Protoplasma 123, 140—151 (1984)

PROTOPLASMA

Cytochemical and Biochemical Observations on the Cell Wall of the Spore of *Glomus epigaeum*

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Received December 28, 1983
Accepted March 21, 1984

Summary

The cell wall of the spore of *Glomus epigaeum* Daniels and Trappe, which has fibrillar subunits regularly arranged in arcs, was studied ultrastructurally and biochemically.

The periodic acid/thiocarbohydrazide/silver proteinate (PATAg) reaction for polysaccharide location (THIÉRY 1967) and the silver methenamine reaction for protein location (SWIFT 1968) were performed on whole spores, progressively alkaline-extracted and autoclaved spores, and untreated and alkaline-extracted cell wall fractions. The cytochemical results and those obtained from frozen sections indicated that the fibrils forming the main structure of the outer and inner wall consist of chitin. Quantitative determinations showed that chitin is the most important component (47%) of the alkali-insoluble residue and represents 27.2% of the whole cell wall fraction. It occurs predominantly as the acetylated form. Cytochemical and biochemical observations showed that the matrix surrounding the fibrils is made of alkali-soluble, PATAg positive polysaccharides (4.98% of the whole cell wall fraction). Monomers were identified by gas liquid chromatography as being γ-lactone of glucuronic acid, and glucose, rhamnose and mannose. Alkali-soluble proteins are an important part of the matrix, being spread mostly throughout the inner wall and constituting a large portion (55.1%) of the alkali-soluble fraction.

From the results we derive a model in which the chemical components are interconnected to build up a macromolecular network, in agreement with electron-microscopic observations.

Keywords: Biochemistry; Cell wall; Chitin; Cytochemistry; *Glomus epigaeum*; Spore.

1. Introduction

Close integration between ultrastructural and biochemical data is necessary to understand cell wall morphogenesis in algae, fungi and higher plants. This includes polymer assembly mechanisms, the morphology of the macromolecular complexes and their site of deposition within the wall (ROBINSON and QUADER 1981, TANNER and LOEWUS 1981).

For fungi, use of cytochemical staining and biochemical analysis has shown the location of polymers such as fibrillar chitin and β glucans in the septal wall of *Schizophyllum commune* (VAN DER VALK *et al.* 1977). WESSEL and SIETSMA (1981) developed a theoretical model of walls of filamentous hyphae in which alkali-insoluble components (chitin or cellulose fibrils) and other alkali-soluble components were linked to produce a wall composed essentially of one layer.

Similar models have been proposed for non-hyphal walls, where multilayered walls can occur. For example, in *Agaricus bisporus* (RAST and HOLLENSTEIN 1977), in *Phytophthora* (HEGNAUER and HOHL 1978) and in *Plasmodiophora* (BUCZACKI and MOXHAM 1983, MOXHAM and BUCZACKI 1983) both fibrillar and amorphous components were morphologically and chemically identified, by examining spore walls or replicas after enzymatic or chemical digestions.

Recently, it has been demonstrated that the sporal wall of *Glomus epigaeum* Daniels and Trappe, a vesicular-arbuscular mycorrhizal fungus, has a complex multilayered wall, part of which contains fibrillar subunits regularly organized in arcs (BONFANTE-FASOLO and VIAN 1984). The present paper reports the results of ultrastructural studies, cytochemical staining and biochemical analysis, limited to polysaccharides and proteins, of the wall of this spore.

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2. Materials and Methods

2.1. Material

Individual sporocarps of *Glomus epigaeum* Daniels and Trappe (2–5 mm in diameter) were collected from the soil surface of pot cultures of *Ginkgo biloba,* as described by BONFANTE-FASOLO and VIAN (1984).

2.2. Electron Microscopy

Fixation and embedding. Isolated spores were fixed at room temperature in a mixture of 3% (v/v) glutaraldehyde (GA) and 3% (v/v) acrolein in 0.1 M sodium cacodylate buffer at pH 7.2, or in 3% GA alone for 3 hours, then post-fixed in 1% osmium tetroxide in the same buffer for 2 hours.

Other spores were subjected to a chemical extraction method: after GA fixation, spores were incubated in 0.5% ammonium oxalate at 60 °C for 48 hours, then in 4% sodium hydroxide at room temperature for 72 hours, and lastly in 17.5% sodium hydroxide for 24 hours. At each stage, some spores were rinsed in distilled water and postfixed in osmium tetroxide (BONFANTE-FASOLO and GRIPPIOLO 1982). A third group of spores were autoclaved at 121 °C for 3 hours in 100 ml of saturated aqueous potassium hydroxide to deacetylate the chitin, according to PEARLMUTTER and LEMBI (1978). After rinsing, the spores were fixed as described above. Alternatively, the treatment with saturated aqueous potassium hydroxide was carried out at room temperature for 1 hour.

All the material used in the investigation was dehydrated through a graded ethanol series and embedded in Durcupan or Spurr (1969) resin mixture.

Staining. In addition to the usual uranyl acetate and lead citrate staining thin sections were stained with 1. silver proteinate (PATAg reaction) for location of polysaccarides (THIÉRY 1967); controls were performed by omitting thiocarbohydrazide and by replacing periodic acid by H_2O_2; 2. silver reagent (SWIFT 1968) for the location of proteins; control sections were treated with benzylthiol and iodoacetate, or the free aldehyde groups were blocked with dimedone (KNIGHT and LEWIS 1977, SCANNERINI and BONFANTE-FASOLO 1979). Frozen sections, prepared by cryoultramicrotomy (BONFANTE-FASOLO 1982), were floated on 4% sodium hydroxide for 2–4 minutes, washed and negatively stained with 1% sodium silicotungstate.

All sections were examined with a Philips EM 300 electron microscope.

2.3. Cell Wall Isolation Procedure

Sporocarps were sonicated 3 times for 5 minutes using a MSE Sonifier.

Isolated spores were washed several times and filtered through 60 µm mesh nylon to remove further soil debris or hyphal remnants. Spores were disrupted with glass beads of two sizes (0.5 and 0.11–0.12 mm) in a Braun apparatus at 4 °C. The treatment was repeated 4 times for 30 seconds at 45-second intervals.

