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Resistance of *Zygorhynchus* Species to Lysis

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Zygorhynchus vuilleminii, a nonmelanin-containing fungus, was not lysed by mycolytic actinomycetes. Several enzymes and *Streptomyces* enzyme preparations digesting walls of other fungi were without appreciable activity on walls of *Zygorhynchus* species. A bacterium able to solubilize a portion of the *Zygorhynchus* wall released little or no reducing sugars from these structures. Fractions of *Z. vuilleminii* walls were resistant to glucanase hydrolysis, but certain fractions were digested by chitinase and microbial enzyme preparations. The walls and several wall fractions were not readily susceptible to degradation by a soil community. Walls of lysis-resistant *Zygorhynchus* species contained glucosamine, fucose, glucuronic acid, and galactose but little or no glucose. Resistant wall fractions were rich in uronic acid and fucose, whereas the readily degradable fractions contained abundant glucosamine. Cultural conditions affected the extent of digestion and composition of the walls. Possible reasons for the resistance of *Zygorhynchus* to lysis in nature are discussed.

The lysis of fungal hyphae is carried out by a variety of microorganisms that are capable of excreting certain enzymes, frequently glucanases and chitinase, which catalyze the digestion of the hyphal wall (24, 27). Yet, despite the abundance of lytic microorganisms in soil, many species of fungi are present and persist in that environment as spores or in the hyphal form. Hence, resistance to lysis, or possibly the avoidance of lytic species, must play an important role in the ecology of terrestrial fungi.

It has been known for some time that many dark-pigmented fungi are particularly resistant to lysis (12), and it is now evident that this resistance frequently can be ascribed to the presence of melanin or melanin-like pigments on the surface components of the refractory structure, the polyaromatic substance protecting digestible polysaccharides from enzymatic destruction (5, 11, 19). In nonmelanized fungi or yeasts, resistance to lysis has been correlated with cell walls containing polysaccharides with monomers in addition to glucose and *N*-acetylglucosamine. Thus, Ballesta et al. (2) found a high content of xylose in the walls of the resistant yeast *Torulopsis aeris*, and Pengra et al. (18) reported that hyphal walls of *Mortierella parvispora*, which is common in soil and resistant to lysis, have fucose-containing polymers.

The present study is concerned with *Zygorhynchus*, a genus of nonpigmented fungi common in soil and notably resistant to lysis. The investigation was conducted to provide additional information to help understand why melanin-free fungi are frequently abundant in natural communities teeming with potential microbial agents of lysis.

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MATERIALS AND METHODS

Z. vuilleminii 6744 and *Pholiota marginata* 14196 were obtained from the American Type Culture Collection. *Z. heterogamus* 1489, *Z. exponens* 1492, and *Z. moelleri* strains 1495, 1500, and 2661 were provided by C. W. Hesseltine, U. S. Department of Agriculture, Peoria, Ill. *Fomes annosus* strains, *Polyporus versicolor*, and *Lenzites trabea* were provided by E. B. Cowling, North Carolina State Univ., Raleigh, and *Colletotrichum lagenarium* by R. L. Millar, Cornell University.

The fungi were grown at 25 C in 2-liter flasks containing 800 ml of 1% malt extract (Difco) in tap water. The flasks were incubated on a rotary shaker operating at 150 rev/min. When a specific carbon source was used, it was added to a basal medium containing 0.8 g of K_2HPO_4, 0.2 g of KH_2PO_4, 0.5 g of $(NH_4)_2SO_4$, 0.2 g of $MgSO_4 \cdot 2H_2O$, 0.01 g of $FeCl_3 \cdot 6H_2O$, and 1.0 liter of distilled water. To obtain the lytic preparation, the basal medium was supplemented with 0.05% yeast extract and 2% of either mycelium or cell walls prepared from 4- to 5-day cultures of *Z. vuilleminii* or *Aspergillus oryzae*. The lytic bacterium or *Streptomyces* was incubated at 30 C on a rotary shaker for 3 or 7 days, respectively. At the end of the incubation period, the culture supernatant was concentrated by lyophilization, filtration through a Diaflo ultrafilter

membrane UM-2 (Amicon Corp.), or by precipitation with $(NH_4)_2SO_4$ at 75% of saturation. The concentrated preparation was dialyzed for 24 hr at 4 C against 0.05 M phosphate buffer (*p*H 6.5) or, in one instance, against 0.05 M acetate buffer, *p*H 4.5. The material remaining in the dialysis bag was centrifuged for 25 min at 12,000 × *g*, the pellet was discarded, and the solution was made up to 0.1 of its original volume by using the same buffer.

