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Cell Walls and Lysis of *Mortierella parvispora* Hyphae

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Walls of *Mortierella parvispora*, *Pullularia pullulans*, *Absidia repens*, *Fusarium oxysporum*, and of several *Penicillium* species varied in their susceptibilities to digestion by glucanase and chitinase. Polysaccharides were present in the residues remaining after enzymatic digestion. Acid hydrolysates of the walls contained glucose, glucosamine, and a small amount of galactose. The walls of *M. parvispora*, which also contained fucose, were the least digested by these two enzymes. Much of the *M. parvispora* wall material was resistant to decomposition by a heterogeneous soil community, and viable hyphae were not lysed by a glucanase-chitinase mixture. Walls of this fungus were fractionated, and the chemical composition of the fractions was determined. The chitin which was abundant in one of the fractions was apparently largely shielded from chitinase hydrolysis by a glucan. The ecological significance of these findings is discussed.

In a natural environment containing a large, heterogeneous microbial community, natural selection will eliminate species unable to cope with the stress imposed by neighboring species occupying the same site. The mechanism of elimination of a species from a natural community may involve competition, toxin production, predation, or parasitism. Moreover, for an organism to remain viable for considerable periods of time when the supply of nutrients in a densely populated environment is discontinuous in time, the organism must produce vegetative or resting structures which not only retain the capacity to reproduce when nutrients are again present but also are resistant to destruction by the predatory, parasitic, or lytic coinhabitants of the ecosystem.

Certain fungi exist in the soil environment in the hyphal form, whereas others persist largely as spores. These vegetative and spore structures produced by the indigenous fungal community must therefore remain viable despite the presence of an abundant lytic flora. It has been shown that vegetative filaments of selected terrestrial fungi are readily lysed because their hyphal walls are largely composed of chitin and a β-1,3-glucan, chitinase and β-1,3-glucanase being responsible for the destruction of mycelial integrity, whereas other fungal species are not destroyed because they produce hyphae, conidia, or sclerotia containing melanin or a melanin-like material in the outer surface structure (1, 4, 9, 11). However, many fungi apparently resist microbiological lysis under natural conditions, although they produce no melanin-containing filaments or resistant structures. The present report concerns one such fungus, *Mortierella parvispora*, an organism which shows marked resistance to digestion by microbial enzymes. Members of this genus are abundant and widespread in terrestrial environments (8). They must, therefore, have physiological or structural traits that enable them to grow and persist in soil communities which contain an abundant and highly heterogeneous microflora.

MATERIALS AND METHODS

A culture of *M. parvispora* was provided by C. W. Hesseltine, two strains of *Penicillium spinulosum* were obtained from the American Type Culture Collection, and *P. brefeldianum* QM1872 and Basidiomycete QM806 were provided by U.S. Army Natick Laboratories. These fungi, as well as *Fusarium oxysporum*, *Absidia repens*, and a nonpigmented strain of *Pullularia pullulans* were grown in a medium containing, per liter: glucose, 15 g; NH_4NO_3, 5.0 g; KH_2PO_4, 5.0 g; $MgSO_4 \cdot 7H_2O$, 0.75 g; sodium citrate, 0.50 g; $FeCl_3$, 40 mg; $ZnSO_4$, 20 mg; $CaCl_2 \cdot 2H_2O$, 20 mg; yeast extract, 10 mg; and $MnCl_2$, 5.0 mg. Solutions containing the glucose and yeast extract, the nitrate and phosphate, and the other salts were autoclaved separately, cooled, and then combined. *M. parvispora* was grown for 7 days at 25 C in Roux bottles containing 100 ml of medium. All other fungi were grown on a rotary shaker in 500-ml flasks containing 200 ml of medium for 4 days at 30 C, except for *P. pullulans*, which was grown at 25 C.

Cell walls were prepared by the technique described

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by Mahadevan and Tatum (7) modified to include a brief high-speed homogenization of the hyphal material with the sodium dodecyl sulfate. The mycelium was allowed to stand overnight in a 1% solution of the detergent at 4 C. The wall preparations were then collected by filtration or centrifugation, washed several times with distilled water until free from detergent, suspended in ethyl alcohol solutions of increasing strength, and dried in a vacuum desiccator over $CaCl_2$.

Soluble carbohydrate was determined by use of anthrone, with a glucose standard (6), a method which does not give a positive reaction with the soluble products formed from chitin by the action of chitinase. Reducing sugars were estimated by the alkaline copper method of Somogyi (12). *N*-acetylhexosamine was determined by the procedure described by Reissig et al. (10). Glucose was measured enzymatically by use of Glucostat reagents (Worthington Biochemical Corp., Freehold, N.J.). Glucosamine was determined by the procedure of Elson and Morgan (3).

