Structure and Composition of Walls of the Yeast Form of *Verticillium albo-atrum*

By M. C. WANG AND S. BARTNICKI-GARCIA

*Department of Plant Pathology, University of California,
Riverside, California 92502, U.S.A.*

(Accepted for publication 11 September 1970)

SUMMARY

Walls of the yeast form of *Verticillium albo-atrum* showed a granular appearance on the outer surface. The granular components could be extracted by alkali, revealing a fibrillar wall fabric. A region of circularly oriented microfibrils with a minute central 'orifice' was commonly found at one of the cell poles and probably represented a bud scar. The material dissolved by alkali was a heteropolysaccharide-protein complex containing mannose, galactose, glucose, glucuronic acid, glucosamine and the common range of amino acids. The alkali-insoluble microfibrillar network was made of a β -linked glucan and chitin. The glucan was digested by endo- β -glucanases yielding glucose, β -1,3-linked glucose oligomers and cellobiose, but no evidence for cellulose was found. Most of the glucan was also soluble in hot acid. The acid-insoluble glucan (hydroglucan) contained β -1,6-links. Acid treatment produced coarse microfibrils resembling those in *Saccharomyces cerevisiae* walls treated similarly. The hydroglucan was soluble in alkali leaving an insoluble microfibrillar network composed mainly of chitin. A small amount of lipid (2.7 to 3.4 %), mostly of the bound type, and traces of phosphate were also found.

INTRODUCTION

Little is known about the biochemical basis of differentiation in *Verticillium albo-atrum*, a fungus which causes wilt diseases of a wide range of economically important plants. Brandt and his associates (Brandt, 1962, 1964, 1965; Brandt & Reese, 1964; MacMillan & Brandt, 1966) have clarified some of the events leading to microsclerotial formation but nothing is known about the biochemistry of vegetative development in this organism.

Verticillium albo-atrum is a dimorphic fungus. In stationary culture media, it grows as a typical mycelium; in shake cultures it can develop chiefly as budding yeast-like organisms (Malca, Erwin, Moje & Jones, 1966). Significantly, the yeast form (also referred to as conidial or spore form) also occurs in infected tissues of cotton plants (Garber & Houston, 1966). The dimorphism of *V. albo-atrum* is somewhat analogous to that of animal pathogens where the parasitic phase is usually the yeast phase. The dimorphic capacity of *V. albo-atrum* plays an important role in pathogenesis; colonization of the upper parts of infected plants is by movement of the yeast form (conidia) through vascular tissue and not by mycelium spreading (Presley, Carns, Taylor & Schnathorst, 1966).

This report describes the walls of the yeast form of *Verticillium albo-atrum* grown in shake culture. This is an initial effort to correlate cellular morphogenesis with wall

structure, as done in the study of morphogenesis of other fungi (Nickerson & Bartnicki-Garcia, 1964; Bartnicki-Garcia, 1968). The ultimate objective of this project is to elucidate the biochemical basis of dimorphism of *V. albo-atrum* and thus contribute to a better understanding of host-parasite relationships in Verticillium wilt.

METHODS

Microbiological techniques. A microsclerotial isolate (v_3H) of *Verticillium albo-atrum* which causes defoliation of cotton (Mathre, Erwin, Paulus & Ravenscroft, 1966) was used. Stock cultures were routinely maintained on yeast extract + potato + glucose-agar slopes at 25°. Slopes were inoculated with single cells to maintain morphological stability. The yeast from was grown in shake cultures in glucose nitrate medium (Malca *et al.* 1966) prepared by dissolving the ingredients, excluding phosphates, in 4/5 final vol. water. A solution of KH_2PO_4 and K_2HPO_4 (pH 6.4) was prepared separately, both were autoclaved 20 min. at 121°, then mixed aseptically. Unless otherwise specified, 50 ml. medium in a 250 ml. Erlenmyer flask was inoculated with 1 ml. of a 7 day liquid culture which had been freed of a small proportion of mycelium by passing through a double-layer of sterile cheesecloth. The yeast suspension which emerged was centrifuged at 1000 g, rinsed with sterile water and suspended to 10^6 to 10^7 organisms/ml. and used as an inoculum. Cultures were incubated in a rotary shaking incubator at 25° for 5 to 7 days. The yeast form was harvested after filtration through nylon mesh (in place of cheesecloth to avoid contamination with cellulose fibres) to remove hyphae; centrifuged at 10,000 g for 10 min. at 1° and rinsed twice with 10 vol. cold 0.05 M-tris-HCl buffer, pH 7.5.

Isolation of walls. The organisms were suspended in 3 vol. cold 0.05 M-tris-HCl buffer, pH 7.5, and the slurry transferred to a 50 ml. Bronwill MSK homogenizer glass container to which an equal vol. of acid-washed glass beads (0.25 to 0.30 mm. in diameter) had been added. After four 15 sec. treatments with 5 sec. intervals for cooling, while liquid CO_2 was flushed continuously around the flask (at the end of a run, the temperature of the container seldom exceeded 15°) the glass beads were allowed to sediment and the broken cell suspension was decanted into a centrifuge tube. The walls were centrifuged at 500 g for 5 min. at 0°. The pellet was suspended in 5 vol. buffer and blended at the top speed of a Virtis 45 homogenizer for 2 min. and centrifuged again at 500 g.