The glucanase was prepared from *Penicillium brefeldianum* QM 1872 as described by Pengra et al. (18). Chitinase, cellulase, and β-glucuronidase were obtained from Worthington Biochemical Corp., Freehold, N.J.; hemicellulase was from Pierce Chemical Co., Rockford, Ill.; and pectinase came from Sigma Chemical Co., St. Louis, Mo. Tests of enzymatic activity were performed at 37 C with a 2-hr incubation period, and the reaction vessels were continuously shaken. Glucanase, cellulase, and hemicellulase were incubated in 0.05 M acetate buffer (*p*H 5.0), chitinase in 0.025 M phosphate buffer (*p*H 6.3), β-glucuronidase in 0.05 M acetate buffer (*p*H 4.5), and pectinase in 0.025 M phosphate buffer (*p*H 7.0). The reaction mixture contained 100 μg of enzyme preparation and 1.0 mg of substrate per ml of buffer. When lytic preparations were used, 1.0 ml of the preparation was incubated with 5.0 mg of walls for 6 hr at 37 C. Reducing sugars were determined by the method of Somogyi (25) with glucose as standard, and *N*-acetylglucosamine was measured by the technique of Reissig et al. (20).

The cell walls were prepared by disrupting an aqueous suspension of mycelium for 10 min at top speed in an ice-cooled Omni-mixer (Ivan Sorvall, Norwalk, Conn.). The residue was washed exhaustively with 1% NaCl and then 0.005 M phosphate buffer, (*p*H 7.0), followed by water; it was then lyophilized. The residue from *Zygorhynchus* walls was treated with 1% sodium dodecyl sulfate at 4 C for about 15 hr, as described by Mahadevan and Tatum (14), but microscopic examination of these walls showed extensive contamination even after three detergent treatments. The contaminating materials could be removed by extraction with an ether-methanol solution (1:1, v/v) for 24 hr at 4 C. The cell walls were finally treated in a sonic oscillator (Bronwill Scientific, Rochester, N.Y.) for 1 hr, sequentially washed with 1% NaCl, 0.05 M phosphate buffer (*p*H 7.0) and water, and then lyophilized. The final wall preparations showed no cytoplasmic contamination when stained with Lugol's iodine solution.

Tests for lysis in agar were carried out in a medium identical to that used for making the lytic preparations, but it was supplemented with 1.5% agar and 2% mycelium. The plates were streaked with the lytic isolates, incubated at 30 C, and checked daily for zones of clearing. The decomposition of the walls in soil was measured by the procedure of Kuo and Alexander (11) by using labeled cell walls obtained from *Z. vuilleminii* grown for 5 days or *F. annosus* grown for 7 days in the malt extract medium supplemented with 25 μCi of glucose-*UL*-^{14}C (Calbiochem, Los Angeles, Calif.) per liter.

To fractionate the cell walls, 300 mg of walls was treated with 30 ml of 2 N NaOH at room temperature for 3 hr. The residue was collected by centrifugation and treated with 30 ml of 2 N NaOH at room temperature for 3 hr. The residue was collected by centrifugation and treated three additional times in an identical manner. The final residue was washed with 30 ml of water, and the water and alkali extracts were pooled and designated as solution A. The residue was again washed with water to a neutral reaction, and this wash water was discarded. The resistant residue was designated residue B. Solution A was neutralized with HCl, it was allowed to stand for 2 hr at 4 C, and the precipitate that developed was collected by centrifugation, washed three times with water, and lyophilized to give fraction 3I. The supernatant remaining from solution A was concentrated to 0.1 of its original volume, dialyzed against water for 18 hr at 4 C, and treated with 3 volumes of ethanol; the precipitate that appeared was dissolved in water, reprecipitated, redissolved, and then lyophilized to give fraction 3S. Residue B was treated four times with 30 ml of 0.5 N HCl for 3 hr at room temperature, and the residue that remained was collected and treated with the acid three more times; this residue was washed until no longer acid, lyophilized, and designated fraction I. The remaining solution was neutralized, brought to 4 C for 2 hr, and then centrifuged at 6,000 × *g* to yield a pellet that was washed well with water and lyophilized (fraction 2I). Fractions 1, 2I, 3I, and 3S accounted for 30, 15, 25, and 10% of the wall weight, respectively.

Total carbohydrate was determined by using the anthrone procedure (8), fucose was measured by the procedure of Tsiganos and Muir (26), and uronic acid was estimated by the method of Bitter and Muir (4). Phosphate was determined by the Fiske and Subbarow method (9) and proteins were determined by the Folin phenol reagent (13). Glucose and galactose were estimated with the Glucostat and Galactostat reagents (Worthington Biochemical Corp.) in hydrolysates prepared by treating walls with 2 N HCl at 100 C for 2 hr in sealed ampoules; 2.0 ml of acid was used per mg of walls. Glucosamine was determined in the 2 N HCl hydrolysate or in a hydrolysate prepared by treating the walls with 6 N HCl for 6 hr at 100 C in sealed ampoules. The hydrolysate was treated with Dowex 50W-X8 (6), and glucosamine was then determined by the Elson and Morgan reaction (22).