Glass beads and unbroken cells were eliminated by low-speed centrifugation. The wall fragments were sedimented and collected on nylon (10–15 µm mesh), the glass dust passing through. The wall fragments were purified by successive washes (5 times with ice cold distilled water, 10 times with ice cold phosphate buffer, 0.1 M pH 6.5, 3 times again with distilled water) and centrifugation at 2,000, 1,500, and 3,000 × g for 15, 10, and 15 minutes respectively.

The purity of the cell wall fraction was checked on fresh material observed by light microscopy after Lugol staining and on material fixed and embedded for electron microscopy as described above for whole spores.

2.4. Cell Wall Treatments

The cell wall fractions were subjected to progressive alkaline extractions in ammonium oxalate and 4 and 17% sodium hydroxide, and a portion was fixed in GA and OsO_4, dehydrated and embedded as described in section 2.2.

The supernatants of the alkaline extractions (ammonium oxalate, 4 and 17% sodium hydroxide) were combined together because of the small quantity of the sample and precipitated by adding methanol to 75%. Several centrifugations at 3,000 × g for 10 minutes and washes with 60% methanol removed the alkali from the pellets, which were dried and weighed.

2.4.1. Hydrolysis

The soluble fraction was hydrolyzed according to DUTTON (1973): dissolution in 20 N H_2SO_4 for 1 hour at room temperature, and then dilution to 2 N for 6 hours at 100 °C in sealed tubes in a nitrogen atmosphere. The hydrolysate, after neutralization with Ba CO_3 and $Ba(OH)_2$, was concentrated by rotary evaporation and fractioned, according to DATEMA *et al.* (1977 a) by ion exchange chromatography, as shown in the following scheme:

Hydrolysate
|
Dowex 50 W × 8 (H^+)
Water | | 1 M HCl
Elution 1 Elution 2
|
Dowex 1 × 8 ($HCOO^-$)
Water | | 1 M $HCOO^-$
Elution 2 Elution 4

The column (0.8 cm in diameter and 5 cm in height) was eluted by 8 volumes of the eluants indicated.

In the scheme Elution 2 (E_2) corresponds to the fraction of aminosugars; E_3 to the neutral sugars and E_4 to the uronic acids.

2.4.2. Quantitative Determinations

The elutions were determined as follows:

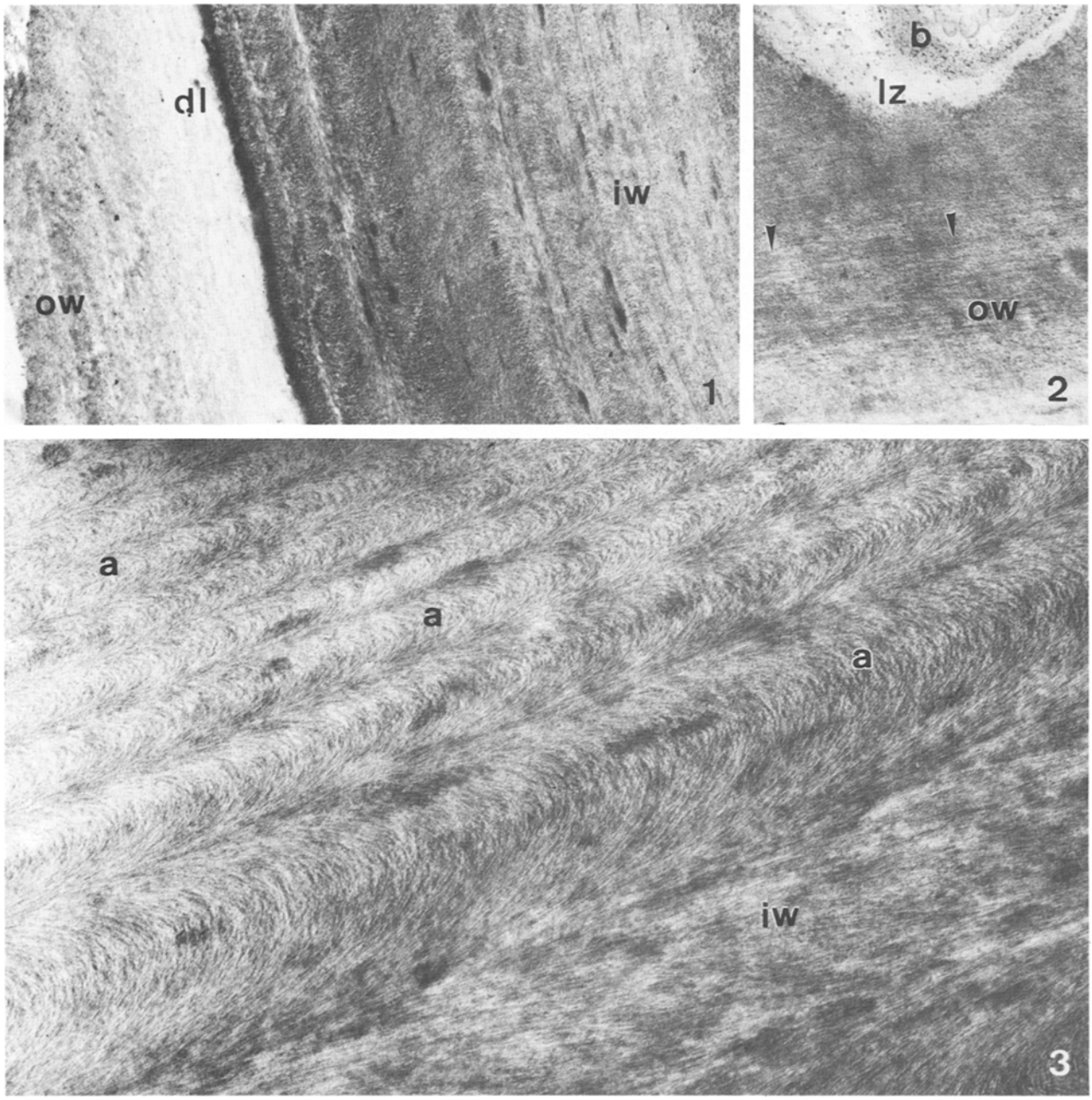
E_2 aminosugars by the Elson-Morgan procedure modified by WAGNER (1979);

E_3 neutral sugars by the phenol-sulphuric reagent (DUBOIS *et al.* 1956);

E_4 uronic acids by ortho-hydroxydiphenyl in concentrated sulphuric acid/tetraborate according to BLUMENKRANTZ and ASBOE-HANSEN (1973).

