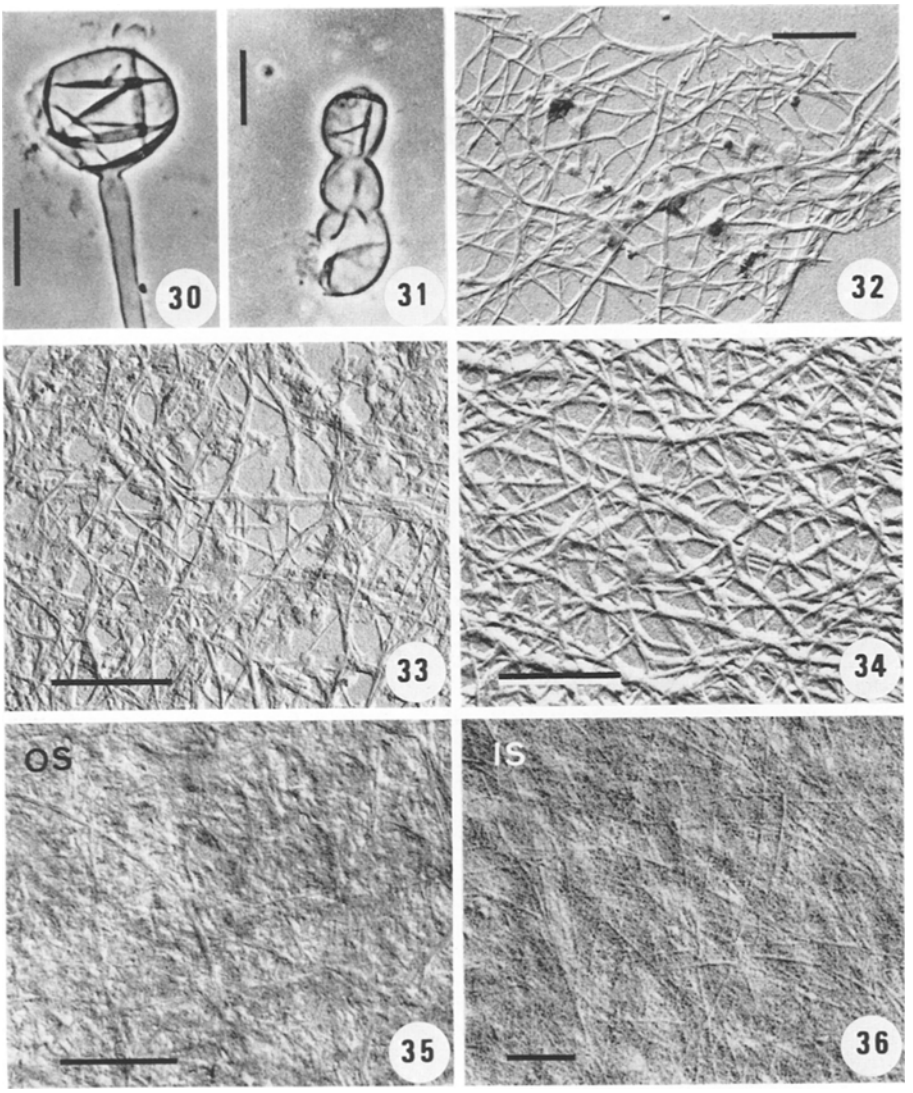
It is known that chitin and α-1,3-glucan are prominent in the walls at this stage of regeneration and that the antibiotic polyoxin D prevents chitin synthesis but not α-1,3-glucan synthesis (DE VRIES and WESSELS 1975). Therefore the effect of this drug on the appearance of the wall components was studied. As can be seen in Fig. 27 regeneration in the presence of polyoxin D (100 μg/ml) resulted in the formation of walls which lacked the microfibrillar component but appeared to be entirely composed of reticulate material that must constitute the α-1,3-glucan.