Lipids were estimated by extracting 200 mg of cell walls with 400 ml of methanol at 55 C for 2 hr. The solution was cooled and then mixed vigorously with 2 volumes of chloroform. The solution was allowed to stand for 16 hr at room temperature, the precipitate was removed by filtration, and the soluble portion was dried under vacuum and redissolved in 10 ml of chloroform; the precipitate was then discarded and the final solution was dried in vacuum and weighed.

The monosaccharides in the acid hydrolysates were identified by chromatography on Whatman no. 1 paper or by thin-layer chromatography on precoated plates of cellulose (Brinkmann Instrument, Inc., Westbury, N.Y.). As solvent systems, *n*-butanol-acetone-water (4:5:1), *iso*-propanol-*n*-butanol-water (7:1:2), and *n*-butanol-acetic acid-water (2:1:2) were used for paper chromatography and ethyl acetate-pyridine-water (2:1:2), and formic acid-methyl ethyl ketone-*t*-butyl al-

cohol-water (3:6:8:3) was used for thin-layer chromatography. Spots were detected using 0.1 M *p*-anisidine phthalate in 95% alcohol for reducing sugars, naphthoresorcinol and trichloroacetic acid in butanol for uronic acids, and the sulfosalicylic acid reagent for sugar phosphates (23). Uronic acids were isolated either from a Dowex 1-X8 column (10) or from chromatograms on Whatman 3MM paper by elution with water.

For the electrophoretic separation of polysaccharides, cellulose polyacetate strips were employed with 0.01 M Veronal buffer, *p*H 9.2. Whatman no. 1 paper strips were used with 0.05 M acetate buffer, *p*H 5.1, to separate uronic acids from sugars. In both instances, good resolution was obtained in 90 min at 300 v.

RESULTS

Resistance in plate tests. Twenty actinomycetes previously shown to lyse selected fungi were tested on a group of unstudied fungi, namely *C. lagenarium*, *F. annosus*, *L. trabea*, *P. marginata*, *P. versicolor*, and *Z. vuilleminii*. Plates were incubated for 5 days at 30 C. Each of these fungi was lysed to some extent, except for *Z. vuilleminii*, a non-pigmented organism. The apparently marked resistance of this fungus prompted a more detailed study.

Resistance to lytic preparations and purified enzymes. A number of species and strains of the genus *Zygorhynchus* were studied to determine the frequency of resistance to lysis in this fungal group. These tests were performed with crude preparations from culture filtrates of *Streptomyces* strains. Various means of concentrating the preparations were employed, and incubations were performed at *p*H 4.5 and 6.5 and at temperatures of 25, 30, and 37 C. The actinomycetes were cultured at 37 C for 7 days in the basal medium supplemented with 4- to 5-day old *Z. vuilleminii* hyphae. Possible digestion of the test fungi was measured by loss in dry weight or the release of reducing sugars after a 6-hr incubation period. In no instance was there detectable activity on *Z. vuilleminii*, and all the *Zygorhynchus* strains showed uncommon resistance to enzymatic hydrolysis. The data in Table 1 reveal the marked resistance of the *Zygorhynchus* strains to digestion. Similar results were obtained with lytic preparations obtained from five other actinomycetes; thus 0, 5.6 to 7.5, 6.3 to 7.1, 13.2 to 14.1, and 75.1 to 91.1 of the wall weights of *Z. vuilleminii*, *Z. moelleri* strains 1495, 1500, and 2661, and *Fusarium* sp., respectively, were solubilized and recovered as reducing sugars.

The resistance of *Zygorhynchus* spp. cell walls to degradation by several enzyme preparations was also assessed. Cellulase, β-glucuronidase, and pectinase were without effect on all *Zygorhynchus* strains tested. Mixtures containing chitinase, glucanase, and hemicellulase showed slight activity on several of the *Zygorhynchus* cultures, but never was the digestion appreciable (Table 2). The enzymes failed to catalyze the release of reducing sugars from *Z. vuilleminii* and *Z. exponens* walls. *Fusarium* sp., by contrast, was extensively digested by these enzyme preparations. Among the *Zygorhynchus* strains, the maximum dry weight loss was noted with *Z. moelleri*, but this amounted to no more than 6 to 8%.

Isolation of lytic bacterium. An attempt was made by enrichment culture techniques, by using the medium described above, to isolate microorganisms active on *Z. vuilleminii*. Enrichments were prepared from soils of different origin. Only a single bacterium, strain 11, yielded a small zone of lysis on agar containing cell walls of a 4- to 5-day old culture of *Z. vuilleminii*.

When this bacterium was grown in the basal medium supplemented with 2% *Zygorhynchus* cell walls as the carbon source, only sparse growth was obtained, but up to about 60% of the wall material was solubilized in 5 days as measured by dry weight loss. The supernatant of such a culture, which was quite active on *Fusarium* sp., showed no action on *Z. vuilleminii* cell walls as measured by the release of reducing sugars, even if the enzymes were concentrated with $(NH_4)_2SO_4$. Some dry weight loss from the walls of *Z. vuilleminii* was evident, however (Table 3). More extensive decomposition of *Z. moelleri* walls occurred, but here too the digestion was never substantial. By contrast, the digestion of *Fusarium* walls was appreciable. Apparently, the bacterium effects a partial destruction of the *Zygorhynchus* wall, with the release of a polymer resistant to further degradation by this microorganism.

