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Botanical fractions of rice straw colonized by white-rot fungi: changes in chemical composition and structure

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Abstract

Three species of white-rot fungi (*Cyathus stercoreus* (Cs) ATCC-36910, *Phanerochaete chrysosporium* (Pc) BKM, and *Pleurotus sajor-caju* (Ps) 537) were grown on leaf blade (leaf) or stem plus leaf sheath (stem) of rice straw for 30 d by solid state fermentation (SSF). Physical and chemical methods were employed to evaluate substrate specificity, substrate composition and histology. Changes in histology of decayed material were evaluated before and after ruminal digestion by scanning electron microscopy (SEM) and transmission electron microscopy (TEM). Control leaf and stem were similar in IVDMD (38%), although leaf was higher in crude protein and lower in cell wall compared to stem (3.7 vs. 2.8%; 73.9 vs. 80.7%, respectively). The changes were due mostly to a higher concentration of silica in leaf compared to stem (17.0 vs. 13.1%). After 30 d of SSF, Cs and Ps increased the IVDMD of leaf from 38.1 to 49 and 46.3%, respectively, by selective degradation of hemicellulose as opposed to cellulose. In contrast, Pc degraded cellulose and hemicellulose indiscriminately in leaf and lowered the IVDMD of leaf to 30.1%. Partially degraded lignin, silica and hemicellulose of leaf were negatively correlated (r) with IVDMD in contrast to cellulose ($r = -0.49$, -0.54, -0.16 and 0.85, respectively). Prediction of IVDMD of fungal-decayed leaf was primarily a function of hemicellulose and cellulose with a coefficient of IVDMD = $-0.155 + 2.14$ (cellulose) $- 0.87$ (hemicellulose);

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$R^2 = 0.98$. Stem decayed by Pc and Cs became less digestible compared to the control (18.5 and 20.3% vs. 39.7%, respectively), although hemicellulose and cellulose of stem were poorly degraded after SSF. Only Ps improved the IVDMD of stem compared to the control (44.1 vs. 39.7%). SEM sections of leaf decayed by Pc showed complete degradation of mesophyll but the more recalcitrant vascular and epidermal tissues resisted rumen degradation and resulted in lower IVDMD. Leaf tissues colonized by Cs and Ps showed presence of all tissues but after 72 h rumen microorganisms completely degraded mesophyll tissue which resulted in a higher IVDMD. Observation of TEM sections showed that fungal treatment facilitated rumen microbial penetration of lignified tissues. Improvement of digestibility of decayed straw depends upon the fungal species, the plant substrates and the botanical fractions.

Keywords: Rice straw; Fungi; Chemical composition; Chemical structure

1. Introduction

Agricultural crop residues, especially cereal straws, contribute a major part of the diet of ruminants in developing countries (Jackson, 1981). This contribution is mainly due to surplus production and their local availability. In contrast, disposal of crop residues is a major problem in industrialized countries because of possible environmental pollution (Oh et al., 1971). Crop residues are lignocellulosic material rich in energy, low in crude protein and poor in palatability. Ruminal microbial utilization of energy-rich cell walls of crop residues is hindered by the presence of nonpolysaccharide compounds such as lignin, phenolic acids and silica in some cereal straws (Besle et al., 1994). Extensive research has been done in the past to understand the role of lignin and phenolic acids in cell wall utilization by rumen microorganisms, the exact mechanism has yet to be fully elucidated.

Research has sought to improve the nutritive value of poor quality crop residues by physical, chemical and enzymatic pre-treatments (Jackson, 1977; Barl et al., 1991). Chemical treatment of poor quality roughages has been extensively studied and well documented (Jackson, 1977; Fahey et al., 1993). However, a potential environmental problem exists in long term usage of chemicals. Chemical pretreatment is expensive, corrosive and possibly toxic to workers. As an alternative to chemical treatments, the use of lignolytic white-rot fungi (WRF) for converting lignocellulosic materials to more digestible feedstuffs has been investigated. Solid state fermentation (SSF) of poor quality crop residues treated with WRF has often been shown to improve in vitro dry matter digestibility (IVDMD) of the decayed material (Zadrazil, 1977; Agosin et al., 1987). Many species of WRF have been screened on a variety of lignocellulosic substrates for their ability to improve the nutritional value of poor quality crop residues for use as a ruminant feedstuff (Zadrazil, 1984; Reid, 1989; Fahey et al., 1993). Of all substrates, biological treatment of wheat straw has been the most widely studied crop residue. However, the SSF of WRF treated crop residues has not always resulted in improvement of IVDMD, and research on these fungi and their substrates is needed.