Negative staining showed that this material actually consisted of thick irregular fibers (fiber diameter 20–30 nm) (Fig. 28). Similar fibers were also observed in preparations of reprecipitated S-glucan from normal hyphal walls (Fig. 29).

3.3. Examination of Shadowed Wall Preparations

Phase-contrast microscopy showed that walls isolated after 3 hours of regeneration were very fragile and easily fell apart but that walls prepared after 9 hours of regeneration mostly retained the shape of the cell (Figs. 30 and 31).

Electron microscopy of shadowed preparations revealed that after 3 hours of regeneration most walls consisted of two components, *viz.* a thin net of microfibrils and aggregates of amorphous material, which partly covered the



Figs. 30 and 31. Phase-contrast micrographs of isolated cell walls of regenerated protoplasts (9 hours of culture). The walls of both reverted protoplasts (Fig. 30), and chain-cells (Fig. 31) retain the shape of the living cell. Scale lines = 10 μm

Figs. 32–36. Surface structure of shadowed isolated wall fragments of regenerating protoplasts

Fig. 32. Non-extracted wall fragment (3 hours of regeneration). The wall consists of a microfibrillar net only. Scale line = 0.5 μm

Fig. 33. As Fig. 32, but in this specimen the wall consists of a microfibrillar net enmeshed and covered with amorphous material. Scale line = 0.5 μm

Fig. 34. KOH-extracted wall fragment (3 hours of regeneration) consisting of microfibrils only. Scale line = 0.5 μm

Fig. 35. Outer surface of a non-extracted wall fragment of a primary cell (9 hours of regeneration) showing microfibrils which are partly covered with amorphous material. Scale line = 0.5 μm

Fig. 36. Inner surface of a non-extracted wall fragment of a primary cell (9 hours of regeneration) showing microfibrils embedded in an amorphous matrix. Scale line = 0.2 μm

microfibrils (Fig. 33). Some wall fragments lacked the amorphous material (Fig. 32). Treatment with alkali, which solubilizes α-1,3-glucan, removed the amorphous material but did not change the appearance of the microfibrils (Fig. 34). A minor fraction of these wall fragments contained three components as shown by the fact that the inner and outer surface of the alkali-resistant fragments were clearly different. This feature became prominent in wall fragments of primary cells isolated after 9 hours of regeneration. The outer surface of the non-extracted wall fragments was rough and microfibrils were partly (Figs. 35, 37, and 39) or completely covered by alkali-soluble amorphous material. Alkali-treatment completely uncovered the microfibrils at the outer surface of the wall of the primary cells (Figs. 38, 40, and 41). The inner surface of the non-extracted walls was rather smooth revealing microfibrils which were embedded in an amorphous alkali-resistant matrix (Fig. 36). Individual microfibrils often could be traced for considerable distances at the inner surface of the wall. Alkali-treatment did not change the appearance of the inner wall surface.

The wall architecture of buds and chain-cells was identical to that of primary cells. An outer layer of alkali-soluble amorphous material covered the

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Figs. 37–44. Surfaces of shadowed walls of regenerated protoplasts (9 hours of culture), showing bud-like structures and hyphal tubes. *B* = bud-like structure; *H* = hypha; *IS* = inner surface; *OS* = outer surface; *PC* = primary cell

Fig. 37. Non-extracted wall of budding cell. The microfibrils of primary cell and bud are partly covered with amorphous material. Scale line = 1 μm

Fig. 38. KOH-extracted wall of budding cell. The microfibrils of primary cell and bud are completely uncovered. Scale line = 1 μm

