APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Sept. 1987, p. 1987–1995
0099-2240/87/091987-09$02.00/0

Vol. 53, No. 9

Mixed Fungal Populations and Lignocellulosic Tissue Degradation in the Bovine Rumen

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Received 26 May 1987/Accepted 2 June 1987

Anaerobic fungi in ruminal fluid from cows eating Bermuda grass hay plus a grain and minerals supplement were evaluated for diversity in sporangial morphotypes and colony growth patterns and for the degradation of various lignocelluloses. In selective cultures containing streptomycin and penicillin, an active population of ruminal fungi colonized leaf blades and degraded fiber at rates and extents almost equal to that of the total ruminal population. Three major sporangial morphotypes were consistently observed on leaf blades: oval, globose, and fusiform. Fungal colonies representing three distinct growth types consistently developed in anaerobic roll tubes inoculated with strained ruminal fluid. Sporangial morphotypes could not be matched to colony types due to multiple sporangial forms within a colony. Under identical growth conditions, one type exhibited a monocentric growth pattern, while two types exhibited polycentric growth patterns previously unreported in ruminal fungi. Mixed ruminal fungi in selective cultures or in digesta taken directly from the rumen produced a massive clearing of the sclerenchyma. Quantitation of tissue areas in cross sections by light microscopic techniques showed that fungal incubations resulted in significant ($P = 0.05$) increases in sclerenchyma degradation compared to whole ruminal fluid incubations. The mestome cell wall was at times penetrated and partially degraded by fungi; the colonization was less frequent and to a lesser degree than with the sclerenchyma. Conversely, ruminal bacteria were not observed to degrade the mestome sheath. Phenolic monomers at 1 mM concentrations did not stimulate to a significant ($P = 0.05$) extent the dry weight loss or fungal colonization of leaf blades; at 10 mM concentrations cinnamic and benzoic acids were toxic to ruminal fungi.

In recent years ruminal fungi have been shown to be significant colonizers of fiber (6, 7, 22, 23) and to have enzymes capable of degrading plant structural carbohydrates (8, 18, 26). In certain systems ruminal fungi responded to sulfur fertilization in plants and were shown to be the predominant degraders of forage fiber (4). In other systems the fungi were comparatively less active in degrading fiber then other microbial components (32).

Four ruminal fungi have been described to date: *Neocallimastix frontalis* (20), *N. patriciarum* (25), *Sphaeromonas communis* (21), and *Piromonas communis* (23). Only *Neocallimastix* spp. have been extensively studied ultrastructurally for classification (14, 19) and for enzymatic activities (18, 24, 26, 33). It has been suggested that this fungus fits best in the order *Spizellomycetales* of the class *Chytridiomycetes* (14, 22), with Heath et al. (14) proposing the new family *Neocallimasticaceae*.

Orpin (22, 23) reported that *N. frontalis* and *P. communis* invade plant tissue. In studies in our laboratory (3) and others (6), sporangia of various morphotypes have been observed to be associated with fiber incubated with ruminal fluid. While it has been reported that sporangial morphology of anaerobic fungi does vary in the colonizing population (20), variations in morphotypes may provide a means to monitor various fungi in mixed populations of microbes colonizing forage leaves.

Lignins within plant cell walls vary based on the predominant monomeric units, e.g., syringyl, coniferyl, and *p*-coumaryl (30, 31). Leaf blade sclerenchyma shows an initial and strong reaction for lignin with chlorine sulfite, while the xylem and metaxylem vessels of vascular bundles stain predominantly for lignin with acid phloroglucinol (2, 30). Stafford (30) showed differences between sclerenchyma and xylem cells in lignin chemistry and attributed the chlorine sulfite reaction to a prevalence of syringyl groups. The chlorine sulfite- and acid phloroglucinol-staining tissues comprise the majority of the undigested fiber residue after ruminal microbial digestion (2). However, the former tissues are less resistant than the latter to microbial attack or to chemical treatment (2). There are clearly differences in the structure of lignified tissues within forages that relate to microbial availability. The xylem cells of the leaf blade are the most resistant tissues observed (2).

Previous work (3, 32) has shown that the addition of streptomycin and penicillin prohibits bacterial fiber degradation by ruminal fluid, although certain non-fiber-degrading bacteria such as methanogens (8) survive. Addition of streptomycin and penicillin to ruminal fluid promotes an increase in fungal biomass and permits evaluation of potential fiber degradation under the confinements of the in vitro system (3).

An in vitro system with antibiotics was used in this study to evaluate an inoculum selective for fungi. The objectives of the work were to (i) evaluate the mixed fungal population for potential fiber degradation, (ii) characterize various components of the mixed fungal population, (iii) evaluate ruminal fungi in in vivo and in vitro studies for their ability to degrade different lignified tissues, and (iv) evaluate the influence of phenolic monomers on digestibility of plant tissue by ruminal fungi.

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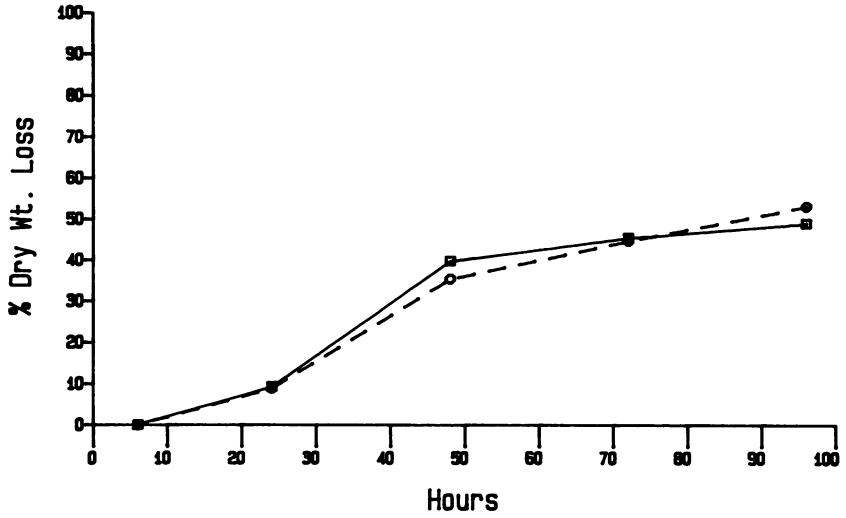

FIG. 1. Percent fiber loss with time due to ruminal fungi, i.e., ruminal fluid plus streptomycin and penicillin (□), and to the whole ruminal population, i.e., no antibiotics (○). Fiber values were corrected for initial washout of soluble material and represent the average of three tubes.