Our initial studies demonstrated that the nutritional value of rice straw and corn stalks, the world's most abundant crop residues, could be substantially improved with the white rot fungus, *Cyathus stercoreus* (Karunanandaa et al., 1992). The mechanism(s)

for the improvement in the quality of fungal treated material remains unclear. Other studies, including ours, indicated selective degradation of substrates by WRF (Rolz et al., 1986). As a result, successes as well as failures have been reported in terms of improvement in the quality of crop residues by WRF. Rice straw differs considerably from other cereal straws such as barley, wheat and oat in that it has a very high proportion of leaf: stem (60 vs. 40%) (Theander and Åman, 1984), and the leaf and stem are similar in digestibility (Walli et al., 1988). Fungus-substrate (whole plants) specificity has been widely reported however, no information is available on fungus specificity for plant parts.

Screening of WRF for their ability to improve the quality of crop residues has been limited to chemical methods. Structural methods, including scanning electron microscopy (SEM) and transmission electron microscopy (TEM), are effective in evaluating digestion of specific cell types and have increased our understanding of the contribution of plant and ruminal microbial factors in digestion of forages (Akin, 1989). The objectives of the present study were firstly to investigate the effect of different fungal species on botanical parts of rice straw for improved digestibility and secondly to determine chemical and structural alterations responsible for the improvement.

2. Materials and methods

2.1. Sample preparation

Rice straw used in this experiment was baled and transported from Louisiana in 1992. Rice straw was hand-separated into leaf blade and stem plus sheath. Since Cherney et al. (1983) reported similar rates of digestion for cell wall of leaf sheath and stem for barley and oat straw, in the present study rice leaf sheath was considered part of the stem. Both leaf and stem fractions of rice straw were ground through a Wiley mill using a 9 mm screen prior to SSF. Representative samples of intact leaf blade and stem fractions were saved for microscopic evaluation.

2.2. Rye spawn

Rye spawn was used as the inoculum to introduce the WRF to the substrates. Rye spawn was defined as a pure vegetative growth of the fungus on sterilized rye (*Secale cereale* L) grain supplemented with hardwood sawdust. Approximately 90 g rye and 15 g hardwood sawdust were placed in a 500 ml flask with 1 g $CaSO_4$ and 120 ml tap water. The material was autoclaved at 121°C for 45 minutes, cooled overnight and inoculated with two agar plugs of mycelium per flask. For a detailed procedure see Karunanandaa et al. (1992). Strains of WRF used in this study are presented in Table 1.

2.3. Solid state fermentation (SSF)

Fifty grams (air dried) of each substrate was placed in 1L Erlenmeyer flasks with 150 ml tap water. The flasks were stoppered with cotton plugs to allow air exchange. Flasks

Table 1
Species, strain number, abbreviation and source of fungi used in this study

Strain number	Species	Abbreviation	Source
BKM-1787	*Phanerochaete chrysosporium*	Pc	Ming Tien, Department of biochemistry, The Pennsylvania State University, PA, USA
6910	*Cyathus stercoreus*	Cs	American Type Culture Collection, 12301 Park Lawn Drive, Rockville, MD, USA
537	*Pleurotus sajor-caju*	Ps	Daniel J Royse, Department of plant pathology, The Pennsylvania State University, PA, USA.

were autoclaved at 121 °C for 35 min, cooled overnight, and inoculated with the rye spawn. Controls were identical samples that were not inoculated with rye spawn. After a 30-day incubation, contents were transferred quantitatively into a tarred paper bag and oven-dried at 40 °C for 48 h. Oven dried samples were weighed and ground through a 1 mm sieve using a laboratory UDY cyclone mill (Ft. Collins, CO, USA).

Chemical analyses were performed on ground samples and reported as a percent of dry matter of the decayed sample. Neutral detergent fiber (NDF), acid detergent fiber (ADF), acid detergent lignin (ADL), and cellulose were determined according to procedures by Goering and Van Soest (1970). Hemicellulose was calculated as the difference between NDF and ADF. Silica was determined as ash remaining after ADF ash was leached with 48% HBr for one and a half hours (Goering and Van Soest, 1970). Neutral detergent solubles were calculated as the percentage of organic matter minus NDF. IVDMD was determined as a 48 h incubation followed by a 24 h acid pepsin digestion as described by Marten and Barnes (1979).