The entire breakage and washing procedure was repeated four or five times to obtain a wall preparation with less than 0.1 % unbroken cells. Cytoplasmic debris was eliminated after 20 to 30 cycles of centrifugation and homogenization. Walls, stained with Lugol's iodine solution, were examined with a Reichert phase-contrast microscope and glass bead fragments were removed by consecutive 1-min. centrifugation at 50, 100 and 200 g. The pellet resulting from each centrifugation was suspended in water and centrifuged again at the same speed, while the supernatant fluid was combined with the residue sedimenting at the next higher speed. Purified walls were lyophilized and stored in a desiccator.

Chemical analyses. Two types of hydrolysate were prepared. *H₂SO₄ hydrolysate*: dry wall samples were soaked with 22.5 N- H_2SO_4 and incubated at 30° for 3 h. The acid was diluted to 0.85 N and heated at 97° for 4 h. *HCl hydrolysate*: dry wall samples were suspended in 6 N-HCl, sealed in ampules under N_2 and hydrolysed at 105° for

different times. The HCl was evaporated to dryness under vacuum in a rotary evaporator, or in a desiccator over NaOH pellets under vacuum at 45° overnight.

H₂SO₄ hydrolysates containing neutral sugars were neutralized with BaCO₃ and de-ionized with a double bed of Dowex-1 (acetate form)+Dowex-50 (H⁺ form). Sugars were separated on paper chromatograms (Whatman 3 MM) irrigated in descending fashion with either butanol+pyridine+water (BPW) (6+4+3, by vol.) or ethyl acetate+acetic acid+water (EAW) (9+2+2, by vol.) for 36 h. or longer. Sugar spots were revealed with aniline phthalate (Dawson *et al.* 1959). For quantitative measurements, unsprayed portions of the chromatogram containing suspected sugars were cut out, eluted, and the sugar estimated by the anthrone method.

For gas-liquid chromatography (g.l.c.), hexose samples were converted to their corresponding alditol acetates. They were reduced to the corresponding alditols by sodium borohydride at room temperature for 3 h.; the resulting mixture was decationized through a Dowex-50 (H⁺ form) column and the borate in the filtrate was evaporated off with methanol+1% (v/v) acetic acid. The alditols were acetylated with acetic anhydride and pyridine (Hause, Hubicki & Hazen, 1962). The alditol acetates were separated by a Perkin-Elmer gas chromatograph, model 881, using a stainless steel column (10 ft × $\frac{1}{8}$ in. o.d.) packed with Gas Chrom Q 60 to 80 mesh, coated with 3% ECNSS-M, an organosilicone polyester phase resulting from the combination of ethylene glycol succinate and a silicone of a cyanoethyl type (Kim, Shome, Liao & Pierce, 1967).

Amino sugars were determined in the HCl hydrolysate by the Elson-Morgan method (Tracey, 1955). Hexosamine was identified as glucosamine by oxidation with ninhydrin (Stoffyn & Jeanloz, 1954) and by paper electrophoresis in 1% borate (Maley & Maley, 1959).

Hexoses were estimated by Dreywood's anthrone method (Bartnicki-Garcia & Nickerson, 1962). Reducing sugars were determined by the method of Somogyi & Nelson (Hodge & Hofreiter, 1962). Glucose was assayed by glucose oxidase reagent (Glucostat 'special'; Worthington Biochemical Corp., New Jersey, U.S.A.).

Uronic acids in the neutralized H₂SO₄ hydrolysate were separated by column chromatography. After neutralization with BaCO₃ the cleared supernatant fluid was placed on a Dowex-1 (acetate form) column. Uronides were eluted in 10 ml. fractions by a linear gradient of acetic acid (0 to 2 M; total vol. 1000 ml.). A modified carbazole reagent (Bitter & Muir, 1962) was used to detect uronic acid. Uronic acids and aldobiouronic acids were identified by reduction to the corresponding hexoses and neutral disaccharides, respectively, by way of the methyl ester methyl glucoside which was reduced with sodium borohydride (Lewis, Smith & Stephen, 1963) and acid hydrolysed to liberate the free monomers.

Total amino acid-N in HCl hydrolysates was determined by ninhydrin after elimination of interfering glucosamine (Bartnicki-Garcia & Nickerson, 1962). Individual amino acids and amino sugars were estimated in a Spinco automatic amino acid analyser, model 120 C. Total phosphate was determined spectrophotometrically (Dryer, Tammes & Routh, 1957). Free and bound lipids were extracted as described previously (Bartnicki-Garcia & Nickerson, 1962).

Physical techniques. Infrared spectra of purified walls in KBr pellets, and of alditol hexa-acetates in chloroform, were determined with a Perkin-Elmer spectrophotometer. Optical rotations were measured with a Bendix automatic polarimeter. X-ray diagrams

of purified wall samples spread on glass microslides were obtained with a Norelco Philips recording diffractometer using $\text{CuK}\alpha$ radiation. Chemically extracted wall residues were shadowed with Pd and examined in a RCA electron microscope, model EMU-3B.

Enzymic digestion. A glucanase preparation from *Streptomyces* sp. QMB 814 containing chiefly β -1,3- and β -1,4-endoglucanases was obtained through the courtesy of Dr E. T. Reese (U.S. Army Quartermaster Research and Engineering Center, Natick, Massachusetts, U.S.A.). Cell walls were digested in 12-ml. glass-stoppered centrifuge tubes at 50° for 36 h. A typical digestion mixture consisted of 5 ml. 0.05 M-sodium citrate buffer (pH 5.75), 1 mg. *Streptomyces* endoglucanases and 10 mg. cell walls. Digestion was stopped by heating at 97° for 5 min., and the mixture centrifuged at 1000 g for 10 min. The supernatant was de-ionized with Dowex resins as indicated above, and the soluble digestion products then separated by paper chromatography.