In experiments involving the use of ^{14}C, 50-g portions of garden soil, *p*H 6.4, were incubated in 250-ml Erlenmeyer flasks with 300 mg of labeled *M. parvispora* walls, with 300 mg of labeled *P. pullulans* walls, or with no amendment. The cultures from which the walls were obtained were grown for 4 days at 25 C with aeration in 3-liter Erlenmeyer flasks containing 2.0 liters of the medium described above, supplemented with 50 μc of uniformly labeled ^{14}C-D-glucose (specific activity, 110 mc/mmole). The soils were aerated and incubated at 25 C. The CO_2 formed was collected by bubbling the air leaving the flasks through 50 ml of 0.1 N NaOH. A 2-ml amount of a saturated $BaCl_2$ solution was added to 5 ml of the alkali solution; the $BaCO_3$ that formed was collected by centrifugation, and the radioactivity of the sample was measured with an Abacus Scaler, model 123 (Baird Atomic, Inc., Cambridge, Mass.). The CO_2 traps were filled with fresh NaOH solution after each sample was taken.

For determination of the products of enzymatic or acid hydrolysis of substances released from heat-treated wall preparations, a descending paper chromatographic system with Whatman no. 1 paper was used. The compounds were located either by dipping the paper in $AgNO_3$ solution (0.1 ml of saturated aqueous $AgNO_3$ in 100 ml of acetone), drying the chromatograms, and then dipping them in 0.5% alcoholic NaOH, or by spraying the chromatograms with a 3% solution of *p*-anisidine hydrochloride dissolved in *n*-butyl alcohol saturated with water. The latter chromatograms were heated at 90 to 100 C for 5 to 10 min (5).

The glucanase was obtained from *P. brefeldianum* grown for 8 to 10 days with aeration at room temperature in 8 liters of a medium containing 0.3% glucose, 0.3% malt extract, and 0.01% yeast extract. The hyphae were removed by filtration and centrifugation, and the supernatant liquid was cooled to 2 C and treated with $(NH_4)_2SO_4$ to 70% of saturation. The solution was allowed to stand overnight at 4 C, and the resulting precipitate was collected by filtration on celite. The protein precipitate was dissolved in distilled water and dialyzed against distilled water at 4 C; the solution was then lyophilized. The preparation (80 mg) was dissolved in 1.0 ml of 0.033 M phosphate buffer, *p*H 6.1, and the solution was placed on a Sephadex G-150 column, 3.1 × 70 cm, previously equilibrated with the same buffer. The column was eluted with the phosphate buffer, and 2.5-ml fractions were collected. Those fractions having high β-1,3- and β-1,6-glucanase activity were pooled and lyophilized.

The glucanase preparation had no chitinase or amylase activity when tested at *p*H 5.0. Activity of the glucanase was assessed by use of 0.1 ml of the enzyme solution, 0.9 ml of 0.1 M acetate buffer, *p*H 5.0, and 1.0 ml of substrate solution containing 1.0 mg of either laminarin or pustulan for the β-1,3- and β-1,6-glucanase, respectively. With laminarin and pustulan as substrates, an amount of the preparation containing 1.0 mg of protein catalyzed the formation of 4.1 and 0.20 μmoles of glucose per min at 37 C and *p*H 5.0 in 0.05 M phosphate-acetate buffer (0.05 M Na_2HPO_4 adjusted to *p*H 5.0 with glacial acetic acid). To obtain enzymatic hydrolysis of the walls, 25 mg of the walls, 23 ml of phosphate-acetate buffer, *p*H 4.5, and 1.0 ml each of the glucanase and chitinase preparations (containing 1.25 mg per ml) were incubated for 48 hr at 37 C with shaking. Merthiolate (0.1 ml of 1:1,000 solution) was added to prevent microbial growth. The insoluble residue was removed by centrifugation. In studies of the time course of enzymatic digestion, 3.0-ml samples were taken at regular intervals during a 48-hr incubation period.

Microslide cultures of the fungi were prepared by pipetting melted, inoculated Potato Dextrose Agar (Difco) under a cover glass supported about 1 mm above the slide by a U-shaped glass capillary tube. The slides were incubated for 7 days at 25 C for *P. pullulans* and at 30 C for *M. parvispora*. To each slide culture was added either 0.1 ml of the glucanase-chitinase mixture (100 μg of each enzyme in phosphate-acetate buffer, *p*H 4.5) or 0.1 ml of buffer. The slides were incubated at 37 C and examined under phase contrast.