The experimental design used in this study was a completely randomized design with 2×4 factorial arrangement of treatments with three replicates. The two factors were morphological fractions (leaf and stem) of rice straw and fungi (three species of WRF (listed in Table 1) and the control). Data were analyzed as a two factorial treatment by using the general linear model (GLM) procedure of SAS (1990). The major sources of variation were due to treatment effects of botanical fractions and fungi. Effects were considered different based on a significant ($P < .05$) F ratio. Treatments means were separated using Duncan's multiple-range test at the 5% level of probability.

2.4. Gas sterilization and sample preparation for microscopic study

Leaves and stems were cut into 1–2 mm pieces before SSF. To prevent any unwanted microbial growth on leaf and stem, samples were gas sterilized as follows: leaf and stem samples were placed separately into two plastic containers and soaked over night with tap water and the excess water was poured off from the containers after 24 h-soaking. This was done to maintain the moisture content of samples around 70–80% prior to SSF. Then the plastic containers were wrapped with a plastic sheet including vials of ethylene oxide (processed according to product instructions; Anprolene, H.W. Anderson Products, Inc., Chapel Hill, NC 27514) and placed in a gas sterilizer for overnight. The following morning, samples were transferred into a 9 cm petridish containing a water

agar medium. The experimental design used in the microscopy study was similar to the earlier experiment described in this paper. Samples were inoculated with fungi (5 rye spawns per petridish) and the petridishes were sealed with parafilm to prevent any contamination of samples. Samples were incubated for 30 d at room temperature (25 °C). At the end of the incubation period, part of the samples were randomly selected and transferred into small bottles containing the glutaraldehyde fixative solution. The fixative contained: 4% (v/v) glutaraldehyde in a sodium cacodylate (.1 M) solution and the final pH was 7.2. The rest of the samples were incubated in the rumen by suspending in nylon bags (20 pieces per bag). Nylon bags were incubated in the rumen for 24, 48, 72, and 96 h. At the end of incubation, bags were hand-washed in cold water and the samples were placed in fixative for further microscopic studies. Samples were prepared for SEM as described by Akin et al. (1983b) and for TEM as described by Akin et al. (1983a).

3. Results

3.1. Chemical composition of fungal decayed material and IVDMD

Chemical composition of control rice leaf and stem (Table 2) revealed that rice leaf had higher concentrations of crude protein, silica, neutral detergent solubles and lower cell wall (NDF) content compared to stem (3.7 vs. 2.8%; 17.0 vs. 13.1%; 9.7 vs. 3.1%; 73.9 vs. 80.7%, respectively). The higher cell wall content found in rice stem compared to leaf was mainly due to the higher concentration of cellulose (43.6 vs 32.7%) in stem compared to leaf. However, the concentration of hemicellulose and lignin were similar

Table 2
Chemical composition of rice leaf and stem after 30 d of solid state fermentation with *Phanerochaete chrysosporium* (Pc), *Cyathus stercoreus* (Cs), and *Pleurotus sajor-caju* (Ps)

	Rice Leaf				Rice Stem				
Variables	Pc	Cs	Ps	Control	Pc	Cs	Ps	Control	SEM
% of Dry Matter									
Organic matter	66.0 [f]	69.5 [c]	69.4 [c]	73.4 [a]	73.8 [a]	74.6 [b]	72.3 [d]	76.1 [a]	0.21
Crude protein	5.1 [b]	5.1 [b]	4.9 [c]	3.7 [e]	3.4 [fg]	3.5 [f]	3.8 [b]	2.8 [h]	0.17
NDF [1]	58.5 [g]	63.0 [f]	63.0 [f]	73.9 [a]	76.3 [a]	78.6 [b]	68.0 [e]	80.7 [a]	0.46
Ash free NDF	42.8 [g]	52.5 [f]	51.1 [f]	63.7 [c]	69.6 [a]	71.0 [b]	61.3 [e]	73.0 [a]	0.48
NDS [3]	23.2 [a]	17.0 [c]	18.3 [b]	9.7 [e]	4.3 [f]	3.6 [fg]	10.9 [d]	3.1 [g]	0.38
ADF [2]	50.2 [f]	54.0 [c]	54.0 [c]	55.0 [b]	58.3 [a]	61.4 [a]	58.7 [b]	61.1 [a]	0.41
Hemicellulose	8.3 [g]	9.1 [fg]	9.0 [fg]	18.9 [a]	18.1 [bc]	17.2 [c]	9.3 [d]	19.5 [a]	0.28
Cellulose	24.7 [g]	33.5 [d]	32.8 [d]	32.7 [d]	38.6 [c]	41.5 [b]	42.6 [ab]	43.6 [a]	0.46
ADL [4]	3.5 [c]	2.5 [d]	2.6 [d]	5.6 [b]	6.2 [a]	6.5 [a]	2.7 [d]	5.4 [b]	0.18
Ash	27.4 [a]	23.6 [b]	23.8 [b]	20.5 [c]	19.9 [c]	19.0 [f]	21.1 [e]	17.9 [g]	0.18
Silica	22.7 [a]	18.8 [c]	19.4 [b]	17.0 [d]	13.9 [f]	13.5 [fg]	15.0 [e]	13.1 [g]	0.18