2.4.3. Gas Liquid Chromatography

Elutions 3 and 4 were analysed by Gas liquid chromatography (GLC) according to SWEELEY *et al.* (1963). The trimethylsilyl derivatives of sugars dissolved in pyridine were analysed on an Intersmat instrument equipped with a fused silica column (25 m length, internal diameter 0.23 mm, film thickness 0.13 µm, liquid phase C.P. Sil 5). The operative conditions were: linear programming of temperature from 130 to 180 °C (1 °C/minute), injector temperature 200 °C, detector temperature 250 °C, flow rate of the carrier gas (N_2) 2 ml/minute.



Transmission electron micrographs of untreated spore wall

Fig. 1. Oblique section, showing the inner wall (*iw*) and the outer wall (*ow*) separated by a dark line (*dl*). × 16,200

Fig. 2. Higher magnification of the outer wall (*ow*), showing an ordered array of the fibrils. A lysed zone (*lz*) produced by soil bacteria (*b*) can be seen in the surface. × 50,200

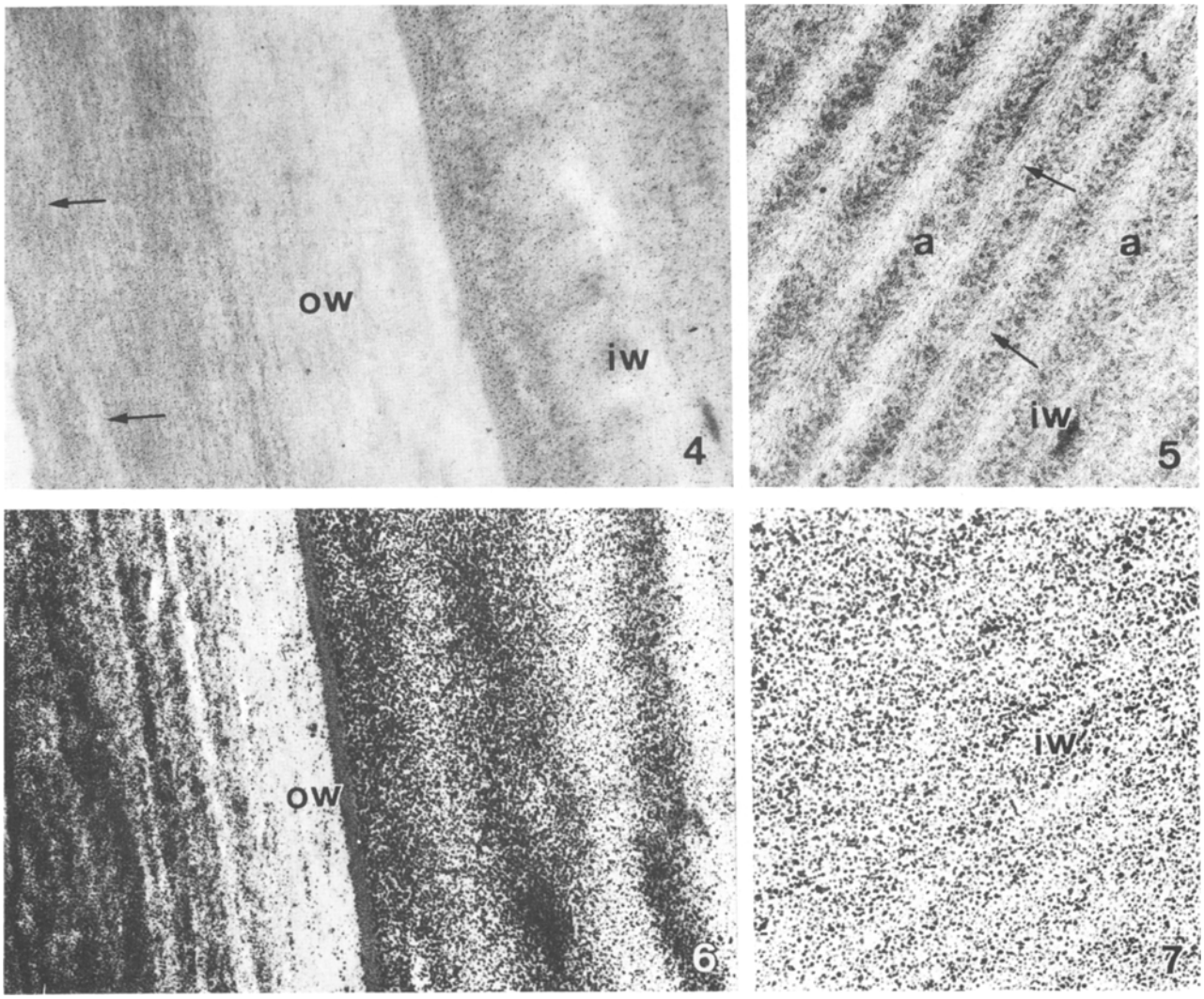
Fig. 3. Bow shaped structures, with arcs (*a*) of decreasing width are shown in the inner wall (*iw*). × 32,000

2.4.4. Analysis of the Insoluble Fraction

The sulphuric acid hydrolysis failed to hydrolyse the residue of the alkaline extractions. Hydrolysis was performed with 6 N HCl for 6 hours at 100 °C. The hydrochloric acid was then eliminated by rotary evaporation and the hydrolysates were stored in a vacuum desiccator over KOH pellets overnight. Samples of the hydrolysates were fractioned on Dowex resins as described above and the elutions determined colorimetrically and analysed by GLC as in sections 2.4.2. and 2.4.3.