Hydrochloric acid hydrolysis was accomplished by heating about 10 mg of the material in 3 N HCl for 3 hr at 100 C. The HCl was removed by evacuation over KOH pellets.

D-Glucose was obtained from Distillation Products Industries, Rochester, N.Y.; galactose, from Difco Laboratories, Detroit, Mich.; D-glucosamine and *N*-acetylglucosamine, from Nutritional Biochemicals Corp., Cleveland, Ohio; fucose, mannose, and xylose, from Calbiochem, Los Angeles, Calif.; and a streptomycete chitinase from Worthington Biochemical Corp., Freehold, N.J. The enzyme preparation employed did not catalyze the deacylation of *N*-acetylglucosamine nor did it contain detectable β-1,3-glucanase activity.

RESULTS

After treatment with a glucanase-chitinase mixture for 48 hr, at which time the release of glucose, *N*-acetylhexosamine, and anthrone-positive materials had essentially ceased, wall preparations of *P. pullulans*, *P. brefeldianum*, *P. spinu-*

losum, *F. oxysporum*, *A. repens*, Basidiomycete QM806, and *M. parvispora* showed various degrees of digestion. Digestion, as measured by the loss of weight resulting from the solubilization of the insoluble wall material, ranged from 85% for the walls of *F. oxysporum* to only 25% for those of *M. parvispora*. Most of the wall constituents which were solubilized by these enzymes were carbohydrates (Table 1); indeed, the amounts of the wall solubilized when measured by weight loss were similar to the sums of the quantity of anthrone-positive carbohydrate and *N*-acetylhexosamine in solution. The enzymes released little free *N*-acetylhexosamine from *P. spinulosum*, *P. pullulans*, Basidiomycete QM806, and *M. parvispora*, whereas much was found in solution after the enzymatic hydrolysis of *F. oxysporum* walls. Considerable glucose was freed from the walls of certain species, although little was liberated from the walls of other fungi. Noteworthy is the marked resistance to digestion by these two enzyme preparations of the walls of the Basidiomycete and *M. parvispora* (Table 1).

TABLE 1. *Release of soluble carbohydrates from fungal wall preparations by a glucanase-chitinase mixture*

Fungus	Wall wt (mg)	Percentage of wall recovered as soluble carbohydrate		
		Anthrone-positive materials	*N*-acetylhexosamine	Glucose
Penicillium brefeldianum	25.1	41.0	20.9	24.4
P. spinulosum 9123	25.8	19.1	14.0	12.0
P. spinulosum 10498	25.7	20.3	7.8	9.3
Pullularia pullulans	24.6	49.4	5.7	39.7
Fusarium oxysporum	24.7	35.4	26.7	14.0
Absidia repens	30.5	30.2	22.1	23.6
Basidiomycete QM806	31.4	13.7	7.0	5.2
Mortierella parvispora	25.2	11.9	8.7	8.4

The insoluble residues remaining after glucanase-chitinase digestion of the walls were hydrolyzed with HCl. Compounds with the chromatographic characteristics of glucose and glucosamine were found in all of the acid hydrolysates, suggesting that at least some of the glucose and glucosamine of the walls was in a form inaccessible to these enzymes or in a polysaccharide not suitable as a substrate for them. The quantity of glucose and glucosamine found varied with the fungus. Other unidentified products were also noted in these digests. Mannose, xylose, and galactosamine were not found in either the enzymatic or acid hydrolysates.

The untreated wall preparations were hydrolyzed with HCl, and the deacidified digests were chromatographed on paper with an isopropanol-water (4:1) developing solvent. Results of a qualitative analysis of these digests are given in Table 2. Glucose and glucosamine were common to all the fungi, but fucose was noted only in *M. parvispora* walls. The apparent galactose spot on all chromatograms was not resolved from that of glucose, except in *M. parvispora* hydrolysates. Xylose, mannose, and galactosamine were not found. The oligosaccharide-like materials had a low relative mobility on the chromatograms, and the slowly moving compounds were apparently not uronic acids since they did not give a typical uronic acid color reaction when sprayed with *p*-anisidine hydrochloride in water-saturated *n*-butyl alcohol or diphenylamine-urea (5).