Degradation of cell wall fractions. The susceptibility to digestion of the fractions obtained from the walls by the procedures described above was tested by the methods used for intact walls. The ability of these fractions of *Z. vuilleminii* walls to support growth of bacterium 11 was also examined by incorporating the fraction, at a concentration of 0.5%, in the basal medium fortified with 0.05% yeast extract and incubating the cultures on a rotary shaker for 5 days at 30 C. The culture was filtered through a glass filter, medium pore size, to remove residual wall material, and the absorbancy of the solution was determined at 600 nm. The lytic preparation from bacterium 11 was obtained from identical culture filtrates after removing the bacteria by centrifugation. The *Streptomyces* 2 preparation was obtained from the culture supernatant after growing the actinomycete for 4 to 5 days in a medium with *Z. vuilleminii* mycelium as sole carbon source. Hemicellulase, β-glucanase, and

TABLE 1. *Digestion of cell walls of species of Zygorhynchus and other fungi by preparations obtained from Streptomyces 2*

Fungus	Dry wt loss (%)[a]		Reducing sugar released (%)[a]			
	A[b]	B	A[b]	B	C	D
Z. vuilleminii	2.2	4.1	0	0	0	0
Z. heterogamus	15	19	13	10	14	11
Z. exponens	6.0	6.5	0	0	0	0
Z. moelleri 1495	10	13	8.6	10	7.5	6.6
Z. moelleri 1500	8.8	7.2	6.3	4.4	7.4	5.3
Z. moelleri 2661	13	14	11	8.5	10	8.4
Fusarium sp.	90	80	91	73	85	75
Fomes annosus T47	50		30			

[a] As per cent of weight of cell wall.

[b] Preparations were made by concentrating the culture filtrate by $(NH_4)_2SO_4$ (A), lyophilization (B), and ultrafiltration (C and D). Reaction mixtures A, B, and C were incubated at *p*H 6.5 and D was incubated at *p*H 4.5.

TABLE 2. *Digestion of fungal walls by several enzyme preparations*

Source of walls	Reducing sugar released[a]		
	Glucanase + chitinase	Chitinase + hemicellulase	Glucanase + chitinase + hemicellulase
Zygorhynchus vuilleminii	0	0	0
Z. heterogamus	5.1	3.8	4.2
Z. exponens	0	0	0
Z. moelleri 1495	2.0	3.3	2.5
Z. moelleri 1500	1	2.3	1
Z. moelleri 2661	5.5	4.2	5.6
Fusarium sp.	54	25	54
Fomes annosus T47	15		

[a] As per cent of wall weight.

TABLE 3. *Digestion of fungal cell walls by a bacterial enzyme preparation*

Source of walls	Lysis by growing bacterium (%)[a]	Activity on cell walls (%)[b]			
		Reducing sugar released		Carbohydrate solubilized	
		A	B	A	B
Zygorhynchus vuilleminii	62.0	0	0	7.6	5.2
Z. moelleri 1500	70.7	4.7	4.6	18.1	14.9
Fusarium sp.	49.8	54.3	60.7	63.3	71.3

[a] Per cent of wall weight remaining after growth of the bacterium for 5 days in a medium containing walls as the only carbon source.

[b] Data expressed as per cent of wall weight. The preparations were derived from the culture filtrates of the bacterium grown on *Z. vuilleminii* (A) and *Z. moelleri* 1500 (B) and precipitated by $(NH_4)_2SO_4$. The assay was performed as done in Table 2.

pectinase had no activity on the four fractions, so only the results of a 2-hr incubation with chitinase are recorded. As shown in Table 4, fractions 1 and 2I but not 3S and 3I were sensitive to the chitinase, bacterial, and actinomycete preparations. Similarly, bacterium 11 grew to some extent on fractions 1 and 2I.

Decomposition in soil. To test the resistance of *Z. vuilleminii* to a heterogeneous microbial community, the decomposition of labeled walls and wall fractions in soil was determined. For this purpose, 213, 419, 63.4, 91.1, 7.8, and 197 mg of fractions 1, 2I, 3I, 3S, a lignin-like fraction (*see below*), and unfractionated walls were added to the soil. To provide an indication of the rate of degradation of an available substrate, 200 mg of glucose was added to one soil sample. The labeled materials had specific activities ranging from 1,000 to 5,590 counts per min per mg. The results presented in Fig. 1 show the amount of radioactivity applied to the soil that was evolved and trapped as CO_2. The data show the marked resistance of unfractionated walls and fraction 3I. The increase in the very slow rate of decomposition of the walls with time may result from the attack on susceptible components that become accessible as resistant, shielding constituents are destroyed. The lignin-like fraction also is slowly attacked, but fraction 2I is readily metabolized.