MATERIALS AND METHODS

Fiber digestion. Coastal Bermuda grass was grown in a well-fertilized (N/P/K = 381:111:111 kg ha^{-1} $season^{-1}$ in three applications) plot near Athens, Ga. Regrowths at 6 to 9 weeks were cut in 1982, and plants were maintained at −10°C until used. Leaf blades were selected from plants, and 1-cm lengths were cut from the central portions of the blades. The 1-cm sections were freeze-dried and stored, and these sections were used in all digestion trials. Groups of 10 leaf blades were freeze-dried, weighed, and placed into incubation tubes (Hungate type; Bellco Glass, Inc.) A 5-ml portion of Caldwell and Bryant (10) basal anaerobic medium was added under a CO_2 atmosphere to each tube, which was then stoppered and autoclaved under fast exhaust. Groups of tubes were prepared without antibiotics or with a combined streptomycin and penicillin solution as described previously (3). Tubes were in triplicate for each treatment.

Ruminal fluid was collected from fistulated Hereford cows eating Coastal Bermuda grass hay plus a grain and minerals supplement (2.5 kg of 59% corn, 33% soybean hulls, 7% soybean meal, 0.3% trace mineral salt, and 0.7% dicalcium phosphate day^{-1}), and the fluid was kept separately for each animal. Ruminal digesta was strained twice through cheesecloth, and 0.5 ml of the strained fluid was inoculated into each Hungate tube, which was incubated at 39°C. After incubation for selected times, leaf blades were retrieved, washed briefly with distilled water, and freeze-dried, and the percent dry weight loss was calculated from the original weight.

Microscopic study of fungi on leaf blades. Sections (3 mm) were cut from fresh frozen Bermuda grass plants. Ten 3-mm sections were distributed into each of the Hungate tubes such that the sections were uniform among tubes containing streptomycin and penicillin and those with no antibiotics. Leaf sections were retrieved after selected incubation times with ruminal fluid and stained with lactophenol cotton blue for light microscopy or prepared for scanning electron microscopy (SEM) and transmission electron microscopy (TEM) as described previously (3, 4). For enumeration by light microscopy, the total number of sporangia on both cut edges of leaf blades was divided by the leaf blade width to give sporangial numbers per millimeter on cut edges of leaf blades. For evaluation of relative numbers of sporangia per site on the leaf surfaces, sporangia within five randomly selected fields (×20 objective lens; area, 0.55 mm^2) on the midrib surface of the blade were counted. Twelve blade sections were evaluated for each treatment. Leaf and stem fragments of Bermuda grass were removed directly from the rumens of cows supplying inoculum fluid and prepared for electron microscopy to evaluate the in vivo relationship of microbes to plant tissues.

Isolation of colonies. Roll tubes were prepared as described before (1) and contained cellobiose (0.2%), xylan (from larch sawdust; 2 or 3%; Koch-Light) or cellulose (1.5 or 2%, MN 300, Macherey-Nagel) with streptomycin and penicillin (2). Strained ruminal fluid was diluted to 10^1 and 10^2, and 0.2 ml of the dilution was inoculated into roll tube media as described previously (1). Fungal colonies developing on various media were counted for total numbers (duplicate tubes and duplicate replications at each dilution), and those on xylan and cellulose were also evaluated for zones of clearing in the opaque media. Colony development was monitored by observation of roll tubes at specific times. For SEM, tubes with colonies growing on agar were filled with 4% glutaraldehyde in 0.1 M cacodylate buffer. After fixation for 24 h, colonies were excised from the tubes, adhered to SEM stubs, and fixed in OsO_4 vapor for 2 h. Colonies were coated with Au-Pd alloy and observed by SEM. For TEM, colonies in agar fixed in glutaraldehyde were postfixed in 1.5% buffered OsO_4 for 2 h, dehydrated in ethanol, and embedded (4).

Quantitation of leaf blade degradation. For quantitation of tissues degraded by microorganisms, 1-μm-thick sections were cut from embedded blocks for TEM containing leaf blades digested for 48 h. The sections were heated briefly on a hot plate in the presence of water to expand the sections, and the sections were then dried at low temperature for 1 h. Sections were stained with 1% (wt/vol) toluidine blue in 1% (wt/vol) sodium borate by laying stain-saturated paper over the sections for 4 min at low temperature. The stained sections were rinsed in water, decolorized for 30 s in 70% EtOH, rinsed again in water, and then decolorized for 60 s in 100% EtOH. Sections were rinsed in water, dried, and mounted in glycerol. The tissues stained blue and were readily recognized with a digitizing monitor associated with