When HCl digests of *M. parvispora* walls were chromatographed on paper with isopropanol-water, a compound was noted that reduced the alkaline silver reagent and moved about 1.3 times as rapidly as glucose. This compound had essentially the same mobility as both authentic

TABLE 2. *Products of acid hydrolysis of wall preparations*

Fungus	Glucose	Galactose	Glucosamine	Fucose	Oligosaccharides[a]	Others[a]
Penicillium brefeldianum	+++	+	++	−	46	134, 158
P. spinulosum 9123	+++	+	+++	−	—	—
P. spinulosum 10498	+++	+	+++	−	—	—
Pullaria pullulans	+++	+	+++	−	51	118, 159
Fusarium oxysporum	+++	+	+++	−	11, 47	156
Absidia repens	+++	+	+++	−	11	127
Basidiomycete QM806	+++	+	+++	−	45	121
Mortierella parvispora	++	+	++	+++	—	138

[a] Figures refer to migration of the compound relative to the migration of glucose.

xylose and fucose. Results with the *p*-anisidine hydrochloride spray reagent indicated that the compound was not a pentose but rather that it was a six-carbon reducing sugar. On the basis of its chromatographic behavior in isopropanol-water (4:1), isopropanol-*n*-butyl alcohol-water (7:1:2), and *n*-butyl alcohol-pyridine-water (9:5:4) solvent systems, the compound was concluded to be fucose.

The cell walls of *M. parvispora* were fractionated by the method of Mahadevan and Tatum (7). Fractions I, II, III, and IV accounted for 17.0, 20.2, 0.9, and 16.2% of the wall weight, respectively. Portions (5 mg) of three of the fractions in 4.9 ml of phosphate-acetate buffer, *p*H 4.5, were shaken for 48 hr at 37 C with 0.1 ml of a solution containing 0.25 mg of the glucanase preparation, 0.25 mg of the chitinase preparation, or both preparations. At the end of the incubation, the insoluble material was removed by centrifugation. The results given in Table 3 show that the enzyme mixture released glucose equivalent to 9.0, 0.08, and 0.5% and *N*-acetylhexosamine equivalent to 1.2, 3.0, and 92.5% of the weight of fractions I, III, and IV. The data suggest that fraction IV contains an appreciable amount of chitin. However, since little *N*-acetylhexosamine was liberated by the chitinase in the absence of the glucanase preparation, the chitin seems to be shielded by a polysaccharide. This shielding constituent, presumably a glucan, does not account for a large part of fraction IV, inasmuch as almost 93% of fraction IV was recoverable as *N*-acetylhexosamine.

Infrared spectroscopy provided further evidence for the presence of chitin in this fraction. The spectra of KBr pellets were obtained with a Beckman infrared spectrophotometer, model IR10 (Fig. 1). The similarity of the infrared spectra of crustacean chitin and fraction IV indicates that *M. parvispora* generates a chitin layer in its hyphal walls.

Further support for an interlayering of polysaccharides or for a chitin-glucan complex is provided by an experiment in which *M. parvispora* fraction IV was incubated with the glucanase preparation at 37 C for 18 hr, after which time the mixture was boiled for 1 min to inactivate the enzyme. The residual wall material was washed and then incubated with chitinase for an additional 18 hr at 37 C. Under these conditions, the amount of *N*-acetylhexosamine released was 12.2% of the weight of fraction IV, a figure essentially identical to the 12.6% released in the same study when chitinase was incubated with a sample of fraction IV which had not been preincubated with the glucanase. In contrast to this sequential treatment, the simultaneous hydrolysis by the chitinase-glucanase mixture solubilized an amount of *N*-acetylhexosamine equivalent to 92.5% of the weight of the fraction.

TABLE 3. *Digestion of fractions I, III, and IV of Mortierella parvispora walls by chitinase and glucanase preparations*

Enzyme prepn	Percentage of fraction converted to glucose			Percentage of fraction converted to *N*-acetylhexosamine		
	I	III	IV	I	III	IV
Glucanase....	6.8	—	0	0	—	0
Chitinase.....	0	—	0	0.3	—	13.0
Glucanase + chitinase...	9.0	0.08	0.5	1.2	3.0	92.5

Results obtained with the fractionation scheme indicated a difference between the walls of *M. parvispora* and *Neurospora crassa*. Thus, in contrast with the susceptibility to enzymatic digestion of *N. crassa* wall fractions, reported by Mahadevan and Tatum (7), the glucanase preparation released no glucose from the small quantity of fraction III obtained, whereas about 7% of fraction I of *M. parvispora* was converted enzymatically to the hexose (Table 3).