Composition of walls and wall fractions. The sugar monomers released from *Zygorhynchus* spp. walls by a 2-hr hydrolysis at 100 C with 0.5, 1.0, and 2.0 N HCl were characterized by paper and thin-layer chromatography in various solvent systems. Fucose, uronic acid, glucosamine, and galactose were major components of *Z. vuilleminii* walls. Glucosamine was present in the 1.0 and 2.0 N but absent from the 0.5 N HCl hydrol-

TABLE 4. *Digestion of Zygorhynchus vuilleminii wall fractions*

Fraction[a]	Growth of bacterium 11 (optical density)	Reducing sugar released by		*N*-acetyl-glucosamine released by chitinase (%)[b]
		Streptomycete prepn (%)[b]	Bacterium 11 prepn (%)[b]	
1	0.14	10	20	30
2I	0.18	6.8	21	16
3S	0.05	0	0	0
3I	0.03	0	0	0

[a] Description of fractions is given in Materials and Methods.

[b] Refers to per cent of wall weight.

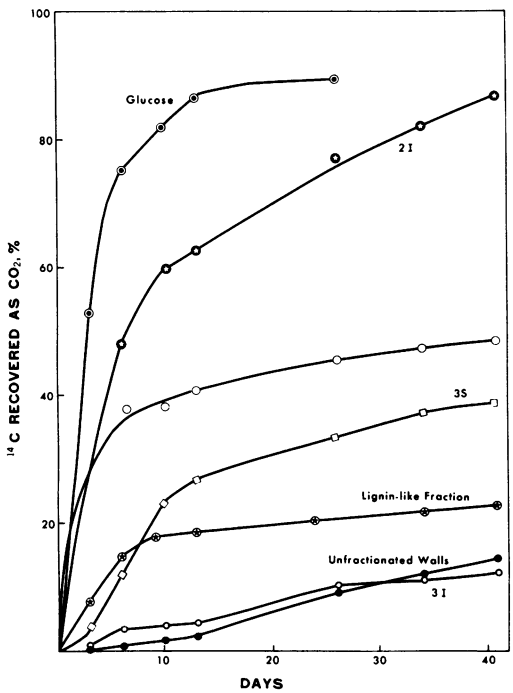

FIG. 1. *Decomposition by a soil community of ^{14}C-labeled cell walls and wall fractions of Zygorhynchus vuilleminii.*

ysates. As shown in Table 5, in which the relative abundance of each compound is assessed by visual comparison of the spots on the chromatograms, essentially the same monomers were found in walls of other strains of the genus. *Fusarium* sp., by contrast, was markedly different, containing much glucose but no detectable levels of fucose or uronic acids. When the residue remaining after hydrolysis with 2 N HCl was hydrolyzed with 6 N HCl for 6 hr at 100 C, glucosamine was the only sugar released from all *Zygorhynchus* strains.

The chemistry of *Z. vuilleminii* hyphal walls was examined further. The data of Table 6 show the composition of the walls of this fungus. The uronic acid, fucose, and phosphate contents are strikingly high. Glucose was not detected. By contrast, *Fusarium* sp. contained 85.2% total carbohydrate, as determined by the anthrone reaction with glucose as standard, 3.1% phosphate, and 2.0% 6 N HCl-resistant constituents.

The possible presence of sugar phosphates was assessed by employing a very mild hydrolysis using 0.01 M acetic acid, *p*H 3.3, as described by Volk (28). This hydrolysis released about 70% of the phosphorus as inorganic phosphate, but no sugar phosphates were detected by paper chromatography with the butanol-acetic acid-water solvent system.

Chemical composition of cell wall fractions. Data on major components of each fraction are presented in Table 6. The walls were obtained from 4-day-old cultures. In this instance, fractions 1, 2I, 3I, and 3S accounted for 28, 16, 26, and 10% of the wall weight, respectively. Significant differences occurred in the distributions of fucose and glucosamine. Most of the amino sugar is in fraction 1, where it probably exists as chitin, as shown by the resistance of this fraction to hydrolysis with 2 N HCl (only 45% of the fraction weight being hydrolyzed) and its sensitivity to chitinase (Table 4). The glucosamine in fraction 2I may be present as a chitosan inasmuch as this fraction, like chitosan, is soluble in acid but insoluble in alkali. Fraction 1 has most

TABLE 5. *Monosaccharide composition of fungal cell walls*

Species	Glucose	Galactose	Mannose	Glucosamine	Fucose	Uronic acid
Zygorhynchus vuilleminii	–	++	Trace	+++++	++	+++
Z. moelleri 1495	Trace	++	+	++++	++	++
Z. moelleri 1500	Trace	++	+	++++	++	++
Z. moelleri 2661	+	++	Trace	++++	++	++
Z. heterogamus	++	++	++	+++	++	+
Z. exponens	Trace	+	+	++++	++	++
Fusarium sp.	+++	+	++	+++	–	–

TABLE 6. *Composition of Zygorhynchus vuilleminii cell walls and wall fractions*[a]