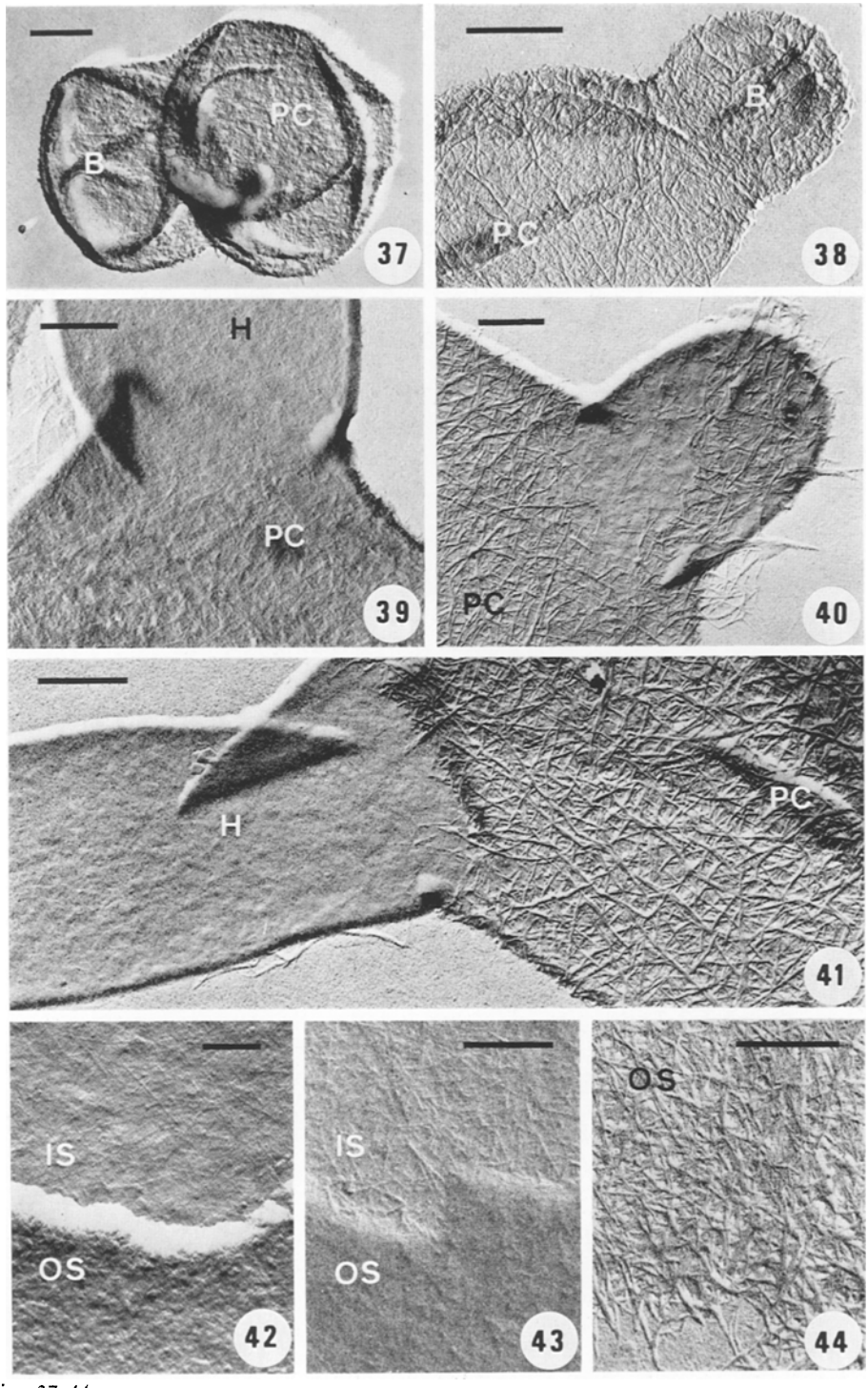
Fig. 39. Non-extracted wall of reverted protoplast showing transition in surface texture at the site of hyphal tube emergence. The surface of the wall of the hyphal tube is smoother than that of the primary cell and does not reveal microfibrils. Scale line = 0.5 μm

Figs. 40 and 41. KOH-extracted walls of reverting protoplasts. The microfibrils at the outer surface of the wall of the primary cells are clearly exposed, whereas those of the wall of hyphal tubes (Fig. 41) and tube initials (Fig. 40) are completely embedded in an alkali-resistant amorphous matrix. The microfibrillar net appears to rupture at the site of hyphal tube emergence (Fig. 40) and remnants of this net are left behind on the surface of these initials. Scale lines = 0.5 μm