RESULTS

At harvest time, our cultures of *Verticillium albo-atrum* had very little mycelium and contained mainly a heterogeneous assortment of single cells in different stages of development. Some of the cells were identical in appearance to conidia or phialospores (Cole & Kendrick, 1969) and may have originated in part from true conidiophores formed on the mycelium prevalent in the first days of incubation. The large majority, however, were 'secondary conidia' produced by a budding process from large single cells, which in turn were derived from the initial conidia. The buds were frequently formed, not on the surface of the mother cell but at the end of a tubular process of variable length, which when well developed, resembled the phialide of a true conidiophore (Pl. 1, fig. 1). Thus the development of these buds has been considered equivalent to true conidiation and the daughter cells named 'secondary conidia' (Buckley, Wyllie & DeVay, 1969). In addition to conidia, a large portion of the culture consisted of single vegetative cells in different stages of budding.

Chemical composition

Chemical fractionation. Purified walls were chemically fractionated into alkali-soluble, acid-soluble, acid-insoluble but alkali-soluble and insoluble residue fractions. The composition of these fractions is summarized in Table 1 and a description of the principal components follows.

Heteropolysaccharide complex. This appeared to be the major component of the alkali-soluble fraction. On hydrolysis with N-acid it yielded D-glucuronic acid, D-mannose, D-galactose and D-glucose. In addition, D-glucosamine (Table 2) and the range of amino acids commonly found in fungal walls were detected in HCl hydrolysates of the alkali-soluble fraction (Table 3). The hexosamine was identified as glucosamine by oxidation with ninhydrin which yielded arabinose; the presence of mannosamine which also yields arabinose was ruled out by paper electrophoresis of the amino sugar in 1 % sodium borate. Up to 2 % glucosamine was detected, but its polymeric state is unknown. To exclude the possibility that this glucosamine arose from chitin, the alkali-soluble fraction was put through a Millipore filter (type HA; pore size 0.45 μm) to remove any contaminating wall fragments. The glucosamine content of the filtrate remained essentially the same.

Neutral sugars were separated by paper chromatography and by g.l.c. after conversion to their corresponding alditol acetates. In both instances, mobility was verified by co-chromatography with authentic samples. The properties of the alditol acetates are summarized in Table 4. Glucose was also identified by the glucose oxidase assay. The relative proportions of the neutral sugars were glucose:galactose:mannose = 3.5:2.6:1.0.

Table 1. *Fractionation of Verticillium albo-atrum walls*

Consecutive fractions	Composition	
	Monomers	Main polymers
1. Alkali-soluble (N-KOH at 60°)	D-Glucose D-Galactose D-Mannose D-Glucuronic acid D-Glucosamine 15 Amino acids	Heteropolysaccharide Protein
2. Acid-soluble (N-HCl at 97°)	D-Glucose D-Glucosamine 15 Amino acids	β -Glucan Protein
3. Acid-insoluble, alkali-soluble (0.75 N-NaOH at 60°)	D-Glucose D-Glucosamine (trace) 15 Amino acids (traces)	β -Glucan (hydroglucan)
4. Insoluble residue	D-Glucose (trace) D-Glucosamine Lysine, histidine	Chitin

Table 2. *Quantitative composition of Verticillium albo-atrum walls**

Component	Wall dry weight (%)
Glucuronic acid†	1.2 to 1.3
Glucose†	41.0 to 56.0
Galactose†	8.0 to 8.5
Mannose†	6.0 to 6.4
Glucosamine (non-chitinous)‡	2.0 to 3.3
Chitin	7.6 to 10.0
Protein	11.0 to 14.0
Lipid, readily extracted	0.4 to 0.6
Lipid, bound	2.3 to 2.8
Phosphate (as H ₃ PO ₄)	0.1 to 0.2
Sum	79.6 to 103.1

* A range of values obtained with at least four different wall preparations.

† Expressed as anhydrosugar.

‡ Computed by subtracting the glucosamine content of insoluble residue (chitin) from that of unextracted wall.

The uronides present in the sulphuric acid hydrolysates were separated by column chromatography with Dowex-I, acetate form (Fig. 3). The separation was performed on the alkali-soluble fraction as well as on a large sample of unextracted walls. Peak A contained mannose, galactose and glucose. Peak B contained an aldobiouronide of D-glucuronic acid and D-galactose. After reduction of the aldobiouronic acid to the neutral disaccharide, followed by acid hydrolysis, glucose and galactose were obtained in a 1:1 ratio and about 50% yield. The compound of Peak C was identified as D-glucuronic acid by: (a) detection of both glucuronic acid and glucuronolactone on the

the paper chromatogram; (b) formation of D-glucose upon reduction of the uronic acid.