Component	Component as per cent of wall or fraction wt				
	Cell walls	1	2I	3I	3S
Fucose	6.8	2.6	5.0	17.1	8.9
Uronic acid	16.0	4.1	13.8	21.5	31.2
Galactose	5.1	0	12	1.5	10
Glucosamine	31.5	55	20	0	1.1
Phosphate	15.5	1.1	4.2	8.9	12.6
Protein	8.5	0	3.1	2.3	6.8
Nonhydrolyzable[b]	8.1	14.3	1.8	5.3	0
Lipids	9.2				
Ash	12.5				

[a] Description of fractions 1, 2I, 3I, and 3S is given in Materials and Methods.
[b] Resistant to hydrolysis by 6 N HCl at 100 C.

of the nonhydrolyzable material. A significant portion of fractions 3I and 3S is made up of fucose, uronic acid, and galactose.

Identification of uronic acid. A large part of the uronic acid appeared in the chromatograms as a fast-moving spot at the R_F value of the lactone form of glucuronic acid. The portion of the Whatman 3MM paper containing this fast-moving spot was cut out, and the compound was eluted with water. Upon rechromatography on Whatman no. 1 paper, two spots were detected in the butanol-acetone-water, *iso*-propanol-*n*-butanol-water, and *n*-butanol-acetic acid-water solvent systems; one behaved chromatographically like the glucuronic acid lactone and the second like the open form of this uronic acid.

By absorption on Dowex 1-X8, the uronic acids were isolated from hydrolysates obtained by treatment of the ^{14}C-labeled walls with 1.0 N HCl for 2 hr at 100 C. The fraction eluted with formic acid by the technique of Gancedo et al. (10) was found to have components identical chromatographically with glucuronic acid and the lactone of glucuronic acid. In addition, two or sometimes three slower moving compounds were noted; these may have been aldobiuronic acids since they reacted with the uronic acid spray and they appeared in the expected positions on the chromatograms.

When the spot corresponding to glucuronic acid was eluted and rechromatographed with authentic glucuronic acid on Whatman no. 1 paper with the butanol-acetic acid-water solvent system, a single, large radioactive spot appeared with the R_F of glucuronic acid. The lactone was evident as a small, radioactive spot. Paper electrophoresis also showed only one spot, and this had the same mobility as authentic glucuronic acid.

Lignin-like components. Precursors of lignin and a pigment based on a phenylpropane skeleton have been reported in Basidiomycetes (7). Since lignin and related polyaromatic compounds are very resistant to degradation in nature, the possibility of such substances accounting for the resistance of *Z. vuilleminii* walls to decomposition was investigated. The lignin extraction procedure of Jayne-Knolle-Rapp (17) was used with 200 mg of walls from a 5-day-old *Z. vuilleminii* or a 7-day-old *F. annosus* T47 culture. The per cent of lignin-like substances, that is material insoluble in concentrated H_2SO_4, was 6.08% in *Z. vuilleminii* and 12.0% in *F. annosus*. On the other hand, melanins were not present in *Z. vuilleminii* walls, as indicated by the absence of 1 N KOH-soluble material that could be precipitated when the alkaline solution was neutralized.

Infrared spectra of cell wall fractions. The infrared spectra of different *Z. vuilleminii* cell wall fractions prepared in KBr pellets reveal clear differences (Fig. 2). The spectrum of fraction 1 shows the characteristic absorption bands of chitin, although the bands were a little more diffuse than those of authentic chitin, probably because of the presence of other substances. A significant feature of the spectra of fraction 3I and of the lignin-like material is the presence of an absorption band at 1,735 cm^{-1}, a band absent from the other fractions. Absorption in the vicinity of 1,735 cm^{-1} is characteristic of carbonyl groups and is also found in some but not all lignins; the remainder of the spectrum of the lignin-like material was quite different from those of other typical lignins (15, 16) and also from the spectrum of a similar material extracted from *F. annosus*. Inasmuch as lignins from various sources differ, the possibility that fraction 3I and the *Z. vuilleminii* nonhydrolyzable material are lignin-like substances cannot be dismissed.

Alterations in resistance. Growth of *Z. vuilleminii* in different media was associated with an

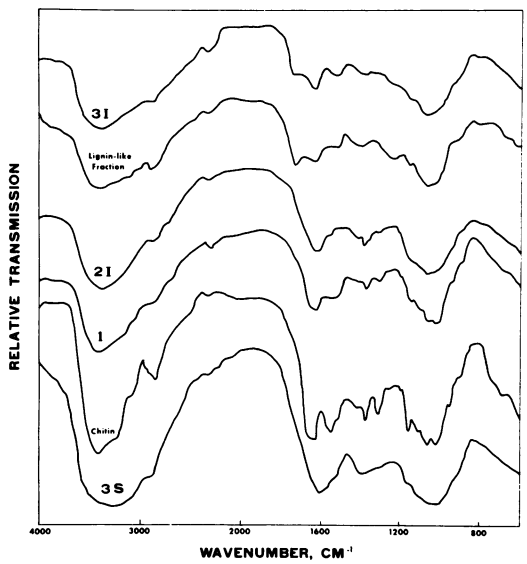

FIG. 2. *Infrared spectra of fractions of Zygorhynchus vuilleminii cell walls and crustacean chitin.*