Fig. 42. Non-extracted hyphal wall. The outer surface of the wall has a rough amorphous appearance. The inner surface is rather smooth and shows microfibrils embedded in an amorphous matrix. Scale line = 0.3 μm

Fig. 43. KOH-extracted hyphal wall. The outer surface of the wall has a smooth amorphous appearance. Microfibrils are not visible. The inner surface shows microfibrils embedded in an alkali-resistant amorphous matrix. Comparison with Fig. 42 shows that alkali-treatment removed the rough wall coat but did not change the appearance of the inner wall surface. Scale line = 0.3 μm

Fig. 44. Hyphal wall after successive KOH/exo-β-1,3-glucanase treatment, showing microfibrils only. Scale line = 0.3 μm



Figs. 37–44

microfibrils which were embedded in an alkali-resistant matrix at the inside only. The microfibrils were hardly visible at the outside without any treatment (Fig. 37) but were clearly exposed after alkali-treatment (Fig. 38).
The wall architecture of hyphae and hyphal initials of reverting protoplasts clearly differed from that of primary cells. The outer surface of these hyphal walls was smoother than that of the walls of primary cells and microfibrils were not visible (Figs. 39 and 42). These walls also contained an outer cover of amorphous material (Fig. 42), presumably α-1,3-glucan. Removal of this cover with alkali revealed a very smooth surface without any microfibrils visible (Figs. 40, 41, and 43). There was a sharp demarcation in wall architecture at the site of hyphal tube emergence (Figs. 40 and 41). At this site the microfibrillar net of the primary cell appeared to be ruptured and remnants of this net were sometimes observed on the surface of the hyphal tube initials (Fig. 40). The inner surface of the hyphal walls was similar to that of the primary cells and buds. Its appearance did not change after alkali-treatment: microfibrils were embedded in an alkali-resistant matrix (Figs. 42 and 43).
Treatment of alkali-resistant wall fragments with hot dilute HCl or exo-β-1,3-glucanase almost completely removed the matrix of both primary cells and hyphae (Fig. 44). Together with its stainability with the Thiéry reagent this suggests that the matrix is similar to the alkali-insoluble R-glucan (β-1,3-β-1,6-glucan) of the normal wall and that the microfibrils represent chitin since chitin is known to be resistant to these treatments.

3.4. X-Ray Diffraction of Isolated Wall Fractions

Fig. 45 shows density tracings of X-ray powder diagrams of isolated wall fractions of regenerating protoplasts (6 hours culture). The diffraction pattern of non-extracted walls (Fig. 45 B) revealed the main reflections of S-glucan (WESSELS *et al.* 1972) indicating the presence of microcrystalline α-1,3-glucan in the native walls. The diffraction pattern of reprecipitated S-glucan prepared from these walls (Fig. 45 A) was identical to that of S-glucan isolated from normal hyphal walls (Fig. 46 A). The residue left after alkali-extraction (Fig. 45 C) showed the reflections of crustacean chitin (Fig. 45 E). The 4.6 Å band of chitin was apparently also present in the non-extracted walls, suggesting the presence of micro-crystalline chitin in the native walls. Treatment of the alkali-resistant wall fraction with exo-β-1,3-glucanase did not change the diffraction pattern (Fig. 45 D).
Fig. 46 compares the X-ray diffraction patterns of reprecipitated S-glucan (A) and "native" S-glucan (B) with that of walls from regenerated protoplasts cultured in the presence of polyoxin D (C). The similarity between the patterns indicates that these preparations all contain microcrystalline S-glucan. Note the absence in these preparations of any reflections that would suggest the presence of chitin.