When unhydrolyzed *M. parvispora* fraction II was chromatographed on paper with isopropanol-water as the developing solvent and the chromatograms were treated with *p*-anisidine or the alkaline silver reagents, spots corresponding to galactose, glucosamine, fucose, and two or three other slowly moving reducing compounds, possibly oligosaccharides, were revealed. Upon acid hydrolysis of this fraction, galactose, glucosamine, and fucose were again apparent. The quantity of these sugars seemed to increase with time of hydrolysis, as indicated by a deepening of the intensity of the reduced silver spots on the

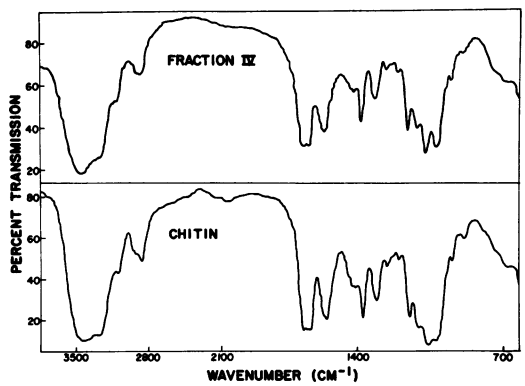

FIG. 1. *Infrared spectra of Mortierella parvispora fraction IV and crustacean chitin.*

chromatogram in relation to the slowly moving components, which seemed to contain less material after acid hydrolysis. Glucose was not observed in the fraction either before or after acid hydrolysis.

Fraction I, produced by alcohol precipitation of a 2 N NaOH extract of the wall material, yielded at least four low-molecular-weight reducing substances when hydrolyzed with HCl. Three of these appeared to be glucosamine, fucose, and glucose on the basis of paper chromatography employing isopropanol-water. A reducing substance that moved 1.43 times as fast as glucose and a component which moved slowly from the origin were also noted. With the use of the same chromatographic conditions, no compounds migrating from the origin were detected when the unhydrolyzed fraction was examined. As indicated above, the glucanase-chitinase mixture liberated *N*-acetylhexosamine and glucose from fraction I in amounts equivalent to 1.2 and 9.0% of the fraction weight, respectively. In contrast to the results with *N. crassa*, only a single ninhydrin-positive component was found in paper chromatograms of HCl hydrolysates of *M. parvispora* fraction I.

The *M. parvispora* wall preparation was autoclaved for 1 hr, and the insoluble material was removed by centrifugation. The supernatant liquid, which accounted for 24.4% of the walls on a weight basis, was found to be rich in soluble carbohydrates when examined by the anthrone method. Paper chromatography (isopropanol-water) of this extract showed that it contained galactose, fucose, four other reducing compounds that migrated more rapidly than fucose, and three constituents that moved more slowly than glucose. When this supernatant liquid was treated with three volumes of absolute ethyl alcohol, 21% of the soluble material was precipitated. Upon incubation of this precipitate with the glucanase-chitinase mixture, a single spot with the R_F of glucose appeared. When 2.0 mg of the ethyl alcohol-insoluble material was incubated for 24 hr with the glucanase preparation (50 μg/ml) in phosphate-acetate buffer, *p*H 4.5, 98.9% was converted to soluble, anthrone-positive materials, and 81.8% was accounted for as free glucose. This suggests the existence of a glucan component in hyphal walls of this fungus.

In a study to determine the susceptibility of ^{14}C-labeled cell walls of *M. parvispora* and *P. pullulans* to degradation by the microbial community of soil, it was observed that $^{14}CO_2$ was evolved from soil treated with labeled walls of both fungi (Fig. 2). The data show that part of the wall material of *M. parvispora* is decomposed rather readily, but that a fraction remains which fails to be degraded microbiologically at an appreciable rate. On the other hand, the walls of *P. pullulans* were more completely decomposed in the test period, and $^{14}CO_2$ was still being produced at the end of the incubation period.