[1] NDF, Neutral detergent fiber. [2] ADF, Acid detergent fiber. [3] NDS, Neutral detergent solubles; NDS = (%OM − %ash free NDF). [4] ADL, Acid detergent lignin. [a,b,c,d,e,f,g], Different letters in rows indicate significant differences ($P < .05$).

Table 3
Losses of organic matter (OM), and cell wall constituents and in vitro dry matter digestibility (IVDMD) of rice leaf and rice stem following 30 d of SSF with *Phanerochaete chrysosporium* (Pc), *Cyathus stercoreus* (Cs), *Pleurotus sajor-caju* (Ps)

	Rice Leaf				Rice Stem				SEM
Variables	Pc	Cs	Ps	Control	Pc	Cs	Ps	Control	
Losses in:	% of Initial Cell Wall Constituents								
OM	25.0 ^{a}	10.0 ^{c}	10.3 ^{c}	–	8.9 ^{d}	5.3 ^{e}	11.9 ^{b}	–	0.20
Hemicellulose	56.1 ^{a}	52.1 ^{a}	52.2 ^{a}	–	7.5 ^{b}	11.8 ^{b}	52.4 ^{a}	–	1.5
Cellulose	24.5 ^{a}	ND	2.1 ^{d}	–	11.4 ^{b}	4.7 ^{c}	2.4 ^{cd}	–	1.0
ADL [1]	37.9 ^{c}	54.7 ^{a}	53.6 ^{a}	–	ND	ND	48.1 ^{b}	–	1.8
IVDMD (% DM)	30.1 ^{d}	49.0 ^{a}	46.3 ^{ab}	38.1 ^{c}	18.5 ^{e}	20.3 ^{e}	44.1 ^{b}	39.7 ^{c}	0.90

[1] ADL, Acid detergent lignin. ^{a,b,c,d} Different letters in rows indicate significant differences ($P < .05$). ND, Not detectable.

in leaf and stem (19.0 and 5.5%, respectively). Since the rice straw was rich in ash, mainly silica, ash-free NDF was determined on decayed samples. Values for NDF were reduced by about 7–10 percentage units, but the relative difference between treatments remained similar.

Chemical composition of fungal decayed material is reported in Table 2. Fungal treatment in general significantly reduced the concentration of cell wall components. The silica content of fungal decayed material increased compared to the control indicating that WRF were unable to remove this element during decay. During the process of fungal growth on substrates, structural carbohydrates are converted into cell solubles for fungal metabolism. In this study, cell solubles of fungal decayed rice leaf were increased by two fold compared to the control leaf indicating successful growth of all three fungi on rice leaf. However in rice stem, except with Ps, the difference in concentration of cell solubles of fungal decayed stem was not high, but statistically significant, from the control stem, suggesting that Pc and Cs failed to grow on the stem fraction. The organic matter content of fungal-decayed leaf is significantly lower compared to the control and ranged from 66 to 69.4%.

Fungal selection of botanical parts of rice straw was determined based on the ability of the fungi to consume organic matter from the substrate during the 30 d of SSF. Organic matter (OM) loss was closely related to the appearance of fungal mass on the substrate at the end of 30 d of SSF. Organic matter loss and IVDMD of fungal decayed material after 30 d of SSF are presented in Table 3. The value for OM loss ranged from 5 to 25%, the lowest loss of OM was in stem decayed by *Cyathus stercoreus* (Cs) while the highest value was for the leaf fraction decayed by *Phanerochaete chrysosporium* (Pc). Pc and Cs preferentially colonized rice leaf compared to stem, while *Pleurotus sajor-caju* (Ps) grew equally well on leaf and stem. Losses in organic matter by fungi during the 30 d of SSF were primarily due to the consumption of carbohydrates which were mostly associated with the cell wall. Therefore, OM loss included the losses in hemicellulose, cellulose and lignin (Table 3). Almost half the amount of total lignin found in rice leaf was degraded by all three fungi during the 30 d of SSF. In contrast, lignin found in stem was degraded only by Ps while the lignin degradation by the other