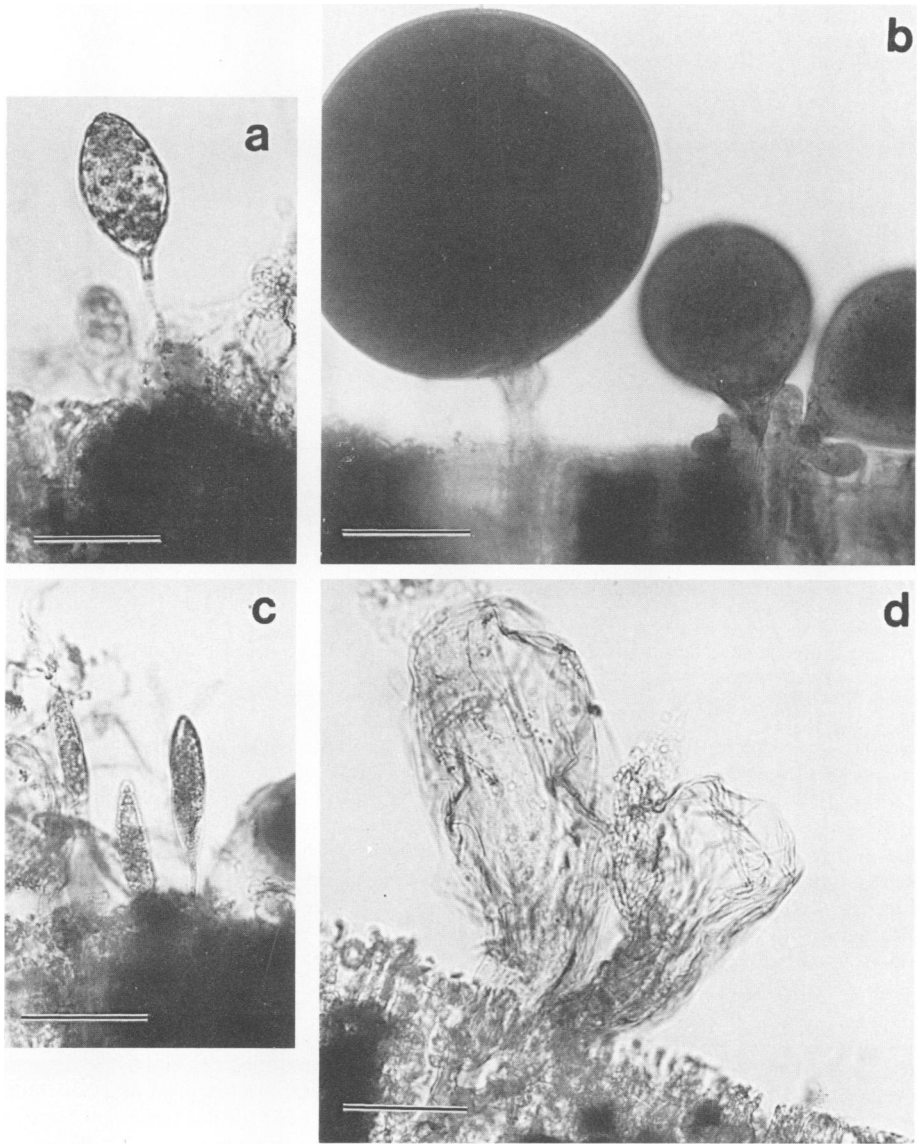

FIG. 2. Sporangial morphotypes associated with leaf cut edge. (a) Oval sporangium with aggregates within, possibly indicating formation of zoospores. (b) Large and small globose sporangia. (c) Fusiform sporangia. (d) Empty sporangia showing collapsed walls. Bar = 50 μm.

a light microscope. The area of sclerenchyma was digitized, with both abaxial and adaxial patches summed for each of the midvein and the two second-order veins to the side of the midvein.

Incubation with phenolic compounds. Phenolic compounds at 1 or 10 mM concentrations were included in the basal medium plus streptomycin and penicillin with 1-cm and 3-mm leaf blades. Compounds included were sinapic acid, syringaldehyde, syringic acid, ferulic acid, vanillin, vanillic acid, *p*-coumaric acid (PCA), *p*-hydroxybenzaldehyde, *p*-hydroxybenzoic acid, hydrocinnamic acid (HCA), and phenylacetic acid (PAA). The first nine compounds, in groups of threes, represent three families of compounds including the cinnamic acid and benzoic aldehyde and acid of dimethoxylated, monomethoxylated, and nonmethoxylated aromatic rings. HCA and PAA are closely related compounds. The pH of the media with these compounds was 6.6 to 6.7. After incubation with phenolic compounds for 72 h, 1-cm leaf blades were evaluated in triplicate for dry-weight loss, and 3-mm blades were evaluated for colonizing fungi as described above.

RESULTS

The data in Fig. 1 show fiber loss with time due to ruminal fungi (i.e., ruminal fluid plus streptomycin and penicillin) or to the whole population. Fiber loss due to the fungi was slightly higher at 24 and 48 h, but was significantly less ($P = 0.05$) at 96 h. Data from a second cow (not shown) were similar. These data indicate that ruminal fungi in this selective culture can potentially degrade plant fiber at similar rates and to near similar extents to that of the bacterial population. Both microbial groups degraded forages faster in the interval between 24 and 48 h in this in vitro system, with digestion tapering off by 96 h to a greater degree with the fungi.

The inclusion of streptomycin and penicillin in the tubes resulted in significantly higher numbers of sporangia com-

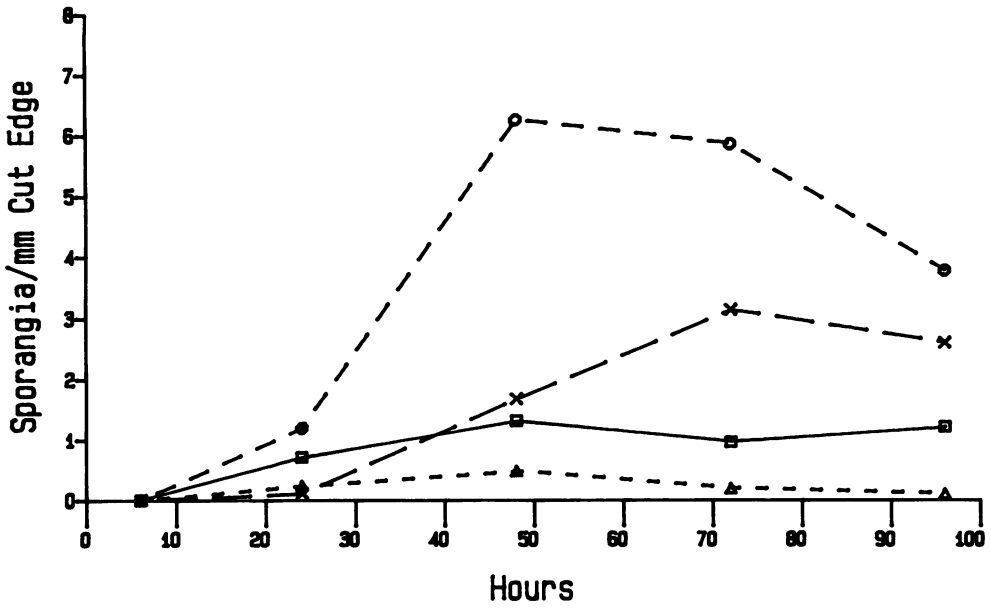

FIG. 3. Sporangial morphotypes developing on leaf cut edge with time. Symbols: □, oval morphotypes; ○, globose morphotypes; △, fusiform morphotypes; ×, empty sporangia.