2.4.5. Chitosan Measurement

The insoluble fraction was also treated with pronase (Koch-Light Laboratories from *Streptomyces griseus*) in order to eliminate residual proteins linked to glucosamine (DATEMA *et al.* 1977 b). The



Evidence for polysaccharide and protein in untreated spores

Fig. 4. PATAg reaction for polysaccharide. The outer wall shows an irregular distribution of Ag grains, that are more abundant in the outer zone, near the surface (arrows). × 50,100

Fig. 5. PATAg reaction for polysaccharide in the inner arched zone (*iw*). silver grains are distributed on the matrix material (arrows), that surrounds the unlabeled fibrils. × 64,000

Fig. 6. Swift reaction for protein. A dense deposition of Silver grains occurs in the inner wall, while the outer wall shows less abundant silver grains. × 30,500

Fig. 7. Swift reaction for protein in the inner arched zone. Silver grains are tightly packed, partly obscuring the arched pattern. × 48,000

material was then degraded with nitrous acid, which only effects deamination and depolymerization if the polymer contains free amino groups. The anhydrosugar resulting from the treatment was colorimetrically determined with 3-methyl-2-benzothiazolone hydrazone hydrochloride (MBTH) and $FeCl_3$, according to RIDE and DRYSDALE (1972). In addition, the same samples of the insoluble fraction were submitted to alkaline hydrolysis (RIDE and DRYSDALE 1972) causing deacetylation of the chitin. The determination was then carried out following Ride and Drysdale's method.

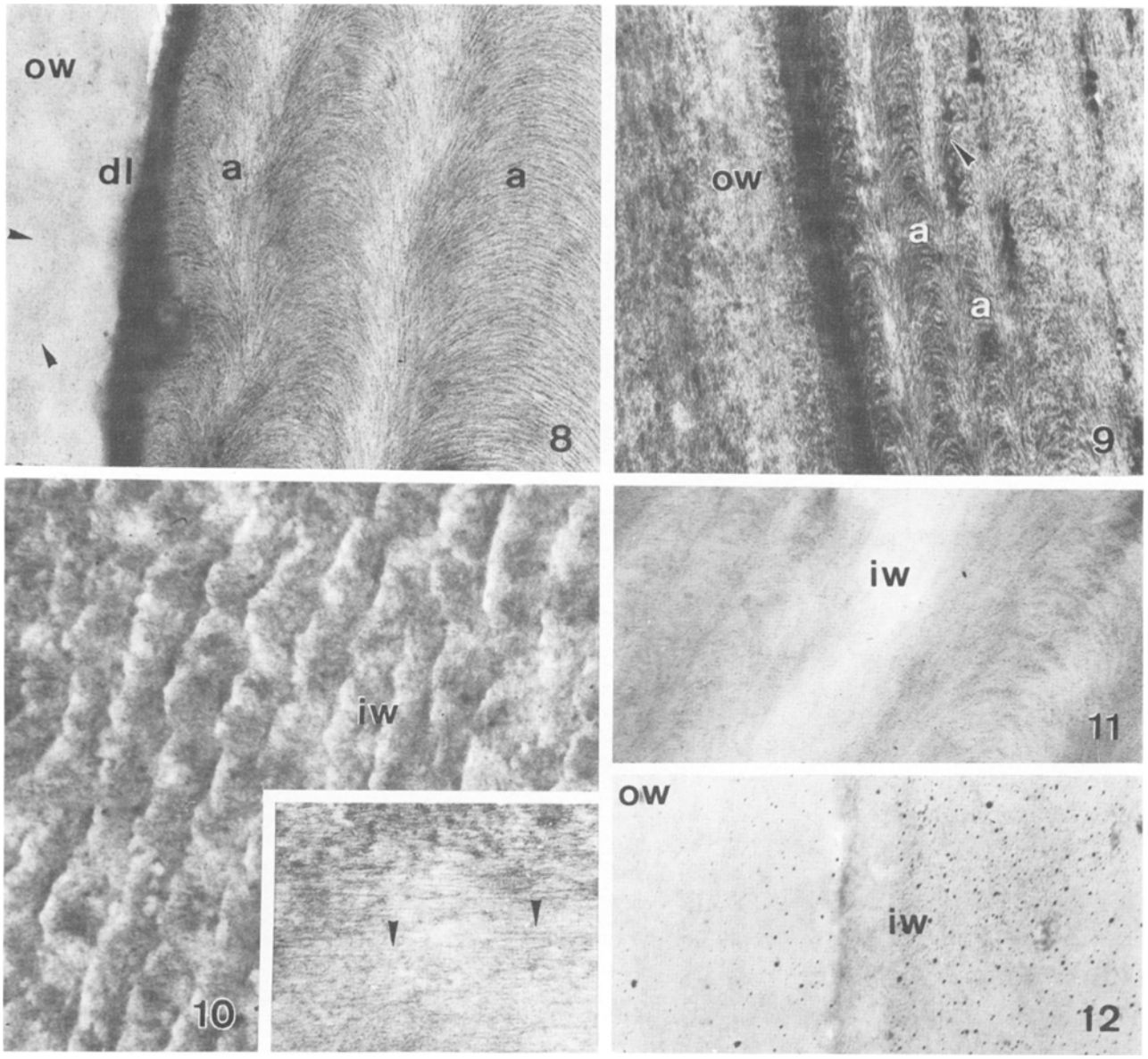
2.4.6. Determination of Proteins

Proteins present in the supernatant of the alkaline extractions and alkaline hydrolysis were determined by the procedure of LOWRY *et al.* (1951) using bovine serum albumin (BSA) as standard. The measurements were made on the supernatants after diluting the alkaline solutions to 0.5 N.

3. Results

3.1. Ultrastructural Observations

The mature spore of *Glomus epigaeum* has a complex multilayered wall, consisting of an outer wall (2–4 μm thick) and an inner wall (5–6 μm thick) separated by an electron-dense line (Fig. 1). While the outer wall



Wall architecture after different alkali extractions

Fig. 8. Ammonium oxalate extraction on the whole spore. Fibrillar arched structures are clearly seen, while fibrils of the outer wall are disordered (arrows). × 24,000

Fig. 9. 17% sodium hydroxide extraction on the wall fraction. The arched architecture is preserved (*a*) even if a little blurred. Single fibrils are evident. × 48,000