Table 4
Correlation coefficients (r) of rice straw components and in vitro dry matter digestibility

Variables	d_f	Rice leaf		Rice stem	
		r	$P <$	r	$P <$
Silica	11	0.54	.07	+0.32	0.30
Lignin	11	0.49	.10	−0.82	0.01
Hemicellulose	11	0.16	.62	−0.53	0.08
Cellulose	11	−0.87	.01	+0.59	0.04
Neutral detergent solubles	11	0.28	.37	+0.73	0.01

The regression equation for leaf digestion coefficient: $y = -0.15 + 2.14x_1 - 0.87x_2$, where x_1 = cellulose, and x_2 = hemicellulose, ($R^2 = 0.98$, $n = 12$). The regression equation for stem digestion coefficient: $y = -0.50 + 2.52x_1 - 4.55x_2$, where x_1 = cellulose, and x_2 = lignin ($R^2 = 0.83$, $n = 12$).

two fungi was not detected. A similar pattern to lignin degradation was found for hemicellulose of rice leaf where all three fungi degraded hemicellulose of rice leaf (average of 53% of the total hemicellulose). However, hemicellulose found in stem was degraded only by Ps, while the other two fungi degraded insignificant amounts of hemicellulose found in stem. Extensive degradation of cell wall components of rice leaf by Pc and Cs, but the lack of breakdown of the same structural components in stem, indicates that the chemical organization within the rice leaf is available to these two fungi. The mechanism for the disparity in growth of the fungi between leaf and stem is not known. Within rice leaf, Pc indiscriminately degraded a greater proportion of hemicellulose and cellulose while the other two fungi selectively degraded hemicellulose and left the major cell wall carbohydrate, cellulose, intact.

Rice leaf and stem were found to be similar in IVDMD (Table 3). The similar IVDMD for leaf and stem found in this study was mainly due to the higher concentration of silica found in leaf compared to stem. After 30 d of SSF, IVDMD of leaf and stem ranged from 18.5 to 49%. The highest value for IVDMD was found in leaf decayed by Cs, and the lowest value was in stem decayed by Pc. Within leaf, Cs and Ps improved the IVDMD equally, in contrast to stem digestibility, which was only improved by Ps, suggesting fungal specificity for the different botanical fractions.

The correlation coefficients (r) of IVDMD and cell wall components of rice straw are reported in Table 4. The r values were determined individually for leaf and stem because of the observed differences in the growth of fungus on leaf and stem; the mechanism especially for the inhibition of IVDMD of stem decayed by Cs and Pc is not known. Within leaf, as expected, a negative relationship for lignin and silica were shown with IVDMD ($P < 0.1$). However these two components failed to explain the major part of the variation found in IVDMD of the leaf fraction. The regression model, with stepwise option, selected hemicellulose and cellulose as the two variables to predict IVDMD of fungal decayed rice leaf with an R^2 of .98. In the prediction equation for IVDMD of leaf (Table 4), hemicellulose and cellulose were negatively and positively correlated with IVDMD, respectively. A negative relationship of hemicellulose with IVDMD may suggest that the remaining hemicellulose, after fungal degradation, became more recalcitrant and resisted ruminal microbial degradation.

3.2. Microscopy of fungal decayed material

Microscopic evaluation of tissues focused primarily on rice leaf, because the chemical analyses of fungal decayed substrates revealed that most of the improvement in IVDMD was from the rice leaf fraction. Control tissues as well as fungal-decayed tissues were evaluated before and after ruminal digestion by scanning electron microscopy (SEM) and transmission electron microscopy (TEM). Five randomly selected samples per treatment of fungal decayed leaves and stems were evaluated by SEM and TEM. Microscopic evaluation of stem was limited to control tissues and stem decayed by Ps.