alteration in the susceptibility of its walls to digestion. The fungus was grown 5 days in (i) 1% malt extract in tap water, (ii) the basal medium supplemented with 0.05% yeast extract and 0.2% glucose plus 0.8% sorbose, and (iii) the basal-yeast extract medium containing 1% glucose and 0.004% griseofulvin. The three media were supplemented with 25 μCi of glucose *UL*-^{14}C per liter. About 50 mg of walls prepared from the hyphae was added to soil. The CO_2 evolved in 30 days at room temperature was trapped in a NaOH solution, the carbonates precipitated as $BaCO_3$, and the radioactivity was determined with a scintillation counter. The per cent of the radioactivity added to the soil which was recovered as CO_2 was 11, 25, and 29% for the walls prepared from fungi grown in malt extract, the griseofulvin medium, and the sorbose medium, respectively.

Griseofulvin did not inhibit the growth of *Z. vuilleminii*, but it caused changes in its cell wall. Indications of such changes were evident in the differences in infrared spectra of the cell walls of cultures grown in the presence of griseofulvin or sorbose on the one hand and walls from malt extract-grown mycelium on the other. The absence of a band at 1,370 cm^{-1} in the walls of griseofulvin- and sorbose-grown cultures was noteworthy. Fraction 3I obtained from the walls of the griseofulvin-grown culture is readily attacked inasmuch as 25% of the radioactivity in labeled fraction 3I was converted to CO_2 in 30 days, and this fraction also was free of the band at 1,370 cm^{-1}. Coinciding with the spectral differences were differences in the wall components. Thus, *Z. vuilleminii* grown in the presence of griseofulvin contained mannose as the major neutral monosaccharide with lesser amounts of fucose and glucose. Mannose was found also in sorbose-grown mycelium, but glucose was absent. Cell walls of hyphae grown in malt extract contained a trace of mannose and no glucose. The walls of mycelium grown in all three media were rich in glucosamine and uronic acid, and they contained some fucose. The walls from the mycelium grown in the griseofulvin and sorbose media contained 10.9 and 9.2% uronic acid.

DISCUSSION

The results show that members of the genus *Zygorhynchus*, and in particular *Z. vuilleminii*, possess characteristics that make them particularly interesting in regard to their susceptibility to microbial attack. The data reveal that the resistance of these fungi to lysis by enzyme preparations or by components of the heterogeneous soil community is associated with a cell wall that displays a unique composition.

Several features distinguish the hyphal walls of these fungi. Especially striking is their high content of uronic acids. The presence of uronic acid in fungal walls is not exceptional, but the concentrations previously detected are quite low (10). The walls of *Z. vuilleminii*, on the other hand, contain 16% glucuronic acid, and this monomer is present in all fractions obtained from the cell wall. Moreover, when the walls became more readily biodegradable, because of growth in media with griseofulvin or sorbose, they were poor in uronic acid. Hence, it is tempting to suggest that uronic acid-containing polysaccharides contribute to resistance. Nevertheless, the fact that readily digestible fractions from the resistant cell walls contain as much uronic acids as the resistant fraction 3I argues against this view.

A second striking feature of the wall polysaccharides of *Z. vuilleminii* is the absence of glucose. A variety of sugars has been reported in fungal cell walls, but with the exception of *Mucor rouxii*, glucans of different types have been noted as significant components of the structure (1). *M. rouxii*, a fungus which is related systematically to the genus *Zygorhynchus*, also has fucose, chitosan, and a high concentration of phosphate in its walls (3). It is likewise possible that polymeric components containing fucose or phosphate may contribute to or be responsible for the resistance to lysis.

The resistant fraction 3I contains an acid-resistant, alkali-soluble material that absorbs at 1,735 cm^{-1}. A substance with similar infrared absorption was found also in the lignin-like ma-

terial, which was quite resistant to degradation, obtained from the unfractionated walls. In support of the view that the component absorbing at 1,735 cm^{-1} may be associated in some way with resistance is the fact that such a band was absent from the readily decomposable walls derived from griseofulvin- and sorbose-grown mycelia as well as from fraction 3I obtained from the fungus grown in the antibiotic-containing medium. This acid-resistant material cannot be the sole determinant of resistance to lysis, however, since walls of *F. annosus* have more of it than *Z. vuilleminii*, yet the former fungus is readily attacked.

Representatives of *Zygorhynchus* are quite common in soil. Robinson (21), for example, cites a species of *Zygorhynchus* which persists in a viable form in soil in the hyphal form. It is not possible to conclude that their abundance is attributable largely or solely to their resistance to lysis, but surely the refractory nature of the hyphae aid the fungus in its struggle for existence. The data show, moreover, that the ability of fungi to withstand elimination by lytic microorganisms has more than one structural basis, melanin serving as the protective device in some fungi while another material has the same ecological role in an organism like *Z. vuilleminii*.