pared with media without antibiotics. Evaluation by light microscopy of fungi associated with leaf blades showed the following general morphotypes of sporangia: oval (Fig. 2a), globose (Fig. 2b), and fusiform (Fig. 2c). Pleomorphic structures apparently representing the sporangial wall of empty sporangia, i.e., those that had lost zoospores or cellular contents, are shown in Fig. 2d. Leaf blades incubated with rumen fluid plus streptomycin and penicillin or plant material removed directly from the rumen and evaluated by SEM revealed that sporangial morphotypes similar to those observed by light microscopy were prevalent (not shown); however, the large rhizomycelium observed in streptomycin and penicillin cultures occurred infrequently in in vivo samples. SEM showed that fungi were associated with resistant tissues and especially the vascular tissues. The distribution with time of these sporangial morphotypes per linear millimeter of leaf cut edge is shown (Fig. 3). The maximum number of sporangia of all types usually occurred at 48 h of incubation. Globose sporangia were clearly the most prevalent morphotype, followed by the oval and then the fusiform morphotypes. The globose category included the large and small sporangia (Fig. 2b), often with the large ones showing a distorted outer wall at later digestion times. At the later incubation times (48 h and longer), many sporangia and especially the oval type were cleaved into smaller structures, possibly indicating zoospore formation (Fig. 2a).

TABLE 1. Ruminal sporangia on the leaf surface of Bermuda grass

Incubation (h)	Sporangia on surface (per site, avg ± SD)[a]	
	+ S and P	− S and P
6	0	0
24	1.2 ± 0.3	0.9 ± 0
48	10.0 ± 1.6	1.6 ± 1.5
72	8.2 ± 4.4	0.6 ± 0
96	4.8 ± 3.4	ND[b]

[a] Five sites for each of 12 leaf blades for two cows examined. S, Streptomycin; P, penicillin.
[b] ND, Not determined.

The total number of sporangia (not listed as different morphotypes) on the leaf surfaces is shown in Table 1. Incorporation of streptomycin and penicillin stimulated the development of sporangia from 24 to 96 h, and the highest numbers occurred at 48 h, as did those on the cut edge. Differences occurred in the localization of the sporangial morphotypes developing on leaf blades. The percentage of oval sporangia was greater on the leaf surface than on the cut edge (41 and 25%, respectively), while the globose sporangia were subsequently lower in percentage on the leaf surface than on the cut edge (51 and 67%, respectively); the fusiform morphotypes were fewer in number than the globose or oval types and similar for the two plant locations. Fungal colonies isolated directly from ruminal fluid onto cellobiose roll tubes consisted predominantly of two types, designated A and B, while a third, type D, was less commonly observed. (A colony initially designated as type C was, upon later inspection, included with the B type.) Examination of the agar surface within a few hours of inoculation showed early development of rhizoidal structures, sometimes with a young sporangium. Further development of the colonies isolated from ruminal fluid was symmetrical and indicative of growth of a single fungus.

Evaluation of the agar surface of the roll tubes at specific times showed variations in growth patterns of the three colony types. In type A, a prominent rhizomycelium without sporangia was evident at 48 h, and irregular rhizoids (i.e., with enlarged areas) were prevalent. SEM of the colony surface confirmed light microscopic observations of irregular rhizoids and lack of sporangia. TEM showed that the extensive rhizomycelium consisted of uneven filaments filled with dense cytoplasm (Fig. 4a). At 96 h and later, type A colonies had developed both oval and small globose sporangia in the rhizomycelium (Fig. 4b).

Type B colonies had large bodies that macroscopically gave a granular appearance by 48 h. Upon light microscopic observation, numerous large globose bodies resembling sporangia were associated within the rhizomycelium (Fig.

5a). Large and small globose sporangia were still present by 96 and 168 h. TEM revealed sporangia with a thick wall, packets with multiple flagella (Fig. 5b), and rhizoidal structures devoid of cytoplasm, indicating a fungus of a different nature or growth rate from type A.

Type D differed from types A and B by having a central cluster of round bodies (presumably sporangia), radiating rhizoids, and a much reduced rhizomycelium (Fig. 6a). Type D was a potent digester of MN 300 cellulose as shown by clearing zones around colonies (Fig. 6a). In individual type D colonies evaluated for several consecutive periods in roll tubes, motile zoospores originating from the colony center swam to nearby sites of undigested cellulose (Fig. 6b) and produced oval and globose sporangia (Fig. 6c) that then released another generation of zoospores to swim to other sites and begin new colonies. At times colonies had a concentric ring of sporangia around the colony origin. This monocentric type of life cycle was different from the more filamentous, polycentric nature of types A and B. TEM confirmed the reduced nature of the rhizomycelium in type D and often showed that bacteria, possibly methanogens (8), were near the rhizoids.

Colony types developing on specific media are shown in

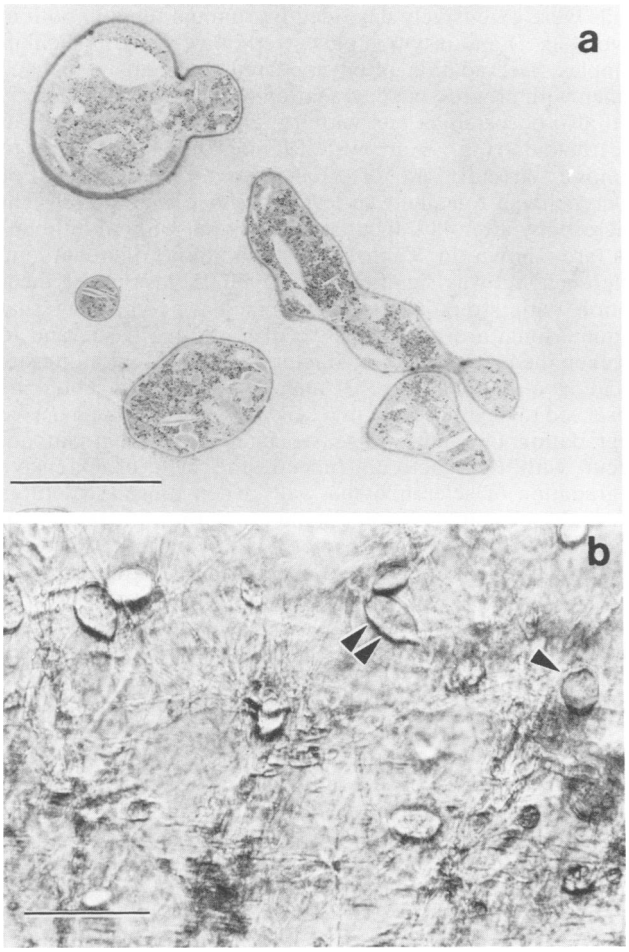

FIG. 4. Colony representing type A growth in cellobiose roll tube medium plus streptomycin and penicillin upon initial development from ruminal fluid. (a) Growth for 2 days. Irregular rhizoids with dense cytoplasm are present. Bar = 5 μm. (b) Growth for 4 days. Globose (arrow) and oval (double arrow) sporangia develop among the rhizomycelium. Bar = 100 μm.