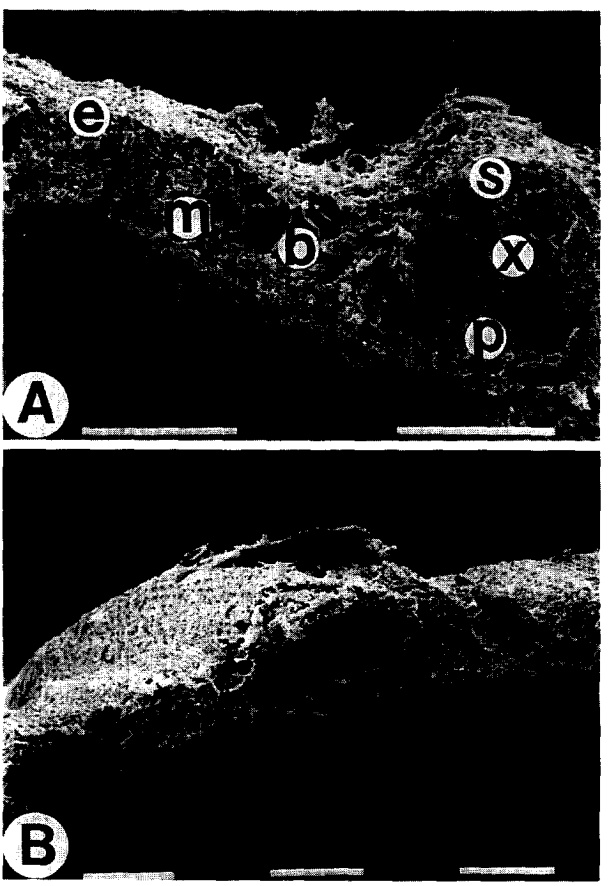

Fig. 1. Scanning electron micrographs of rice leaf blade cross sections. (A) Control leaf not incubated with rumen fluid. Tissues present are epidermis (e), sclerenchyma (s), phloem (p), xylem (x) and mesophyll (m). Presence of bulliform cells (b) are also evident. Bar, 0.1 mm. (B) Control leaf incubated for 72 h in situ. All tissues are intact except phloem and minimal amounts of mesophyll tissues which were degraded. Bar, 0.1 mm.

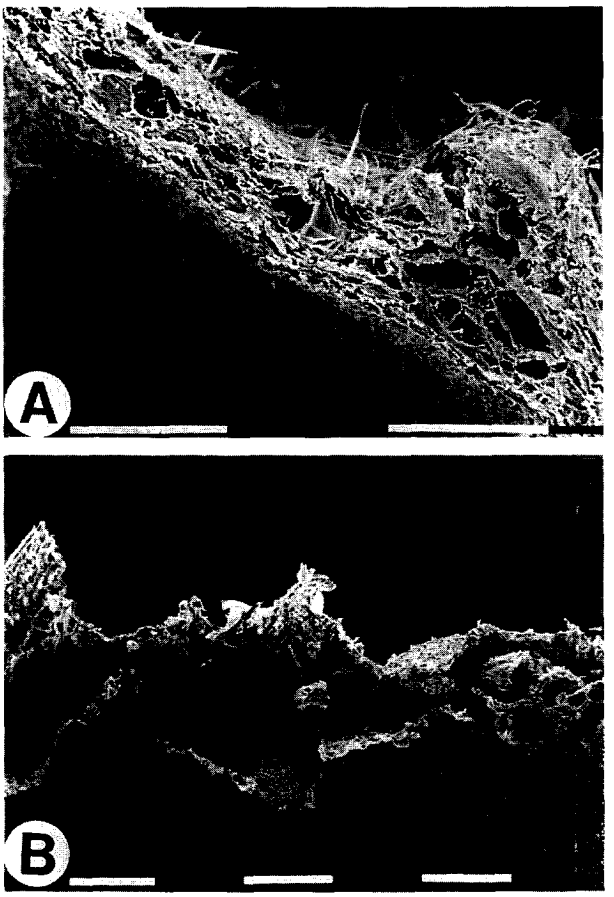

Fig. 2. Scanning electron micrographs of rice leaf sections after 30 d of SSF with *Cyathus stercoreus*. (A) Fungal treated leaf not incubated with rumen fluid. All tissues are intact but distorted and the rigidity of the tissues was reduced. Cell walls included lignified cells of the vascular bundle appeared altered. Bar, 0.1 mm. (B) Fungal treated leaf incubated for 48 h in situ. Extensive degradation of mesophyll, phloem and sclerenchyma tissues occured. Vascular bundles resisted ruminal microbial digestion but were detached from the epidermal tissue due to the removal of the supporting sclerenchyma tissue. Vascular bundle cells are partially degraded. Bar, 0.1 mm.