4. Discussion

The results show that no morphologically identifiable wall remnants are present on the surface of isolated protoplasts of *S. commune*. This excludes the possibility that gross wall fragments act as primers during cell-wall regeneration. However, carbohydrates appear to be associated with the plasmalemma of both isolated and regenerating protoplasts as evidenced by the Thiéry stain. The plasmalemma of normal mycelium of *S. commune* also

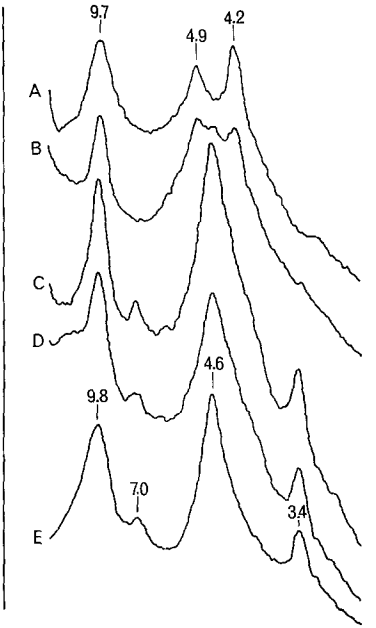


Fig. 45. Radial density tracings of X-ray powder diagrams of isolated wall fragments of regenerating protoplasts (6 hours of culture). *B:* Non-extracted walls. *C:* Residue after extraction with KOH. *A:* Reprecipitated S-glucan. *D:* Residue after consecutive KOH/exo-β-1,3-glucanase treatment. *E:* Crustacean chitin (Fluka). Numbers indicate the crystal spacings (Å)

reacts positively with this stain (VAN DER VALK, unpublished). This shows that the stainability of the plasmalemma is not lost during protoplast formation. In fact, the association of carbohydrates with the plasmalemma appears to be a general feature of various types of eukaryotic cells (*cf.*, COOK and STODDART 1973, ROLAND and VIAN 1971) and does not appear to be specifically related to cell-wall synthesis. Nevertheless, the possibility remains that some of these carbohydrates are cell-wall specific and act as primers during wall regeneration.

The cytoplasmic ultrastructure of protoplasts prior to and during wall regeneration shows the features common for mycelium of *S. commune* (NIEDERPRUEM and WESSELS 1969, RAUDASKOSKI 1970). Conspicuous is the

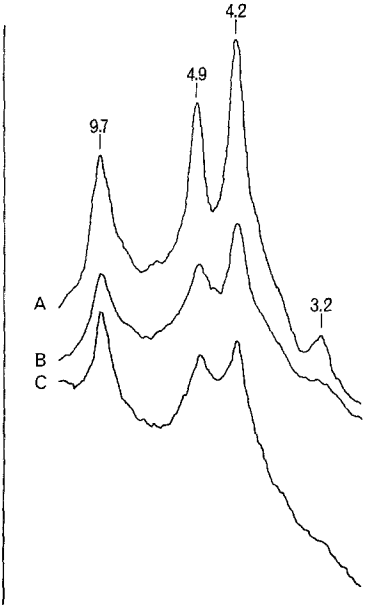


Fig. 46. Radial density tracings of X-ray powder diagrams of different S-glucan preparations. *A:* Reprecipitated S-glucan from normal hyphal walls. *B:* "Native" S-glucan (residue after exhaustive treatment of hyphal walls with *T. viride* enzymes according to DE VRIES and WESSELS (1973). *C:* Isolated walls of regenerating protoplasts, cultured in the presence of polyoxin D (50 µg/ml). Numbers indicate the crystal spacings (Å)