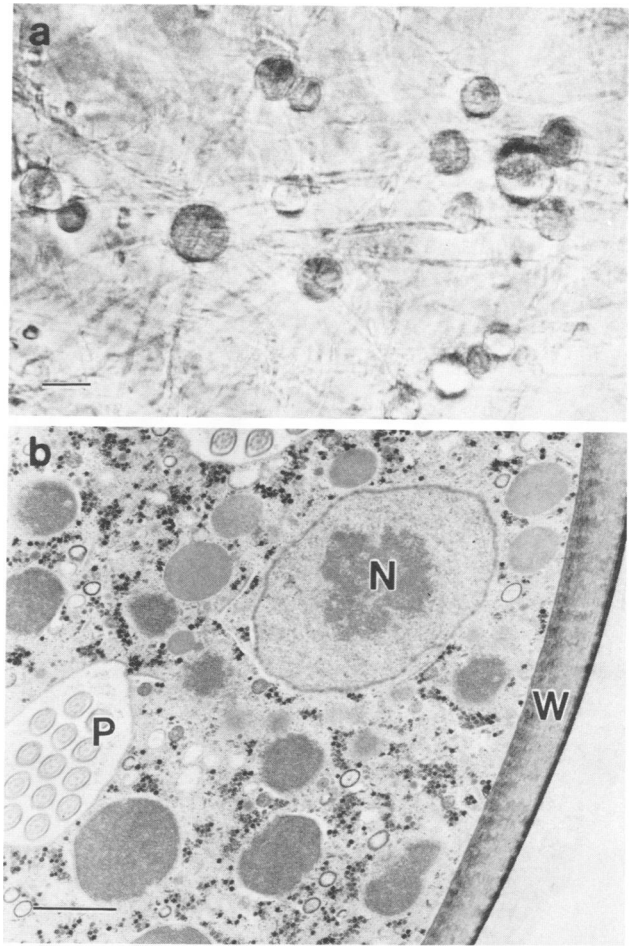

FIG. 5. Colonies representing type B growth in cellobiose roll tube medium plus streptomycin and penicillin upon initial development from ruminal fluid after 2 days of growth. (a) Light microscopy showing numerous large sporangia. Bar = 100 μm. (b) Portion of a sporangium with a thick cell wall (W), one of the many nuclei (N), and packets of flagella (P) indicating polyflagellated zoopores. Bar = 1 μm.

Table 2. Colonies with type A growth made up a large portion of the fungi developing on cellulose and xylan medium. With 1.5% cellulose (Table 2) distinguishable colony centers were not present in type A, and sporangia and rhizoids were present throughout the cleared zone. Zones of clearing were large, but translucent. Type D, the other significant colony type in cellulose medium, produced small, transparent, clearing zones (Fig. 6a). Type D colonies were

TABLE 2. Activity on cellulose and xylan roll tube media of fungal colony types initially isolated from ruminal fluid

Medium	Colony type	No. of colonies sampled	Colony diam (mm)	Zone of clearing (mm)[a]
Cellulose	A	11	9.4 ± 3.2	9.4 ± 3.2
	D	4	0.2 ± 0.2	1.4 ± 0.5
Xylan	A	9	4.8 ± 1.9	10.1 ± 4.8
	B	6	8.9 ± 5.0	13.4 ± 4.5

[a] Zone of clearing includes diameter of clearing plus colony within the clearing after 72 h of incubation.

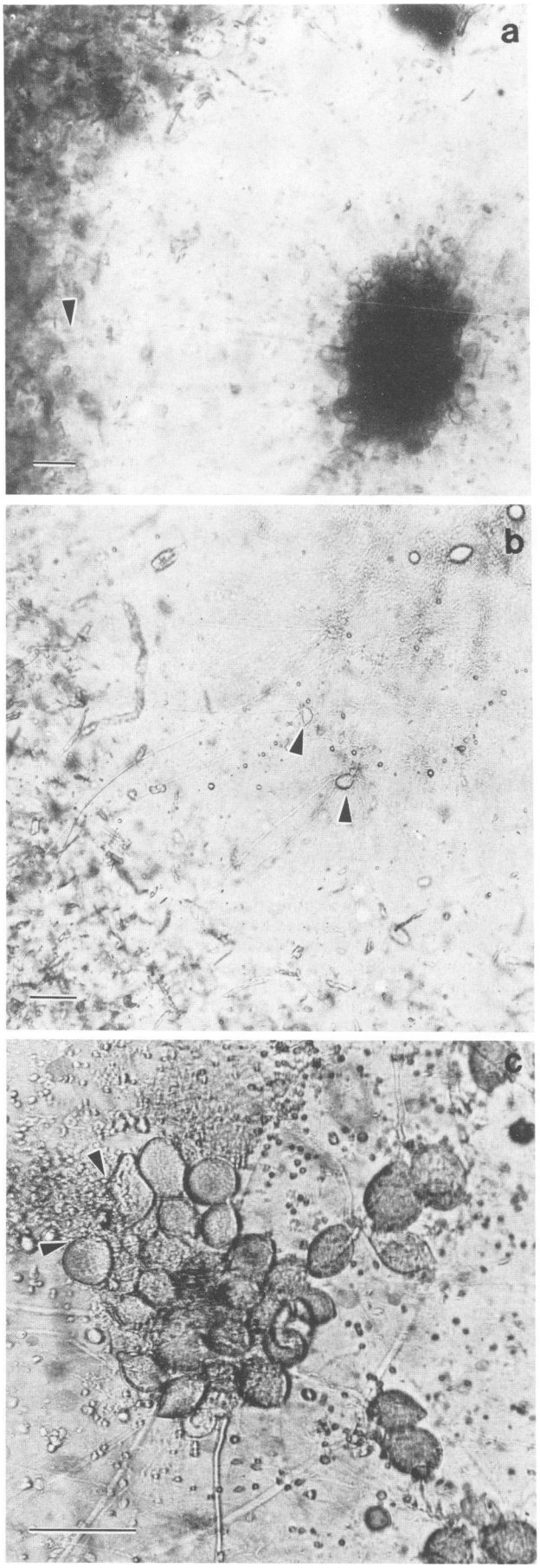

TABLE 3. Area of sclerenchyma remaining in leaf blades incubated with ruminal fungi (plus streptomycin and penicillin) or total rumen populations (no antibiotics) for 48 h