Glucan(s). After removing alkali-soluble components, chiefly heteropolysaccharide and protein, wall residues consisted mainly of glucan(s) and chitin. Two glucan fractions were separated (Table 5). By treatment with hot N-HCl, 25 % (wall basis) glucan

Table 3. *Amino acid analysis of Verticillium albo-atrum walls and fractions thereof**

Amino acid	% μ moles				
	Alkali-soluble	Acid-soluble	Acid-insoluble, alkali-soluble	Insoluble	Whole cell walls†
Lysine	3.9	3.6	T	35.4	4.8
Histidine	3.2	3.4	T	64.6	2.2
Arginine	1.5	1.0	T	—	0.9
Aspartic acid	10.5	8.7	T	—	8.2
Threonine	8.7	12.7	T	—	14.0
Serine	6.4	5.3	T	—	8.6
Glutamic acid	11.3	10.6	T	—	8.5
Proline	12.5	15.7	T	—	13.2
Glycine	10.9	7.1	T	—	10.0
Alanine	12.5	10.9	T	—	7.9
Cystine	T	T	T	—	1.9
Valine	8.1	10.5	T	—	8.4
Methionine	—	—	—	—	T
Isoleucine	3.3	3.2	T	—	2.7
Leucine	5.1	3.2	T	—	2.9
Tyrosine	X	2.9	T	—	3.8
Phenylalanine	2.1	1.1	T	—	1.9
Total amino acid mg./g. cell wall	32.02	44.94	Traces	0.56	90.80†
Glucosamine mg./g. cell wall	1.36	16.88	Traces	65.56	—

* Average of two determinations made on a 6N-HCl hydrolysate at 105° for 12 h. Fractions obtained as indicated in Table 1.

† The wall sample was hydrolysed with 6 N-HCl at 105° for 24, 48 and 72 h., respectively. Per cent μ moles of each amino acid were determined from either average, maximum, and/or extrapolated values.

T = Traces. X = Inseparable from glucosamine residue.

Table 4. *Alditol acetates prepared from the H₂SO₄ hydrolysates of Verticillium albo-atrum walls**

Compound	Melting point		[α] _D ²⁵ in CHCl ₃		G.l.c. retention time (min.)
	Observed	Reference	Observed	Reference	
Glucitol hexa-acetate	99	98	+9.1 (c = 2.05)	+10.0	44
Mannitol hexa-acetate	124	124 to 125	+25.5 (c = 2.59)	+25.2	33
Galactitol hexa-acetate	169	168 to 169	—	—	38

* Reference values from Lohmer (1949).

was solubilized. Glucose was the only hexose detected in the acid hydrolysate (0.85 N-H₂SO₄ at 105° for 10 h.) by paper chromatography using the BPW solvent system. This finding was confirmed by glucose oxidase assays. The remaining acid-insoluble glucan was essentially all dissolved by extraction with alkali (0.75 N-NaOH at 60° for 30 min.). Evidently the acid treatment not only dissolved most of the glucan but also rendered

the rest alkali soluble. This alkali-soluble glucan reprecipitated on acidification. Following two cycles of solution and precipitation, the glucan was examined in the X-ray diffractometer (Fig. 2B). The spectrum was similar to that of the 'hydroglucan' of *Saccharomyces cerevisiae*, with a strong reflexion in the 1.3 to 1.4 nm. region (Hou-wink & Kreger, 1953) which was not seen in the unextracted walls or alkali-extracted walls of *Verticillium albo-atrum*. It appeared when the walls were treated with hot acid

Fig. 1. Separation of uronides on a Dowex-1 (acetate form) column from an unextracted sample (○—○) and a N-KOH soluble fraction (△—△) of *Verticillium albo-atrum* walls. A gradient of 0 → 2 N-acetic acid was applied beginning with tube no. 16 for the unextracted walls and tube no. 24 for the alkali-soluble fraction.

Table 5. *Distribution of hexose polysaccharides in Verticillium albo-atrum walls*

Consecutive fractions	Hexosan* % wall dry weight
1. Alkali-soluble (N-KOH at 60°)	24.2
2. Acid-soluble (N-HCl at 97°)	25.1
3. Acid-insoluble, alkali-soluble (0.75 N-NaOH at 60°)	9.1
4. Insoluble residue	1.8
Sum	60.2
Unfractionated walls	66.0

*Determined with anthrone using glucose as standard; values expressed as % anhydrosugar.

(compare Fig. 2A with 3E), and disappeared upon subsequent alkali extraction (compare Fig. 2A with 3F). Glucose was the only sugar obtained after complete acid hydrolysis of the 'hydroglucan' with 22.5–0.85 N-H₂SO₄. The 'hydroglucan' optical activity was $[\alpha]_D^{25} = -10.5^\circ$ ($c = 0.36$ in 0.1 N-NaOH).

On partial acid hydrolysis the 'hydroglucan' yielded glucose and gentiobiose,

indicating the presence of β -1,6-linked residues. As with the acid treatment, a substantial (28 % wall dry weight) amount of glucan(s) was also dissolved by treatment of the alkali-extracted cell walls with the β -glucanase mixture from *Streptomyces* sp. The solutions resulting from enzymic hydrolysis were de-ionized and chromatographed on Whatman 3 MM paper irrigated by BPW (Table 6). The major products were β -1,3-linked oligosaccharides in addition to glucose and cellobiose. Cellobiose and laminaritriose, which moved at the same rate ($R_{\text{glu}} = 0.65$), were eluted and separated by chromatography with the EAW system. Glucose was identified by purified glucose

Table 6. *Digestion of the alkali-insoluble fraction of the walls of Verticillium albo-atrum with Streptomyces QMB 814 glucanases in the presence of [^{14}C]glucose**

Compound	Relative abundance†	Specific activity (d.p.m./ $\mu\text{g.}$)
Glucose	13.9	2081
Laminaribiose	13.2	17
Cellobiose	25.1	2
Laminaritriose	28.6	6
Laminaritetraose	19.2	0.7

* The alkali-insoluble wall (56.8 mg.) incubated with 1.9 μCi of [^{14}C]glucose (0.63 $\mu\text{Ci}/0.28 \mu\text{moles}$). The oligosaccharides were separated by paper chromatography with BPW (6+4+3, by vol.) at 24° for 24 h. followed by elution with H_2O and by rechromatography with EAW (9+2+2, by vol.) at 24° for 48 h.