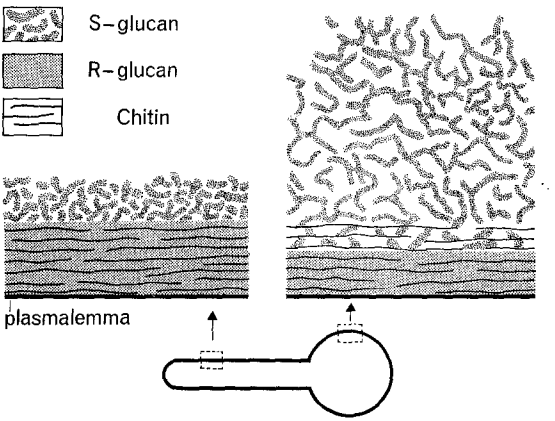


Fig. 47. Model of the ultrastructural location of wall polymers in primary cells and hyphae of reverted protoplasts of *Schizophyllum commune*

abundant synthesis of glycogen during wall regeneration. The observed association of glycogen with lipid bodies has also been noted in chlamydospores of *S. commune* (KOLTIN *et al.* 1973). Unusual are the floating septal pore caps apparently detached from the septa during protoplast formation.

Such floating pore caps have also been detected in common-*A* mycelium of *S. commune* (VAN DER VALK, unpublished) in which septa are continuously degraded.

Plasmalemma invaginations, which have been called plasmalemmasomes when they attain a certain complexity (EDWARDS and STEVENS 1963), are common in the mycelium of *S. commune*. They are absent in naked protoplasts but reappear during wall regeneration. Similar observations have been made by POJNAR *et al.* (1967) and CARBONELL *et al.* (1973), who studied wall regeneration of protoplasts isolated from tomato fruits and yeast cells of *Histoplasma capsulatum*, respectively. These authors believe these structures to play an important role in wall synthesis. Since plasmalemmasomes are absent from the tips of rapidly growing hyphae of *S. commune* (VAN DER VALK, unpublished) a specific role of these structures in wall synthesis seems unlikely and we rather favour the idea that plasmalemma invaginations arise as a result of superfluous membrane synthesis as has been suggested by others (HEATH and GREENWOOD 1970, HEMMES and HOHL 1971, MARCHANT and MOORE 1973). In the absence of wall synthesis the protoplasts continuously grow and extend their plasmalemma surface (VAN DER VALK and WESSELS 1973). The deposition of the chitinous network around the protoplasts probably checks this growth but synthesis of plasmalemma may continue for some time resulting in membrane invaginations.

Noteworthy is the scarcity of vesicles in regenerating protoplasts of *S. commune*. They only are abundant at the site of bud and hyphal tube emergence and in the apex of extending hyphae. The possible role of these vesicles in hyphal extension has been extensively discussed (GIRBARDT 1969, GROVE and BRACKER 1970, BARTNICKI-GARCIA 1973) but remains unresolved. There is evidence that the vesicles fuse with the plasmalemma. In this way they would contribute to the extension of the plasmalemma and the contents discharged could somehow contribute to wall synthesis. Our own observations do not seem to support the latter notion because of the scarcity of vesicles during formation of a wall around the protoplasts. The validity of this argument may be questioned, however, because light microscopic autoradiography after feeding of N-(acetyl-^{3}H)-glucosamine has shown that wall synthesis per unit surface area is rather slow in primary cells as compared to the hyphal tips of reverted protoplasts (WESSELS *et al.* 1976).

DE VRIES and WESSELS (1975) showed that alkali-soluble S-glucan (α-1,3-glucan) and chitin are the first wall components to be synthesized by regenerating protoplasts of *S. commune*, whereas the synthesis of the alkali-insoluble R-glucan lags behind. The synthesis of the latter component becomes prominent after about 5 hours. Indeed our ultrastructural analysis reveals two wall components in most cells during the early stage of regeneration and X-ray diffraction shows the presence of both microcrystalline S-glucan and chitin. Moreover, at this stage the wall of most cells does not

react with the Thiéry stain, which is in accordance with the fact that α-1,3-glucan and chitin consume little periodate (WESSELS *et al.* 1972). Little if any R-glucan is yet present at the 3 hours regeneration stage because this substance would stain with the PA-TCH-SP technique due to the presence of β-1,6-linkages. The absence of Thiéry staining also seems to rule out the presence of mucilage that normally surrounds hyphae and which also contains β-1,6-linkages (WESSELS *et al.* 1972).