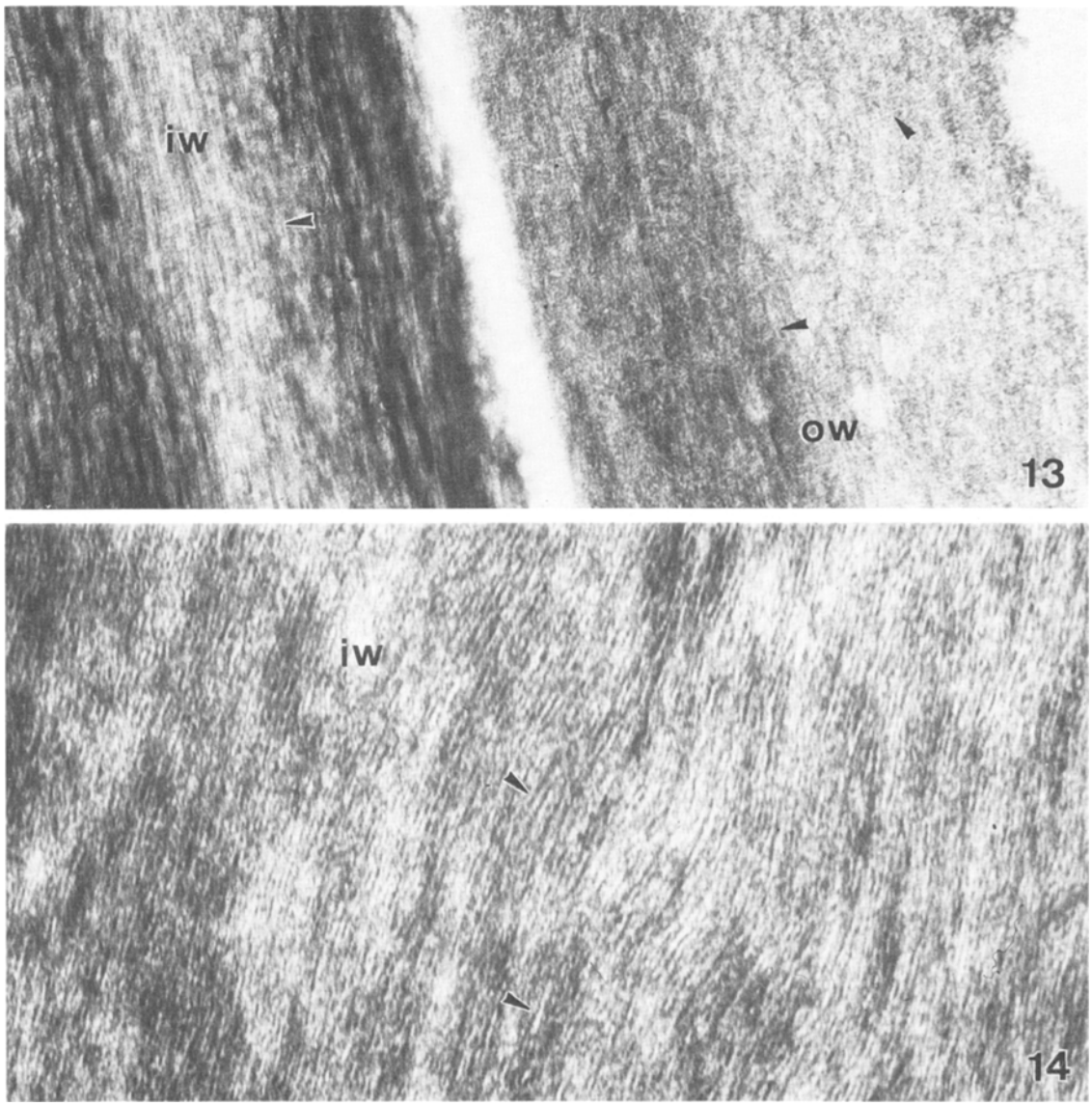
Fig. 10. In the autoclaved spores, the ordered arched structure is no longer recognizable. × 48,000. Inset. At higher magnification fibrils are clearly distinguishable and resistent to the drastic treatment. × 60,000 (arrows)

Fig. 11. PATAg reaction for polysaccharides on the spore subjected to alkali extraction. Silver grains are no longer recognizable. × 77,000

Fig. 12. Swift reaction for protein on the alkali-extracted cell wall fraction. A loose distribution of silver grains is still evident in the inner wall. × 30,500

showed a fibrillar texture with an ordered parallel orientation (Fig. 2), the inner wall displayed an arc-like texture due to the occurrence of layers in which regularly organized fibrils are rotated through a small angle with respect to the previous layers (Fig. 3) (for a complete description, see BONFANTE-FASOLO and VIAN 1984).

After the PATAg reaction for polysaccharides was performed, the outer wall displayed two different depositions of silver grains (Fig. 4). These were abun-



Frozen sections treated with 4% sodium hydroxide

Fig. 13. A section through the whole wall shows that the outer wall has a fibrillar structure in which ordered fibrils alternate with disordered and crossed ones (arrows). In the inner wall, fibrils appear parallel and longitudinally oriented. × 77,500

Fig. 14. Higher magnification of the fibrils belonging to the inner wall. × 182,000

dant towards the surface of the spore, where they delimited unstained fibrils, and disappeared in the deeper zone, near the electron-dense line. The inner wall showed silver grains regularly deposited on the matrix material, surrounding unlabeled fibrillar units (Fig. 5). All the control sections were devoid of silver grains.

The Swift reaction for sulphur-rich proteins led to an irregular deposition of coarse silver grains on the outer wall (Fig. 6), whereas a homogeneous and heavy distribution of coarse Ag grains occurred throughout the inner spore wall, without displaying a clearly arc-like structure (Fig. 7). Control sections treated with dimedone to block free aldehyde groups did not show strong changes in silver distribution, while after benzyl-thiol and iodoacetate treatment, the reaction was largely blocked.

Progressive extraction with alkali (ammonium oxalate and 4% sodium hydroxide) (Fig. 8) or treatment with cold saturated aqueous potassium hydroxide resulted in more evident fibrils, the matrix material appearing less abundant in the inner wall. In some spores, the regular fibrillar organization disappeared in the outer

wall, fibrils appearing crossed and disordered (Fig. 8).

Following 17% sodium hydroxide extraction, the spore wall conserved its architecture, as could also be seen in the cell wall fraction (Fig. 9). In the outer wall, fibrils were no longer easily recognizable, and in the inner wall the fibrillar arched structures were a little more confused. However, the fibrillar units were clearly evident and well defined even at high magnification. The electron-dense line was always present.