† Determined by anthrone with glucose as standard; values are % of the sum of the five components.

oxidase reagent (Glucostat 'special'). The oligomers were identified by: (i) paper co-chromatography with authentic compounds in at least two solvents; (ii) susceptibility to a purified β -glucosidase (Worthington Biochemical Corp.) with negligible α -glucosidase activity; (iii) lead tetraacetate oxidation (Perlin, 1955). In the latter, oxidized β -1,3-sugars gave glucose and arabinose after acid hydrolysis; erythrose and glucose were the major products from cellobiose. The identity of cellobiose was confirmed through the preparation of cellobiose octa-acetate (m.p. and mixed m.p. = 196°).

To exclude the possibility that cellobiose may have arisen by trans-glucosidation during the enzymic digestion, such as that noted in β -glucosidase (Reese, Maguire & Parrish, 1967), digestion of the alkali-insoluble wall residues was repeated in the presence of [^{14}C]glucose. The low specific activity of each of the resulting oligosaccharides indicated that cellobiose and other β -1,3-linked oligosaccharides were not likely transglucosylated products (Table 6).

Despite the presence of cellobiose, no evidence for cellulose was obtained. Extraction with 17.5 % NaOH (under N_2) (commonly used to separate hemicelluloses from α -cellulose in plant materials; Corbett, 1963), did leave a substantial amount of insoluble glucan (50.5 %) in the walls of *Verticillium albo-atrum*. However, this result did not show that the alkali-insoluble glucan was α -cellulose since other β -glucans from fungal walls are known to be equally insoluble (Northcote & Horne, 1952; Bartnicki-Garcia, 1966). Furthermore, other more specific tests for cellulose were negative: (a) Schweizer's reagent dissolved nearly the same amounts of carbohydrate as NaOH (17.5 %) but no cellulose was regenerated upon neutralization; (b) absence of X-ray reflexions of crystalline cellulose (I or II) from any of the cell wall fractions (Fig. 2, 3).

Chitin. The reflexions of crystalline chitin were not clearly evident on the diagrams of the unextracted walls (Fig. 3D) but the two broad peaks, about 0.95 nm. and 0.45 nm., probably corresponded to the major reflexions (0.98 nm. and 0.45 nm.) of authentic chitin. The reflexions remained essentially unchanged after removal of the alkali-soluble components from the wall (Fig. 3E), but became more pronounced following consecutive extractions by hot N-acid and again by alkali (Fig. 3F). Seemingly, this last residue consisted almost exclusively of chitin. The chitin content was estimated

Fig. 2. 'Hydroglucan' in *Verticillium albo-atrum* walls. X-ray diagrams of alkali-extracted walls after treatment with hot N-acid (A); purified 'hydroglucan' from *V. albo-atrum* (B) and from *Saccharomyces cerevisiae* walls (C).

directly from the acid hydrolysate of the acid and alkali-insoluble residue in order to exclude glucosamine residues from non-chitinous constituents; consequently, the figures shown in Table 2 probably represent minimum estimates, since they do not account for losses incurred during both fractionation and hydrolysis.

Protein. Values of overall protein content estimated from total amino acid-N determinations ranged from 11.0 to 14.0 % (Table 2) and were higher than those calculated from the sum of individual amino acids by the automatic analyser (Table 3). The latter analyses showed that about 42 % of the total amino acid content was associated with

the heteropolysaccharide in the cold alkali-soluble fraction. The rest appeared together with the glucan in the acid-soluble fraction. Only traces of amino acids were recovered from the acid-insoluble alkali-soluble fraction (hydroglucan). Only lysine and histidine residues were detected in the hydrolysate of the insoluble fraction comprised almost exclusively of chitin fibrils.

Lipids. Extraction of dry walls with ethanol+ether (1+1, v/v) followed by chloroform yielded 0.4 to 0.6 % of 'readily extracted lipid' (Table 2). An additional 2.3 to

Fig. 3. Chitin in *Verticillium albo-atrum* walls. X-ray diagrams of unextracted walls (D); walls after removal of alkali-soluble components (E); final residue after consecutive alkali, acid and alkali extractions (F); purified lobster chitin (G).

2.8 % of 'bound lipid' was extracted after a mild hydrolysis of the walls in acidic ethanol+ether (1 vol. 12 N-HCl in 100 vol. ethanol+ether, 1+1, v/v mixture) at 50° for 5 h., followed by extractions with ethanol+ether and with chloroform.

Phosphate and ash. Wall samples were ignited at 450° in an electric muffle furnace; a slightly grey ash comprising 0.1 to 0.5 % of the initial weight was obtained. The total phosphate content of spore walls accounted for 0.1 to 0.2 % of the wall dry weight.