The distinct microfibrils of the wall must represent chitin since 1. they are resistant to alkali and dilute acid as is authentic chitin, 2. X-ray diffraction spectra of preparations of these microfibrils show the typical reflections of chitin and 3. such fibrils are absent from the walls when regeneration is carried out in the presence of polyoxin D.

The material constituting the fluffy coat of the primary cells and chain-cells must represent S-glucan because 1. it is alkali-soluble and upon precipitation yields the typical X-ray diffraction pattern of S-glucan, 2. it is the only component discernable in the walls of regenerated protoplasts grown in the presence of polyoxin D. Our X-ray diffraction data confirm the earlier chemical data (DE VRIES and WESSELS 1975) showing that S-glucan is the only wall component detectable in these cells.

The ultrastructure of S-glucan depends somewhat on the method of preparation for electron microscopy. After shadowing and freeze-etching the S-glucan appears as amorphous material with indications of thick irregular fibers. However, in negatively stained preparations of reprecipitated S-glucan and in the S-glucan walls of regenerated protoplasts grown in the presence of polyoxin D thick fibers were clearly visible. Thick irregular α-1,3-glucan fibers of similar width and appearance as those of *S. commune* have also been observed in wall preparations of other fungi (CARBONELL *et al.* 1970, SAN-BLAS and CARBONELL 1974, CARBONELL *et al.* 1973).

The appearance of PA-TCH-SP sensitive material at the inside of the wall during continued wall regeneration occurs at the time that R-glucan synthesis increases (DE VRIES and WESSELS 1975). The location of this material closely corresponds to that of the alkali-insoluble matrix that embeds the chitin microfibrils at the inside of the wall as seen in shadowed preparations. In the primary cells and chain-cells, however, it does not surround the outer chitin microfibrils. That this material is in fact R-glucan is also indicated by the fact that it can be removed by dilute HCl and by exo-β-1,3-glucanase, treatments that solubilize a major portion of authentic R-glucan.

Bud and hyphal tube emergence appears to be accompanied with rupture of the wall of the primary cell in such a way that the bud and hyphal tube wall is continuous only with the inner wall portion of the primary cell. Wall rupture has been observed during bud formation in certain yeasts (MARCHANT and SMITH 1967, KREGER-VAN RIJ and VEENHUIS 1971, MCCULLY and BRACKER 1972, GROVE *et al.* 1973) and during germination of a variety of

fungal spores (*cf.*, GROVE *et al.* 1973) including encysted zoospores (HEGNAUER and HOHL 1973).

Our observations on sections and shadowed preparations show that there are striking differences in the wall architecture of buds and hyphae arising from primary cells. The walls of the buds are quite similar to those of the primary cells in that the S-glucan layer has a fluffy appearance and the chitin microfibrils are embedded in R-glucan at the inside only. The architecture of the walls of the hyphal tubes that arise from the primary cells is different in that these walls have a compact layer of S-glucan and the chitin microfibrils are completely embedded in R-glucan. Even the very inception of the hyphal tubes could be detected because of this difference in wall architecture.

The fact that alkali-treatment does not change the appearance of the inner surface of the wall of both the primary cells and emerging hyphae suggests that S-glucan is virtually absent from the inner wall surface, but does not exclude that some S-glucan penetrates the R-glucan/chitin complex. A model of the location of wall polymers in primary cells and emerged hyphae is given in Fig. 47.

A change in wall architecture at the site of hyphal tube emergence similar to that in *S. commune* has also been observed during reversion of protoplasts of *Pythium acanthicum* (SIETSMA *et al.* 1975) and during germination of encysted zoospores of *Phytophthora palmivora* (TOKUNAGA and BARTNICKI-GARCIA 1971, HEGNAUER and HOHL 1973) and *Allomyces macrogynus* (FULTZ and WOOLF 1972). At least in the two former cases the nature of the microfibrils and probably also the matrix is different from those of *S. commune*. This stresses the importance of the wall architecture rather than the chemical composition of the wall for hyphal morphogenesis.

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