Inclusion of streptomycin and penicillin	Area (μm^2) of sclerenchyma tissue remaining in[a]:	
	Midvein	Second-order vein
−	1,542.7b	120.4b
+	355.8c	15.8c

[a] Area includes the total of abaxial and adaxial sclerenchyma from four leaf blades (no drug inclusion) and three blades (drugs included). Values within columns followed by different letters differ ($P = 0.05$) in the t test.

not prevalent on xylan medium and were found in only one of three inoculations. Characteristics typical of those for type B were not detected for colonies developing on 1.5 or 2.0% cellulose, but type B did develop well and produce clearing zones on xylan medium.

Lignocellulosic tissues were evaluated by TEM for degradation by particular microbial groups. Rhizoidal forms with and without cytoplasm as observed in colony types A, B, and D were near lignified cell walls undergoing degradation. A particular rhizoidal structure was not consistently associated with degradation of a specific tissue. Sclerenchyma cell walls were extensively degraded by ruminal fungi in both in vivo (Fig. 7) and in vitro (plus streptomycin and penicillin) samples. Degradation often appeared to begin in the cell lumen with progressive degradation of the plant wall (Fig. 7) and also occurred as pits within the wall at anchor sites of the rhizoids (Fig. 7, arrows). Ruminal fungi were able to remove virtually all of the cell wall. The areas of sclerenchyma remaining in leaf sections examined by light microscopy after 48-h incubation with and without antibiotics are shown in Table 3. The amount of remaining sclerenchyma was significantly ($P = 0.05$) less in the incubation with streptomycin and penicillin, confirming that ruminal fungi are better able to degrade this tissue and to weaken the plant structure. Bacteria were present at the site in in vivo samples (Fig. 7) and, while bacteria could be observed to degrade most of a cell wall at times, the massive degradation of plant walls associated with fungi did not occur with the bacteria. Indeed, the lack of extensive degradation of sclerenchyma walls when fungal structures were absent and the requirement for direct attachment to sclerenchyma by ruminal bacteria before degradation verified that ruminal fungi were responsible for the massive destruction of the sclerenchyma.

The mestome sheath was partially degraded at times by ruminal fungi (Fig. 8). Pitting and splits occurred in the plant wall and occasionally rhizoidal structures penetrated the entire wall (Fig. 8, inset). The mestome sheath appeared to be less degraded than the sclerenchyma based on the number of cells colonized and the degree of cell wall removal. Bacteria were not observed to colonize or degrade the mestome wall even after extended times (Fig. 9).

In vitro studies carried out without streptomycin and penicillin indicated that fungi and bacteria both were associated with the breakdown of sclerenchyma (not shown).

FIG. 6. Light microscopy of type D colony in cellulose roll tube. (a) Round bodies clustered in center surrounded by radiating rhizoids to the end of the zone of clearing (arrow). (b) Development of rhizoidal structure representing monocentric growth (arrows). (c) Release of zoospores from globose and oval sporangia (arrows). Bar = 100 μm.

Bacteria resembling the ruminoccocci or *Bacteroides succinogenes* (15) colonized the periphery of the sclerenchyma and caused pits and erosion zones in the cell wall, while fungal-like structures were more often associated with the major portion of the tissue resulting in extensive clearing and cell wall removal.

Few bacteria were observed by electron microscopy in in vitro inocula containing streptomycin and penicillin, but the number of fungal structures was markedly increased. Fungi penetrated the cuticle occasionally, possibly taking advantage of a rupture in the layer, and thus showed the potential to invade tissue in this manner (Fig. 10). Fungal growth and development were prevalent in the in vitro system and within plant tissue, as shown by developing sporangia and the formation of sporangia typical for those seen in type B colonies.

A variety of phenolic compounds representing different families at 1 and 10 mM concentrations were tested for their effect on ruminal fungi (Table 4). At the 1 mM level, dry-weight losses were not significantly affected ($P = 0.05$) by any of the phenolic compounds. However, several compounds tended to increase dry-matter loss, with syringaldehyde, *p*-hydroxybenzoic acid, HCA, and PAA giving increases over controls of 6.3, 8.6, 5.6, and 4.0 percentage units, respectively. At 10 mM concentrations, decreases in dry-weight loss occurred with the cinnamic acids and benzoic aldehydes. The lowest value (11.4%) for dry-weight loss occurred with PCA. None of the compounds at the 10 mM level resulted in dry-weight losses higher than the control. The addition of 1 mM concentrations of the phenolic compounds resulted in no significant differences ($P = 0.05$) from controls in total numbers of sporangia (Table 3) or in individual sporangial morphotypes (not shown) per millimeter of leaf cut edge. The nonsignificant increase of 27% with ferulic acid was due to a significant ($P = 0.05$) increase of oval sporangial morphotypes. PCA resulted in significant reductions in oval, globose, and fusiform types. At the 10 mM levels, the cinnamic acids and aldehydes of each phenolic family plus vanillic acid and HCA significantly ($P = 0.05$) reduced sporangial numbers on the cut edge. The fewest numbers of sporangia per millimeter of cut edge occurred with sinapic acid (0.1), vanillin (1.0), PCA (0.6), and *p*-hydroxybenzaldehyde (1.5). No phenolic compounds resulted in an increase in sporangia (Table 4) at the 10 mM concentration. At the 10 mM levels, the cinnamic acids sinapic, ferulic, and PCA and aldehydes syringaldehyde, vanillin, and *p*-hydroxybenzaldehyde were consistently influential in reducing dry-weight loss and sporangial numbers. *p*-Hydroxybenzoic acid and PAA were consistently the least inhibitory compounds tested.