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LITERATURE CITED

1. Aronson, J. M. 1965. The cell wall, p. 49-76. *In* G. C. Ainsworth and A. S. Sussman (ed.), The fungi, vol. 1. Academic Press Inc., New York.
2. Ballesta, J. P. G., F. Uruburu, and J. R. Villanueva. 1969. Resistance of *Torulopsis aeris* cells to enzymatic lysis. Trans. Brit. Mycol. Soc. **52**:1-7.
3. Bartnicki-Garcia, S., and W. J. Nickerson. 1962. Isolation, composition, and structure of cell walls of filamentous and yeast-like forms of *Mucor rouxii*. Biochim. Biophys. Acta **58**:102-119.
4. Bitter, T., and H. M. Muir. 1962. A modified uronic acid carbazole reaction. Anal. Biochem. **4**:330-334.
5. Bloomfield, B. J., and M. Alexander. 1967. Melanins and resistance of fungi to lysis. J. Bacteriol. **93**:1276-1280.
6. Boas, N. F. 1953. Method for the determination of hexosamines in tissues. J. Biol. Chem. **204**:553-563.
7. Bu'lock, J. D., and H. G. Smith. 1961. A fungus pigment of novel type and the nature of fungus 'lignin'. Experientia **17**:553-554.
8. Chung, C. W., and W. J. Nickerson. 1954. Polysaccharide syntheses in growing yeasts. J. Biol. Chem. **208**:395-407.
9. Fiske, C. H., and Y. Subbarow. 1925. The colorimetric determination of phosphorus. J. Biol. Chem. **66**:375-400.
10. Gancedo, J. M., C. Gancedo, and C. Asensio. 1966. Uronic acids in fungal cell walls. Biochem. Z. **346**:328-332.
11. Kuo, M.-J., and M. Alexander. 1967. Inhibition of the lysis of fungi by melanins. J. Bacteriol. **94**:624-629.
12. Lockwood, J. L. 1960. Lysis of mycelium of plant-pathogenic fungi by natural soil. Phytopathology **50**:787-789.
13. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265-275.
14. Mahadevan, P. R., and E. L. Tatum. 1965. Relationship of the major constituents of the *Neurospora crassa* cell wall to wild-type and colonial morphology. J. Bacteriol. **90**:1073-1081.
15. Michell, A. J. 1966. Infrared spectra of lignin model compounds and of lignins from *Eucalyptus regnans*. Aust. J. Chem. **19**:2285-2298.
16. Michell, A. J., and G. Scurfield. 1967. Composition of extracted fungal cell walls as indicated by infrared spectroscopy. Arch. Biochem. Biophys. **120**:628-637.
17. Pearl, I. A. 1967. The chemistry of lignin. Marcel Dekker Inc., New York.
18. Pengra, R. M., M. A. Cole, and M. Alexander. 1969. Cell walls and lysis of *Mortierella parvispora* hyphae. J. Bacteriol. **97**:1056-1061.
19. Potgieter, H. J., and M. Alexander. 1966. Susceptibility and resistance of several fungi to microbial lysis. J. Bacteriol. **91**:1526-1532.
20. Reissig, J. L., J. L. Strominger, and L. F. Leloir. 1955. A modified colorimetric method for the estimation of *N*-acetylamino sugars. J. Biol. Chem. **217**:959-966.
21. Robinson, R. K. 1967. Ecology of fungi. English Universities Press, London.
22. Rondle, C. J. M., and W. T. J. Morgan. 1955. The determination of glucosamine and galactosamine. Biochem. J. **61**:586-589.
23. Runeckles, V. C., and G. Krotkov. 1957. The separation of phosphate esters and other metabolites by ionophoresis and chromatography on paper. Arch. Biochem. Biophys. **70**:442-453.
24. Skujins, J. J., H. J. Potgieter, and M. Alexander. 1965. Dissolution of fungal cell walls by a streptomycete chitinase and β-(1 $\rightarrow$ 3) glucanase. Arch. Biochem. Biophys. **111**:358-364.
25. Somogyi, M. 1952. Notes on sugar determination. J. Biol. Chem. **195**:19-23.
26. Tsiganos, C. P., and H. Muir. 1966. Estimation of pentoses and methylpentoses in biopolymers, in particular of fucose and xylose. Anal. Biochem. **17**:495-501.
27. Villanueva, J. R. 1966. Protoplasts of fungi, p. 3-62. *In* G. C. Ainsworth and A. S. Sussman (ed.). The fungi, vol. 2. Academic Press Inc., New York.
28. Volk, W. A. 1968. Isolation of D-galacturonic acid-1-phosphate from hydrolysates of cell wall lipopolysaccharide extracted from *Xanthomonas campestris*. J. Bacteriol. **95**:782-786.