Physical structure

Walls were extracted consecutively and examined under the electron microscope in shadow-cast specimens; the following observations were recorded:

(1) The outer surface of unextracted yeast walls of *Verticillium albo-atrum* had a coarsely and irregularly granulated appearance (Pl. 2, fig. 6); there was only a vague microfibrillar pattern underlying the surface granulation.

(2) After extraction with N-alkali (at 97° for 30 min.) the surface granules disappeared and the wall texture became fibrillar (Pl. 2, fig. 7). The microfibrils were randomly oriented except at one of the cell poles where there was a region of circularly arranged microfibrils with a raised outer annulus and a minute central orifice or depression. These areas resemble the bud scars of *Saccharomyces cerevisiae* (Houwink & Kreger, 1953). The central 'orifice' might be the septal pore observed by Buckley, Wyllie & DeVay (1969) in thin sections.

(3) Upon subsequent extraction with hot N-acid (at 97° for 30 min.), the interwoven network of microfibrils became more sharply defined as individual microfibrils had become thicker (Pl. 3, fig. 8). The fibrillar structure resembled the 'hydroglucan' network obtained from *Saccharomyces cerevisiae* walls (Houwink & Kreger, 1953) (Pl. 3, fig. 10). The increased thickness of the microfibrils in *S. cerevisiae* and in *Verticillium albo-atrum* probably resulted from aggregation of the original microfibrils during the acid treatment (Houwink & Kreger, 1953). In *V. albo-atrum* the microfibrillar network, at this stage in the extraction sequence, consisted of roughly equal proportions of chitin and glucan. It was not possible, however, to distinguish two types of microfibrils in the electron micrographs.

(4) Upon another extraction with 0.75 N-alkali (at 60° for 30 min.) the glucan ('hydroglucan') was dissolved and the final residue contained almost exclusively randomly oriented chitin microfibrils (Pl. 3, fig. 9). The soluble 'hydroglucan' was reprecipitated twice (see above) and the resulting purified microfibrils are shown in Pl. 3, fig. 11.

DISCUSSION

Although the cultures studied contained some cells that may be regarded as true conidia, we prefer the term yeast to describe the overall appearance and developmental behaviour prevalent in our cultures. Buckley, Wyllie & DeVay (1969) were of the opinion that the process of 'secondary conidium' formation was not budding. However, since the cytological details of budding differ considerably among genuine yeasts (Streiblová & Beran, 1965), we feel justified in using the term budding to describe the multiplication of single cells in *Verticillium albo-atrum*. By using the term yeast we seek specifically to contrast this development with mycelial growth, and to point out that the duality of vegetative morphogenesis of *V. albo-atrum* falls within the realm of mycelial-yeast dimorphism, a widespread phenomenon among fungi (Romano, 1966).

The walls of *Verticillium albo-atrum*, like the walls of other fungi, have a complex chemical composition consisting of polysaccharides, proteins and lipids. The polysaccharides, which are the principal components, are built from different monosaccharides: hexoses, amino sugar and uronic acid. The present work provides a further example of the existence of uronic acid in fungal walls, a fact which was only recently

recognized (Gancedo, Gancedo & Asensio, 1966; Bartnicki-Garcia & Reyes, 1968). At least three different polysaccharides are present in the walls of *V. albo-atrum*: an alkali-soluble heteropolysaccharide of D-glucuronic acid, D-glucose, D-mannose and D-galactose, β -linked glucan(s) and chitin. Similar heteropolysaccharides have been found in the walls of other fungi. A heteropolymer of D-glucuronic acid, D-glucose, D-mannose and D-galactose was isolated from *Aureobasidium pullulans* (Brown & Lindberg, 1967) and Mahadevan & Tatum (1965) detected a heteropolysaccharide of glucose, galactosamine, and glucuronic acid in *Neurospora crassa*. The heteropolysaccharide and protein components extracted by alkali from the walls of *V. albo-atrum* were probably surface and/or interfibrillar components. Thus upon alkali treatment the external surface of the walls lost its granular appearance and a randomly interwoven network of microfibrils was revealed.

This microfibrillar network consisted of β -glucan(s) and chitin. The glucan appeared to be mainly β -1,3-linked with some β -1,6-linkages as is true of most other fungi (Bartnicki-Garcia, 1968). The glucan could be separated into two fractions by treatment with hot acid. However, there was no conclusive evidence that two distinct glucans were present. Conceivably, the glucan fraction dissolved by the hot acid was that portion susceptible to acid hydrolysis, while the hydroglucan represented the acid-resistant core of the glucan molecule. A similar behaviour was noticed for the alkali-insoluble wall glucans of *Saccharomyces cerevisiae* (Houwink & Kreger, 1953), *Endomyces* (Kreger, 1954), *Phytophthora cinnamomi* (Bartnicki-Garcia, 1966) and *Schizophyllum commune* (Wessels, 1965), all of which formed 'hydroglucan' upon acid treatment. The coarsely fibrillar appearance of the 'hydroglucan' might be an artefact (Houwink & Kreger, 1953) resulting from chain aggregation after removal of the acid-soluble portion (side branches?) of the glucan molecule.