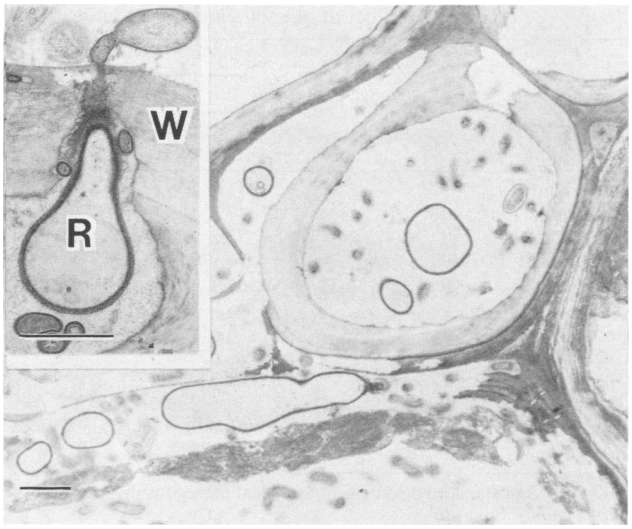

FIG. 8. TEM of microbial degradation of mestome sheath cell walls removed directly from the rumen. Splitting and zones of erosion occurred next to rhizoidal structures devoid of cytoplasm. The inset shows complete penetration of a mestome cell wall (W) by rhizoids (R). Bar = 1 μm.

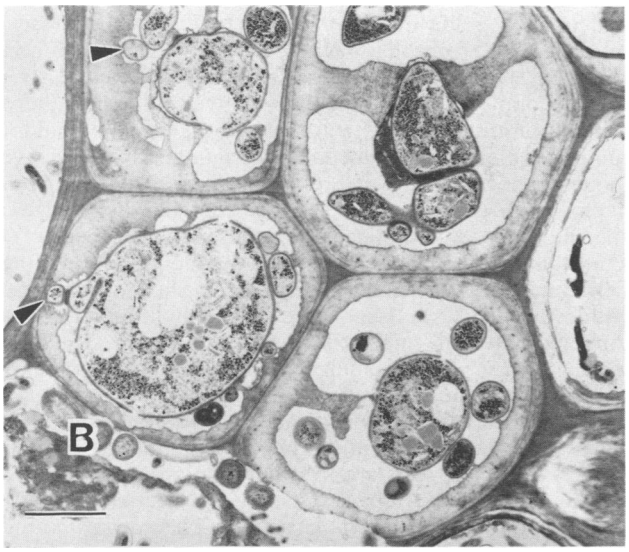

FIG. 7. TEM of microbial degradation of sclerenchyma tissue removed directly from the rumen. Structures consistent with those of fungal rhizoids were associated with sclerenchyma and resulted in massive clearing of plant cell walls. Zones of cell wall clearing occur around rhizoids (arrows). Bacteria (B) were outside the area of major degradation, being found mostly at the periphery of the tissue. Bar = 2 μm.

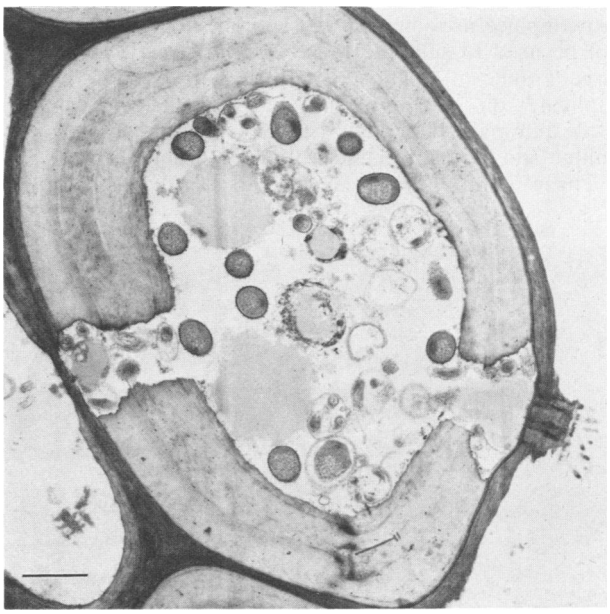

FIG. 9. TEM of leaf blade incubated for 96 h in vitro without antibiotics, showing the presence of bacteria but no plant cell wall degradation. Pit fields are present. Bar = 1 μm.

TABLE 4. Effect of phenolic monomers on dry-weight loss and number of sporangia attached to leaf cut edges[a]

Phenolic monomer	Concn			
	1 mM		10 mM	
	% Dry-wt loss	Sporangia per cut edge	% Dry-wt loss	Sporangia per cut edge
None (control)	51.0 ± 5.6	30.6 ± 12.9	51.0 ± 5.6	30.6 ± 12.9
Sinapic acid	47.2 ± 7.6	26.3 ± 6.7	17.7 ± 13.4[b]	0.1 ± 0.1[b]
Syringaldehyde	57.3 ± 0.7	24.7 ± 5.1	25.5 ± 3.7[b]	0.7 ± 0.6[b]
Syringic acid	54.9 ± 2.3	27.9 ± 18.0	38.2 ± 3.2[b]	17.8 ± 7.0
Ferulic acid	53.1 ± 6.4	39.0 ± 12.3	15.2 ± 13.0[b]	3.7 ± 1.8[b]
Vanillin	46.9 ± 9.1	32.7 ± 17.4	12.8 ± 9.8[b]	1.0 ± 1.5[b]
Vanillic acid	49.1 ± 2.7	31.6 ± 10.9	44.7 ± 10.6	24.7 ± 5.1[b]
PCA	33.0 ± 12.8	23.2 ± 6.5	11.4 ± 9.6[b]	0.6 ± 0.7[b]
p-Hydroxybenzaldehyde	53.8 ± 9.4	28.9 ± 15.5	14.5 ± 3.9[b]	1.5 ± 1.4[b]
p-Hydroxybenzoic acid	59.6 ± 9.5	29.6 ± 21.5	47.9 ± 5.1	18.3 ± 10.2
HCA	56.6 ± 0.2	33.3 ± 6.0	50.3 ± 6.1	9.1 ± 4.5[b]
PAA	55.0 ± 3.2	19.8 ± 4.0	49.8 ± 5.2	18.0 ± 7.4

[a] Average and standard deviation of three leaf blades for dry-weight loss and six blades for sporangia counts. Leaf blades were incubated with streptomycin and penicillin.