Scanning electron micrographs of control leaf, before and after ruminal digestion for 72 h, shows that most types of tissues of rice leaf remained intact after ruminal digestion (Fig. 1(a) and 1(b)). After 30 d of SSF, SEM of leaf decayed by Cs shows presence of all tissues of leaf including mesophyll, sclerenchyma and phloem, but the rigidity of the tissue was reduced and cell walls appeared altered (Fig. 2(a)) compared to control leaf (Fig. 1(a)). When the same fungal-decayed tissue was incubated in the rumen for 24 h, rumen microorganisms completely digested mesophyll tissue and disrupted sclerenchyma tissue (Fig. 2(b)). Even lignified vascular walls were partially degraded. In contrast, SEM of leaf decayed by Pc shows extensive degradation of mesophyll tissue

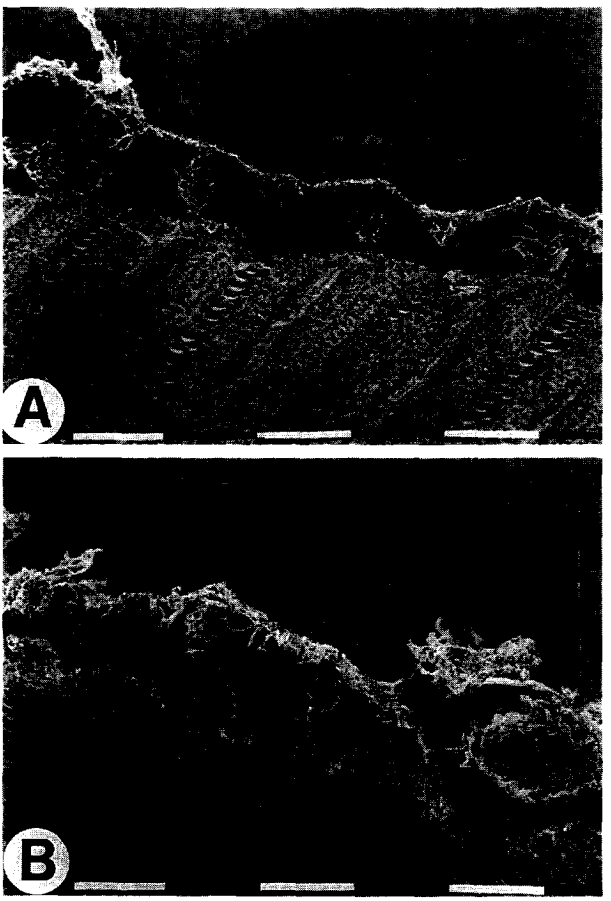

Fig. 3. Scanning electron micrographs of rice leaf sections after 30 d of SSF with *Phanerochaete chrysosporium*. (A) Fungal treated leaf not incubated with rumen fluid. Mesophyll tissue was extensively degraded by the fungus but vascular (bundle sheath cells and xylem) and epidermal tissues were intact. Bulliform cells are still present and appear to be firmly attached to the epidermis. Bar, 0.1 mm. (B) Fungal-treated leaf incubated for 72 h in situ. Bar, 0.1 mm. After a longer incubation with ruminal fluid, no further extensive (except epidermis) digestion beyond that by Pc occurred due to ruminal microorganisms.

(Fig. 3(a)) compared to the control leaf (Fig. 1(a)) leaving recalcitrant substrates. Ruminal digestion of the same tissue for 48 h shows little further digestion of leaf tissues by rumen microorganisms (Fig. 3(b)) compared to leaf decayed by Pc, with epidermal walls being partially degraded by rumen microorganisms. SEM of Ps-decayed rice leaf showed a similar pattern to that by Cs (Fig. 4(a) and 4(b)).

Scanning electron micrographs of control rice stem, before and after ruminal digestion, are presented in Fig. 5(a) and 5(b). Most of the parenchyma tissue of the stem was digested in the rumen by 72 h. The outer vascular bundles and the sclerenchyma resisted ruminal microbial digestion. After 30 d of SSF of rice stem with Ps, the sclerenchyma ring was eroded away and the vascular bundles were freed from the outer epidermis