Although cellobiose was isolated from the enzymic digestion of the alkali-insoluble glucan of *Verticillium albo-atrum*, the polymeric nature of β -1,4-linked glucose units remained uncertain. Protein was found associated with the heteropolysaccharide and with the alkali-insoluble network of chitin-glucan microfibrils. Presumably the walls contained polysaccharide-glucosamine-protein complexes analogous to those described for the walls of yeast (Nickerson, Falcone & Kessler, 1961; Sentandreu & Northcote, 1968). The final insoluble residue made of chitin contained only two amino acids, lysine and histidine. These amino acids might represent the linking bridge between protein and chitin chains.

In view of the presence of alkali-soluble glucosamine-containing components, estimates of chitin content based on the total amount of glucosamine (Isaac & Milton, 1967) may have to be revised.

It was recently noted that the wall composition of any fungus could be correlated with its taxonomic position (Bartnicki-Garcia, 1968). Euscomycetes (and also Homobasidiomycetes and Chytridiomycetes) were characterized as fungi with the chitin- β -glucan type of cell walls. The present findings on *Verticillium albo-atrum* agree with this generalization.

This investigation was supported by CSRS grant 716-15-4 from the U.S. Department of Agriculture.

REFERENCES

- BARTNICKI-GARCIA, S. (1966). Chemistry of hyphal walls of Phytophthora. *Journal of General Microbiology* **42**, 57-69.
- BARTNICKI-GARCIA, S. (1968). Cell wall chemistry, morphogenesis, and taxonomy of fungi. *Annual Review of Microbiology* **22**, 87-108.
- BARTNICKI-GARCIA, S. & NICKERSON, W. J. (1962). Isolation, composition and structure of cell walls of filamentous and yeastlike forms of *Mucor rouxii*. *Biochimica et biophysica acta* **58**, 102-119.
- BARTNICKI-GARCIA, S. & REYES, E. (1968). Polyuronides in the cell walls of *Mucor rouxii*. *Biochimica et biophysica acta* **170**, 54-62.
- BITTER, T. & MUIR, H. M. (1962). A modified uronic acid carbazole reaction. *Analytical Biochemistry* **4**, 330-334.
- BRANDT, W. H. (1962). Manganese stimulation of melanin synthesis and microsclerotia development in *Verticillium*. *Plant Physiology* **37**, xxx.
- BRANDT, W. H. (1964). Morphogenesis in *Verticillium*: effect of light and ultraviolet radiation on microsclerotia and melanin. *Canadian Journal of Botany* **42**, 1017-1023.
- BRANDT, W. H. (1965). Morphogenesis in *Verticillium*: reversal of the near-UV effect by catechol. *BioScience* **15**, 669-670.
- BRANDT, W. H. & REESE, J. E. (1964). Morphogenesis in *Verticillium*: a self-produced diffusible morphogenetic factor. *American Journal of Botany* **51**, 922-927.
- BROWN, R. G. & LINDBERG, B. (1967). Polysaccharides from cell walls of *Aureobasidium* (*Pullularia*) *pullulans*. *Acta chemica scandinavica* **21**, 2383-2389.
- BUCKLEY, P. M., WYLLIE, T. D. & DEVAY, J. E. (1969). Fine structure of conidia and conidium formation in *Verticillium albo-atrum* and *V. nigrescens*. *Mycologia* **61**, 240-249.
- COLE, G. T. & KENDRICK, W. B. (1969). Conidium ontogeny in hyphomycetes. The phialides of *Phialophora*, *Penicillium*, and *Ceratocystis*. *Canadian Journal of Botany* **47**, 779-789.
- CORBETT, W. M. (1963). Determination of alpha-cellulose content of cotton and wood cellulose. In *Methods in Carbohydrate Chemistry*, vol. 3, p. 27. Edited by R. L. Whistler. New York: Academic Press.
- DAWSON, R. M. C., ELLIOTT, D. C., ELLIOTT, W. H. & JONES, K. M. (1959). *Data for Biochemical Research*. London: Oxford University Press.
- DRYER, R. L., TAMMES, A. E. & ROUTH, J. I. (1957). Determination of phosphorus and phosphatase with *N*-phenyl-*p*-phenylenediamine. *Journal of Biological Chemistry* **225**, 177-183.
- GANCEDO, J. M., GANCEDO, C. & ASENSIO, C. (1966). Uronic acids in fungal cell walls. *Biochemische Zeitschrift* **346**, 328-332.
- GARBER, R. H. & HOUSTON, B. (1966). Penetration and development of *Verticillium albo-atrum*. *Phytopathology* **56**, 1121-1126.
- HAUSE, J. A., HUBICKI, J. A. & HAZEN, G. G. (1962). Determination of sorbitol as its hexa-acetates by gas-liquid chromatography using an ionization detector. *Analytical Chemistry* **34**, 1567-1570.
- HODGE, J. E. & HOFREITER, B. T. (1962). Determination of reducing sugars and carbohydrates. In *Methods in Carbohydrate Chemistry*, vol. 1, p. 380. Edited by R. L. Whistler & M. L. Wolfrom. New York: Academic Press.
- HOUWINK, A. L. & KREGER, D. R. (1953). Observations on the cell walls of yeasts. An electron microscope and X-ray diffraction study. *Antonie van Leeuwenhoek* **19**, 1-24.
- ISAAC, I. & MILTON, J. M. (1967). Response of *Verticillium* species to griseofulvin. *Journal of General Microbiology* **46**, 273-283.
- KIM, J. H., SHOME, B., LIAO, T.-H. & PIERCE, J. G. (1967). Analysis of neutral sugar by gas-liquid chromatography of alditol acetates: application to thyrotropic hormone and other glycoproteins. *Analytical Biochemistry* **20**, 258-274.
- KREGER, D. R. (1954). Observations on cell walls of yeasts and some other fungi by X-ray diffraction and solubility tests. *Biochimica et biophysica acta* **13**, 1-9.
- LEWIS, B. A., SMITH, F. & STEPHEN, A. M. (1963). Metal hydride reductions. In *Methods of Carbohydrate Chemistry*, vol. 2, pp. 68-77. Edited by R. L. Whistler & M. L. Wolfrom. New York: Academic Press.
- LOHMER, R. (1949). The hexitols and some of their derivatives. *Advances in Carbohydrate Chemistry* **4**, 211-242.