In the light microscope the walls of the autoclaved spores appeared altered without the lamellations observed in the untreated samples (BONFANTE-FASOLO and VIAN 1984). At electron microscope level, the wall did not show the ordered arched architecture, but there was an apparent collapse of the layers which appeared blurred (Fig. 10). Notwithstanding the loss of the ordered architecture, fibrils were always clearly recognizable (Fig. 10, inset). In these samples, the electron-dense line appeared less dense.

In all the extracted samples, the silver grain deposition of the PATAg reaction progressively disappeared and only sporadic grains remained on the matrix material (Fig. 11). Deposition of silver grains after the Swift reaction was not changed in the GA-fixed and then alkali-extracted spores. In contrast, the reaction was strongly reduced in the sections of cell wall fractions treated with alkali, before the GA fixation (Fig. 12). In the autoclaved spores also, some sporadic silver grains were still observed after the PATAg and Swift reactions.

In frozen sections, where fibrils appeared surrounded by an electron dense matrix (BONFANTE-FASOLO and VIAN 1984), a weak alkali extraction (4% sodium hydroxide for 2–4 minutes) improved fibril definition, both in outer and inner wall (Fig. 13). Fibrils clearly stood out as electron-transparent units and allowed use of higher magnifications (Fig. 14).

3.2. Biochemical Results

The dry weights of the cell wall fractions obtained from *G. epigaeum* spores by alkali treatment are given in Tab. 1.

The soluble fraction contained mostly proteins and only a small amount of polysaccharides, the dry weights and yields of which are given in Tab. 2, A. The total amount of polysaccharides is given by the sum of the quantities obtained in the elutions E_2, E_3, E_4 (Tab. 2, B). After sulphuric acid hydrolysis, the alkali-soluble fraction was completely hydrolyzed and the sugars were determined after separation on Dowex resins (Tab. 2, B). In the alkali-soluble polysaccharides, glucosamine occurred in a small quantity, whilst uronic acids and neutral monosaccharides formed the major part of the fraction. Uronic acids were identified by gas liquid chromatography as being the γ-lactone of glucuronic acid (Fig. 15), and the neutral monosaccharides as being glucose, mannose and rhamnose (Fig. 16).

The insoluble residue was resistent to sulphuric acid hydrolysis, whereas it was hydrolyzed by 6 N HCl. The residue mostly consisted of glucosamine. Tab. 3, A, gives the dry weights of the components. It is evident that chitin, as estimated from glucosamine, represents an important portion of the spore wall. To be certain that the glucosamine determined after acid hydrolysis originally occurred as chitin (*i.e.*, the acetylated poly-

Table 1. Dry weights of the cell wall fractions [1]

	Dry weight mg	Yield %
Total wall	12.09	100
Soluble fraction	5.02	41.5
Insoluble residue	6.865	56.8
Unaccounted for	0.205	1.7

[1] All the values are an average of four determinations.

Table 2

	Dry weight mg	Yield %
A. Proteins and polysaccharides in the soluble fraction [1]		
Soluble fraction	6.45	100
Proteins as BSA	3.556	55.1
Soluble fraction	2.736	100
Polysaccharides	0.3346	12
	Dry weight µg	**Yield %**
B. Composition of the polysaccharide components [1]		
E_2 (glucosamine)	42	12.5
E_3 (neutral sugars as glucose)	174.6	52.2
E_4 (glucuronic acid)	118	35.3
	334.6	

[1] All the values are an average of three determinations.

mer) and not as chitosan (the deacetylated polymer), the latter was estimated after pronase/nitrous acid treatment (Tab. 3, B). Chitosan occurred in a small quantity. In addition, a determination of chitosan in the

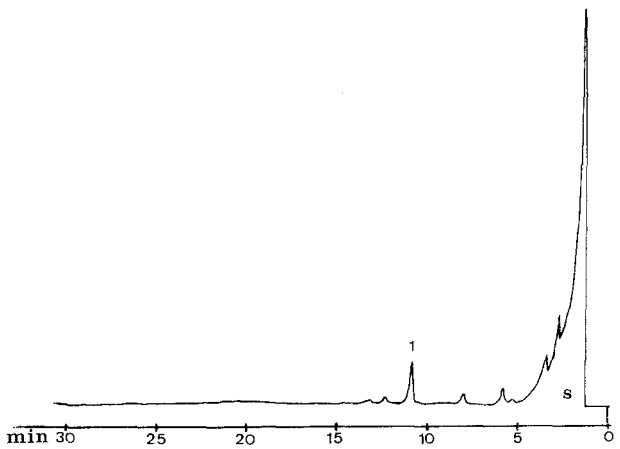


Fig. 15. Gas chromatogram of E_4 fraction, released from *G. epigaeum* cell wall fraction by sulphuric hydrolysis. *S* solvent peak; *1* γ lactone of glucuronic acid

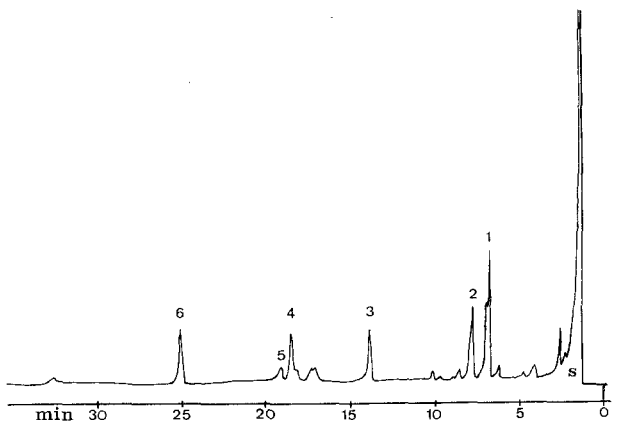


Fig. 16. Gas chromatogram of the neutral fraction (E_3), released from *G. epigaeum* cell wall fraction by sulphuric hydrolysis. *S* solvent peak; *1* and *2* α and β rhamnose; *3* and *5* α and β mannose; *4* and *6* α and β glucose

residue was performed after an alkaline autoclave hydrolysis had been used to deacetylate the acetyl-glucosamine. The results (Tab. 3, C) show a value very close to that obtained from the acid hydrolysis (Tab. 3, A) and that glucosamine arising from hydrolysis is present in the polymer to a very large extent as N-acetyl-glucosamine.