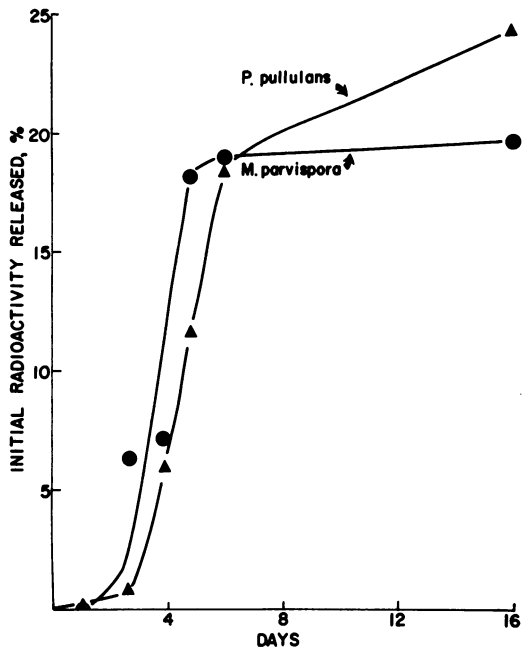

FIG. 2. *Release of $^{14}CO_2$ from labeled Mortierella parvispora and Pullularia pullulans walls incubated in soil.*

These observations on the relative digestibility of the hyphal walls of these two fungi are in accord with microscopic observation of the rate of digestion of the viable hyphae by a chitinase-glucanase mixture. Thus, when observed in microslide cultures, extensive lysis of *P. pullulans* mycelium was observed in 3 hr, and no intact cells remained after incubation for periods of 24 hr or less. By contrast, no visible indications of lysis of *M. parvispora* filaments were detected even at 24 hr.

DISCUSSION

Undoubtedly, many traits underlie or determine fitness in the natural selection of microbial species colonizing or introduced into environments potentially invaded by numerous different microbial types. Many of these fitness characters are associated with the active growth of the species, permitting it to exploit nutrients, conditions, or ecological niches to its advantage and to the detriment of co-colonizers of the locale or microenvironment. On the other hand, morphological and physiological attributes associated with survival mechanisms are also of considerable significance to natural selection in those ecosystems in which continuous vegetative growth is not

possible or in which the community contains an abundance of parasites or predators.

In our previous investigations, it was shown that melanins may endow fungal hyphae or resting structures with a distinct ecological advantage, the melanized surface serving to protect the chitin and glucan components of the wall from enzymatic hydrolysis and thus preventing significant lysis of fungi by their parasitic neighbors (1, 4, 9). As many fungi which do not produce melanin are also capable of surviving in a habitat containing mycolytic bacteria and actinomycetes, a study was initiated to determine whether the hyphal walls of certain of these fungi might also be significantly resistant to enzymatic destruction. *M. parvispora* seemed a logical choice in view of the facts that it is abundant in the soil community and that it often appears to exist in this habitat in the hyphal form (13–15).

In confirmation of previous findings, a major portion of the walls of several fungi were solubilized by a preparation containing chitinase and β-1,3-glucanase, suggesting that chitin and a glucan are important structural components of the hyphal wall. *M. parvispora* walls, by contrast, are notably resistant to digestion whether tested by incubating them with the crude enzyme preparation or with the heterogeneous microbial community of soil. The latter procedure is a more rigorous test of susceptibility to digestion because it allows for the enrichment of any lytic organism capable of using the wall constituents. Similarly, the viable mycelium was not lysed by a chitinase glucanase preparation. The finding that *P. spinulosum* walls are also resistant to the glucanase-chitinase mixture is of interest since Williams (13) reported that this fungus was abundant in soil and presumably existed therein in a vegetative state. The data therefore suggest that walls of mycelia which do not produce melanin, such as *M. parvispora* and *P. spinulosum*, may be endowed with some constituent which makes them refractory to enzymatic hydrolysis, a constituent which may allow for the prolonged viability of the species in habitats in which forces of natural selection would have resulted in the elimination of less fit microorganisms.

The identity of the resistant constituent or components has not yet been resolved. The fractionation revealed that both chitin and a glucan are present in *M. parvispora* walls, but either they are shielded from hydrolytic enzymes when present in the normal filament or their loss does not materially affect hyphal integrity and viability of the organism. The release of some $^{14}CO_2$ from labeled *M. parvispora* walls introduced into soil indicates that portions of the surface structure are indeed readily susceptible to microbial attack. It is possible that resistance in this microorganism is associated with a fucose-containing polymer or a heteropolysaccharide which, because of the different monomers and possibly different linkages, is hydrolyzed only slowly or requires a number of enzymes produced by different organisms, each of which must alight upon the mycelium existing in a nonfluid environment. The finding of fucose in several fractions of this fungus is particularly noteworthy in this regard. Fucose is known to be present in walls of several species of fungi (2), and recent evidence indicates that the fucose-containing fractions derived from *Zygorhynchus vuilleminii* walls are not acted upon significantly by microbial enzymes (J.-P. G. Ballesta and M. Alexander, Bacteriol. Proc., p. 39, 1968).

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