[b] Value differs from control ($P = 0.05$), using the t test.

DISCUSSION

Incorporation of streptomycin and penicillin into ruminal fluid inocula or roll tube media suppressed bacterial growth and markedly increased fungal colonization of leaf blades as has been shown earlier (3). Evaluation of the various morphotypes provided some measure of variability and indicated optimal time of development and variation in sites of colonization in this in vitro system. Sporangial numbers on cut edges or surfaces of Bermuda grass leaf blades have been shown to relate closely to the material solubilized with chitinase (unpublished data). However, sporangial morphology does not provide an absolute characterization of different fungi. Orpin (20, 23) reported that sporangial morphology varied from oval to cylindrical to conical in *N. frontalis* and from cylindrical to oval in *P. communis*. Bauchop (6) reported variations in sporangial size due to age. The consistent finding of both small (<60 μm) globose and oval sporangia on rhizoids of colonies exhibiting type A and D growth patterns indicated that sporangial morphology could not be used to separate these colony types. However, the large (>60 μm) globose sporangia observed in pairs or triplicates on a single rhizoid and without any evidence of oval sporangia indicated that type B represented a type unique and distinguishable from type A and D.

These colony types were consistently isolated from ruminal fluid over several experiments and appeared to represent single fungal colonies by symmetry of colonial growth, reproducible isolation, and purity upon observation of subsequent cultures. Absolute colony purity (e.g., by growth from single zoospores) must be established to verify that each colony type represents a single fungus. Type D had a growth pattern on cellulose medium typical for other anaerobic fungi named *N. frontalis* (see Fig. 6 of reference 24). Fungi represented by types A and B grew in a polycentric form, which was distinctly different from the monocentric growth pattern of type D even though growth conditions were identical.

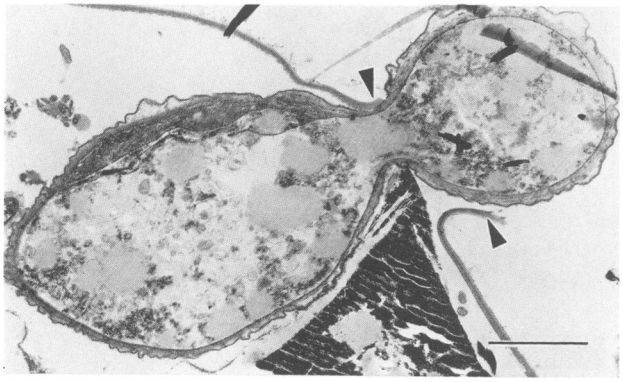

FIG. 10. TEM of leaf blade incubated for 48 h in vitro with streptomycin and penicillin, showing penetration of fungi through the cuticle (arrow). Bar = 2 μm.

Our data indicated that the fungal population was suppressed by the bacteria in the in vitro system and suggested that their activity may be suppressed inside the animal. The fungi, in the absence of bacteria, were potentially able to degrade quantitatively almost as much fiber as the whole population. However, microscopic evaluation has revealed that fungi degrade different tissues (e.g., lignocellulose in sclerenchyma) from that degraded by the bacteria. In other studies (32) fungal populations (i.e., ruminal fluid plus streptomycin and penicillin) have been shown to degrade fiber to a significantly lesser extent than whole ruminal fluid or ruminal bacteria. The reduced dry-weight loss by fungi, even though lignocellulose was broken down to a greater extent, likely occurred because relatively few fungi colonize at specific sites (e.g., lignocellulosic tissue) and secrete enzymes that act on plant cell walls in the vicinity of the fungi. Other sites without fungal colonization show no digestion with even the fragile tissues (e.g., mesophyll) intact. Therefore, increased dry-matter loss would occur in systems in which enough fungi colonized leaf blade to degrade the lignocellulose and nearby fragile tissues.

Our studies have shown further that mestome and xylem cells, which histologically appear to contain coniferyl groups, are more resistant to ruminal microorganisms than are sclerenchyma walls (2). Other research (11) on the lignin-degrading fungus *Phanerochaete chrysosporium* indicated that coniferyl lignin was more resistant than syringyl lignin to degradation. Our studies indicated that the degradative action on lignocellulose appeared to occur by the action of secreted cell wall-degrading enzymes, which caused massive clearing of sclerenchyma cell walls, and by the ability of extremely fine fungal rhizoids to penetrate, split, and weaken cell walls more resistant to microbial

degradation. The present study, while confirming the greater resistance to degradation of mestome cells, did show an important feature of ruminal fungi in that they could attack and strategically weaken the most resistant tissues. Such characteristics may be important in weakening digesta or reducing plant particle size, thus having a positive influence on forage quality by improving particle flow through the digestive system and increasing consumption of fiber by ruminants (4, 27).

The propensity for fungal colonization of lignin-containing tissues by ruminal fungi suggests that these microorganisms might be more tolerant of phenolic compounds known to be toxic to ruminal bacteria (9) and even suggests that a positive influence could be exerted by phenolic compounds. HCA and PAA were included in this study because of their close structural relationship to phenolic compounds in cell walls and for their reported ability to stimulate cellulolysis by ruminal bacteria (28, 29) and growth of aerobic fungi (12). Even at concentrations of 1 mM, phenolic compounds were not stimulatory to a significant degree for dry-weight loss or sporangial colonization of the cut edge. Indeed the general toxic nature of phenolic acids and aldehydes at 10 mM levels to ruminal bacteria (9) was confirmed for ruminal fungi also. Apparently, the fungi are protected against the cell wall phenolic compounds with which they come into contact during colonization and degradation. Possibly, the compounds are polymerized or linked to other carbohydrates (13, 17) such that their toxicity is diminished, but other work is needed to clarify the role of phenolic compounds in the interaction of ruminal fungi and lignified cell walls.

ACKNOWLEDGMENTS

We thank D. Smith for technical assistance and M. S. Fuller for suggestions on the fungal descriptions.

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