The presence of other sugars in the residue was also investigated in the same way followed for the soluble fraction. However, uronic acids were below the limits of

Table 3. *Proteins and polysaccharides in the insoluble residue* [1]

	Dry weight mg	Yield %
A. Determination of chitin, after hydrochloric hydrolysis		
Insoluble residue	4.11	100
Glucosamine	1.937	47
Non-hydrolysate residue	1.03	24.6
B. Determination of chitosan after pronase digestion		
Insoluble residue	5.175	100
Glucosamine	0.245	4.7
C. Determination of chitosan after alkaline hydrolysis		
Insoluble residue	8.7	100
Glucosamine	4.268	49
D. Determination of proteins from the supernatant of the alkaline hydrolysis		
Insoluble residue	8.7	100
Proteins as BSA	0.406	4.6

[1] All the values are an average of three determinations.

Table 4. *Principal components of the cell wall fraction of Glomus epigaeum spore*

Chitin	27.2%
Glucosamine (chitosan?)	3.3%
Alkali-soluble polysaccharides	5%
Proteins	25.4%
Insoluble residue after alkali extraction and acid hydrolysis	14%

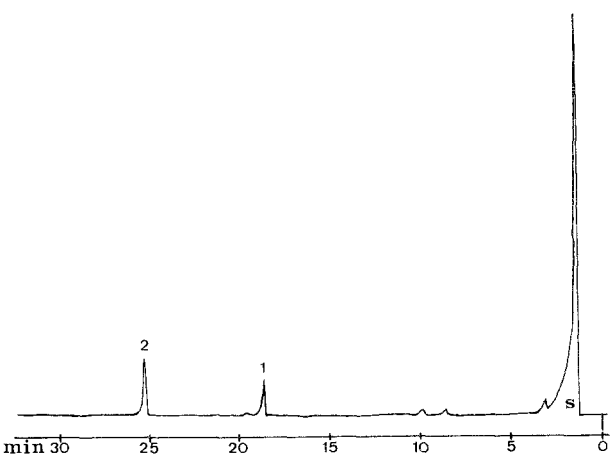


Fig. 17. Gas chromatogram of the neutral fraction (E_3), released from *G. epigaeum* cell wall fraction by hydrochloric hydrolysis on the residue. *S* solvent; *1* and *2* α and β glucose

reliability of the colorimetric method. In the neutral fraction, the only sugar present was identified as glucose (Fig. 17). However, these quantitative determinations are not reliable, because the conditions of the hydrochloric acid hydrolysis are too drastic for the acidic and neutral fractions of polysaccharides (DUTTON 1973).

The proteins still linked to the insoluble fraction represented a small portion of it (Tab. 3, D).

All the results of the analytical tests are summarized in the Tab. 4.

4. Discussion

The results show that a combined cytochemical and chemical study allows the identification of some components in the spore wall of *Glomus epigaeum*.

The fibrillar components are easily recognizable by electron microscopy both in resin sections of whole spores or cell wall fractions and in frozen sections. The ultrastructural, cytochemical and chemical characteristics of the fibrillar components of the wall show that fibrils consist of chitin.

They are electron-transparent, like native chitin (PEARLMUTTER and LEMBI 1978) and are negative to the periodate/thiocarbohydrazide/silver proteinate test for polysaccharide, as chitin would be since it lacks vicinal diols (VAN DER VALK *et al.* 1977). Like chitin (BARTNICKI-GARCIA and NICKERSON 1962), they are resistant to alkali extractions; this is seen both in whole spores, where the alkali extraction effect could be attenuated by the variable thickness of the outer layer (BONFANTE-FASOLO and VIAN 1984) and in the cell wall fractions and the frozen sections. On the latter, even brief alkali treatment is effective, as it acts directly on unembedded material only 80 nm thick. Also, observations on the samples subjected to strong autoclaving show that the single fibrils are not altered, but rather the whole architecture is affected.

In conclusion, the ultrastructural studies show that in *G. epigaeum* chitin exists in a microfibrillar state, different from that found in some other fungi, where it is non-fibrillar (BONFANTE-FASOLO 1982, GOW and GOODAY 1983, POLLACK *et al.* 1983). The X-ray diffraction pattern of the cell wall fraction of *G. epigaeum*, corresponding to that of authentic chitin, confirms our interpretation (Dr. G. GAZZONI, personal communication).

WESSEL and SIETSMA (1981) suggest caution, when claiming the existence of fibrillar chitin in native walls, where the crystallites or fibers become evident after alkali or acid treatments, that can modify the chemical structures of the polymer and change their states of aggregation. However, in the spore studied here, it is possible to state that the ordered units of fibrillar chitin occur in native walls and are not caused by any preparation artifact, being present before and after all the different treatments. This is well evident in the frozen sections where it is most likely that macromolecular structures are present in a relatively undisturbed state (SJØSTRAND and BERNHARD 1976).

The chemical analysis show that chitin represents 47% of the insoluble residue, and 27.2% of the whole spore wall. This high proportion of chitin is comparable with that found in other spore walls, as for example those of *Plasmodiophora brassicae* where chitin represents 25% (MOXHAM and BUCZACKI 1983). However, the *Mucorales*, the order to which *G. epigaeum* belongs, have chitosan, not chitin, as the most important component (BARTNICKI-GARCIA 1968). In mucoraceous fungi, nascent chitin is an intermediate in the biosynthesis of chitosan (BARTNICKI-GARCIA and DAVIS 1983). Treatment with nitrous acid allows us to exclude the possibility that the fibrillar components of *G. epigaeum* consist of chitosan, since they are not affected by the treatment.

Our results are confirmed by the only report regarding the biochemical composition of vesicular-arbuscular mycorrhizal spores. WEIJMAN and MEUZELAAR (1979) showed that *G. convolutus, macrocarpus,* and *mosseae* possess glucosamine polymerized as chitin. The deacetylated form, occurring in small amounts in *G. epigaeum* (3.3% of the whole wall), could be randomly spread throughout the wall, as an integrated part of the chitin fibril (STIRLING, COOK, and POPE 1979) or it could be present as chitosan fibrils, mostly in the outer wall. In this site, ultrastructural observations in fact suggest that fibrils are sometimes affected by alkali treatment, appearing disordered and blurred.