- MACMILLAN, P. C. & BRANDT, W. H. (1966). Possible role of a peroxidative system in melanin synthesis in *Verticillium*. *Antonie van Leeuwenhoek* **32**, 202-211.
- MAHADEVAN, P. R. & TATUM, E. L. (1965). Relationship of the major constituents of the *Neurospora crassa* cell wall to wild-type and colonial morphology. *Journal of Bacteriology* **90**, 1073-1081.
- MALCA, I., ERWIN, D. C., MOJE, W. & JONES, B. (1966). Effects of pH and carbon and nitrogen sources on the growth of *Verticillium albo-atrum*. *Phytopathology* **56**, 401-406.
- MALEY, F. & MALEY, G. F. (1959). The enzymic conversion of glucosamine to galactosamine. *Biochimica et biophysica acta* **31**, 577-578.
- MATHRE, D. E., ERWIN, D. C., PAULUS, A. O. & RAVENSCROFT, A. V. (1966). Comparison of the virulence of isolates of *Verticillium albo-atrum* from several of the cotton-growing regions in the United States, Mexico and Peru. *Plant Disease Reporter* **50**, 920-933.
- NICKERSON, W. J. & BARTNICKI-GARCIA, S. (1964). Biochemical aspects of morphogenesis in algae and fungi. *Annual Review of Plant Physiology* **15**, 327-344.
- NICKERSON, W. J., FALCONE, G. & KESSLER, G. (1961). Polysaccharide-protein complexes of yeast cell walls. In *Macromolecular complexes*, pp. 205-228. Edited by M. V. Edds, Jun. New York: Ronald Press.
- NORTHCOTE, D. H. & HORNE, R. W. (1952). The chemical composition and structure of the yeast cell wall. *Biochemical Journal* **51**, 232-236.
- PERLIN, A. S. (1955). Structure of reducing disaccharides by lead tetra-acetate oxidation. *Analytical Chemistry* **27**, 396-399.
- PRESLEY, J. T., CARNS, H. R., TAYLOR, E. E. & SCHNATHORST, W. C. (1966). Movement of conidia of *Verticillium albo-atrum* in cotton plants. *Phytopathology* **56**, 375.
- REESE, E. T., MAGUIRE, A. H. & PARRISH, F. W. (1967). Glucosidases and exo-glucanases. *Canadian Journal of Biochemistry* **46**, 25-34.
- ROMANO, A. H. (1966). Dimorphism. In *The Fungi*, vol. 2, pp. 181-209. Edited by A. S. Sussman & G. C. Ainsworth. New York: Academic Press.
- SENTANDREU, R. & NORTHCOTE, D. H. (1968). The structure of a glycopeptide isolated from the yeast cell wall. *Biochemical Journal* **109**, 419-432.
- STOFFYN, P. J. & JEANLOZ, R. W. (1954). Identification of amino sugars by paper chromatography. *Archives of Biochemistry and Biophysics* **52**, 373-379.
- STREIBLOVÁ, E. & BERAN, K. (1965). On the question of the vegetative reproduction in apiculate yeasts. *Folia Microbiologica* **10**, 352-356.
- TRACEY, M. V. (1955). In *Modern Methods of Plant Analysis*, vol. 2, p. 264. Edited by K. Paech & M. V. Tracey. Berlin: Springer-Verlag.
- WESSELS, J. G. H. (1965). Morphogenesis and biochemical processes in *Schizophyllum commune* Fr. *Wentia* **13**, 1-113.

EXPLANATION OF PLATES

PLATE 1

- Fig. 1. Yeast cells of *Verticillium albo-atrum* from a 5 day liquid culture. Marker = 10 μ m.
- Fig. 2. Isolated yeast walls of *V. albo-atrum*. Arrow points at a cell with a phialide-like tube. Magnification same as in fig. 1.

PLATE 2

- Fig. 6. External surface of unextracted walls of the yeast form of *Verticillium albo-atrum*. $\times 23,000$.
- Fig. 7. Microfibrillar texture and 'budding scars' in alkali-extracted walls of *V. albo-atrum*. $\times 16,000$.

PLATE 3

- Fig. 8. Microfibrillar network of *Verticillium albo-atrum* walls following consecutive alkali and acid extractions. $\times 27,000$.
- Fig. 9. Microfibrillar network of *V. albo-atrum* walls following consecutive alkali-acid-alkali extractions. $\times 31,000$.
- Fig. 10. Microfibrillar network of *Saccharomyces cerevisiae* walls following same treatment as that in Fig. 8. $\times 26,000$.
- Fig. 11. Purified 'hydroglucan' microfibrils of *V. albo-atrum*. $\times 34,000$.

M. C. WANG AND S. BARTNICKI-GARCIA