However, chitin and chitosan are not easily distinguished morphologically since fibrils observed in the autoclaved spores (and therefore deacetylated) are not different from those in untreated ones. Native chitosan, like chitin, is fibrillar, consisting of electron-transparent fibrils (unpublished results). Only a cytochemical test, using a lectin such as wheat germ agglutinin, specific for the N-acetyl-glucosamine group (CEDERBERG and GRAY 1979) or the use of specific enzymes such as chitinase and chitosanase linked to colloidal gold, as already done for other polysaccharides (VIAN *et al.* 1983) could allow *in situ* differentiation of chitin from chitosan.

In the alkali-soluble fraction, polysaccharides represent

only a small part, while proteins represent the most important component. These results completely agree with the cytochemical observations on polysaccharide location, where silver grains are regularly but loosely spread throughout the wall, delimiting unstained fibrils in the inner and outer wall. After the alkali extractions, the silver grains progressively disappear, showing that polymers of neutral sugars and uronic acids (found in the chemical analysis of the soluble fraction) can be responsible for the deposition, therefore forming part of the matrix material.

Uronic acids have already been found in the walls of *Zygomycetes* for example in *Mucor rouxii* (BARTNICKI-GARCIA and LINDBERG 1971). DATEMA *et al.* (1977a) studied the occurrence of glucuronic acid in *Mucor mucedo* and found that it was associated with mannose, galactose and fucose to form a heteropolymer. The polymer was linked to chitin and chitosan with non-covalent linkages. The sugars, occurring in the alkali-soluble fraction of *Glomus epigaeum* suggest that in this fungus also there is a heteropolysaccharide containing glucuronic acid and neutral sugars.

Even though no cytochemical test of completely proved specificity for proteins is available for electron microscopy (KNIGHT and LEWIS 1977), the heavy deposition of silver grains after the Swift reaction agrees well with the large proportion of protein occurring in the soluble fraction. Proteins also occur in the insoluble residue, giving an explanation for the residual silver grain deposition after the Swift reaction on the alkali-extracted cell wall fractions. They could be strongly bound to chitin, as already described in *Mucor mucedo* (DATEMA *et al.* 1977b) via the aminogroup of glucosamine, or their presence could be due to an incomplete alkaline extraction.

It is interesting to note that the structure of *G. epigaeum* wall, mostly consisting of chitin fibrils in a protein matrix, is more easily comparable to that described in insect cuticle. In the latter, where arched structures were first observed, chitin fibrils are generally described as linked to a protein matrix (NEVILLE and LUCKE 1969). In addition, BLACKWELL (1982) observed that in *Megarhyssa ovopositor* both chitin and protein are ordered, showing a helical array of protein subunits around the chitin fibril.

In conclusion, in the case of *Glomus epigaeum* it is possible to construct a model in which all the polymers revealed by the biochemical analysis are interconnected to build a macromolecular network corresponding to the images observed by electron microscopy (Fig. 18).

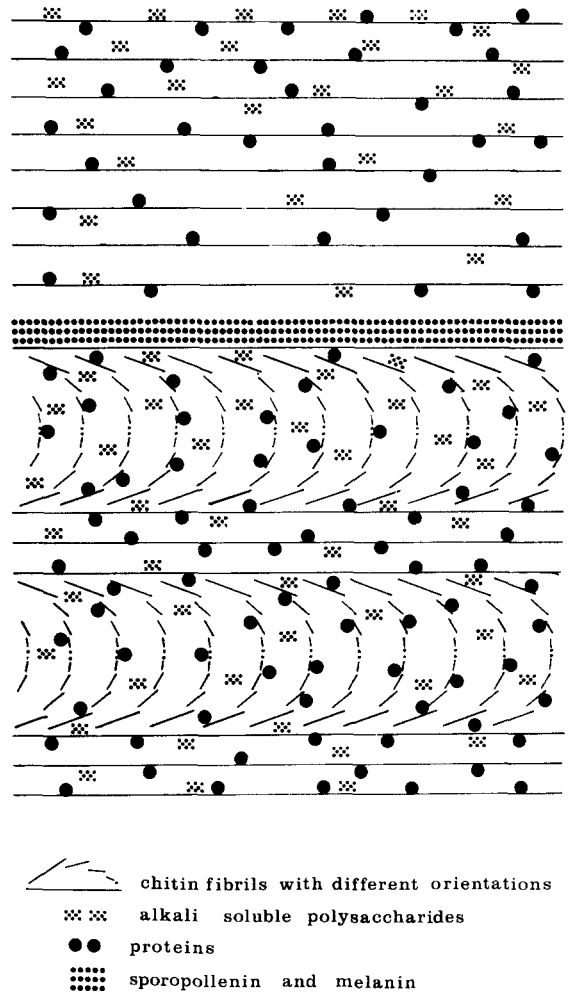


Fig. 18. All the wall components revealed by biochemical and cytochemical tests are shown. In the outer wall, fibrils (chitin or chitosan?) with a linear arrangement, are surrounded by a scarce matrix of alkali-soluble polysaccharides and proteins. In the inner wall, an abundant matrix surrounds fibrils of chitin, regularly organized in arcs

The scheme suggests that the outer and inner wall, separated by the electron-dense line probably consisting of sporopollenin and melanin-like pigments (unpublished results), are morphologically and chemically different. Proteins and alkali-soluble sugars seem to be irregularly distributed in the outer wall, being more abundant in the inner wall. Chitin fibrils occur throughout the whole wall, but show a different texture. Moreover, they might have a different nature, since in the outer wall deacetylated chitin fibrils might occur.

All these differences could be linked to the different roles played by the outer primary wall in comparison with the inner secondary wall: while the primary wall

allows growth and extension of the spore surface, the secondary wall with its chitinous arcs and the sporopollenin line, could provide good resistance to the breakdown processes caused by soil microorganisms.

Acknowledgements

The authors wish to thank Prof. C. MONTANT and his staff (Laboratoire de Cryptogamie, Université Paul Sabatier, Toulouse) for teaching biochemical techniques and for facilities made available to Dr. R. GRIPPIOLO during a stage in Paul Sabatier University.
They are also grateful to Dr. G. GAZZONI (Istituto di Mineralogia e Cristallografia dell'Università di Torino) who kindly performed the X-ray diffraction.
Research work supported by CNR, Italy. Special grant I.P.R.A.-Subproject 1. Paper N. 93.

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