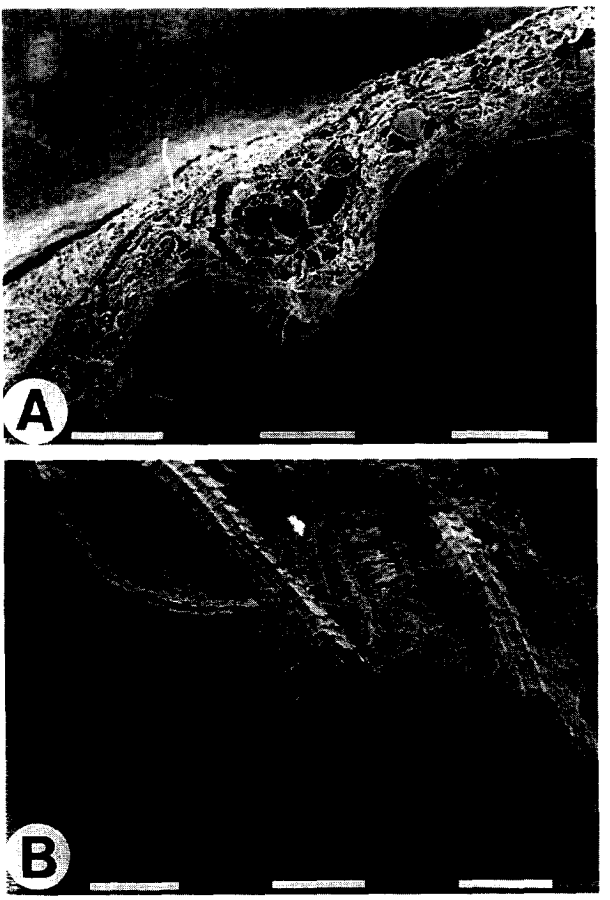

Fig. 4. Scanning electron micrographs of rice leaf sections after 30 d of SSF with *Pleurotus sajor-caju*. (a) Fungal treated leaf not incubated with rumen fluid. Degradation pattern of leaf tissues were similar to that of *Cyathus stercoreus* treatment. Bar, 0.1 mm. (b) Fungal treated leaf incubated for 72 h in situ. Bar, 0.1 mm. No sclerenchyma tissues were found near vascular bundles. This led to the collapse of vascular tissues and facilitated extensive degradation of mesophyll tissue. The fungal effect was similar to that of *Cyathus stercoreus* treatment.

(Fig. 6(a)). Presence of collapsed parenchyma, epidermis and fungal mycelia were evident. After 96 h of ruminal digestion of rice stem decayed by Ps, most of the vascular bundles and the supporting tissues (sclerenchyma and parenchyma) were eroded away (Fig. 6(b)).

Cross sections of tissues of the epidermis, mesophyll, sclerenchyma and mestome were evaluated under TEM for cell wall degradation by the fungus as well as subsequent digestion by ruminal microorganisms. Although a larger number of micrographs were obtained, only a limited number are presented here. We have selected Cs as the fungus to study leaf tissue degradation because of its effectiveness in improving IVDMD. Transmission electron micrograph of the control epidermis cell (Fig. 7(a)) shows a thick

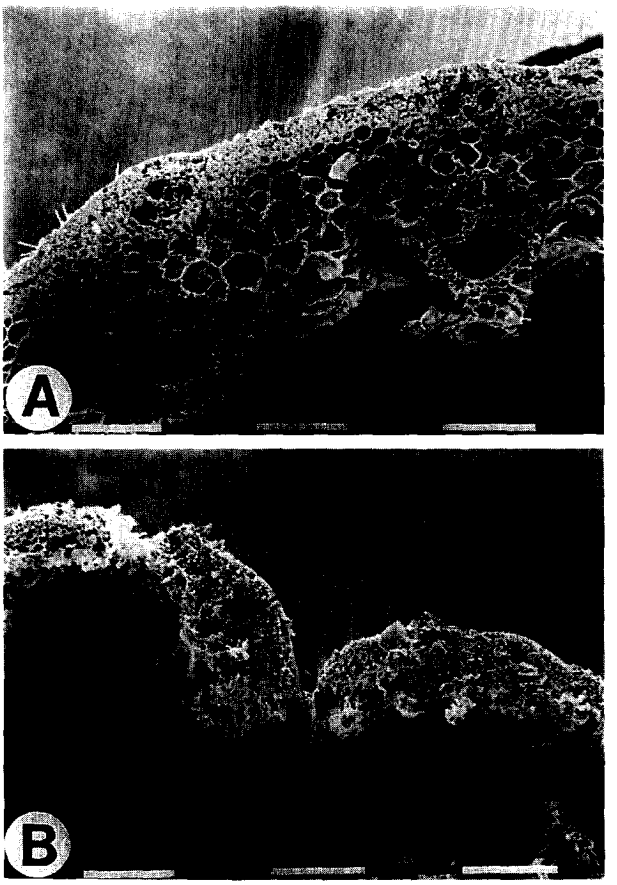

Fig. 5. Scanning electron micrographs of sections of control rice stem. (a) Control stem not incubated with rumen fluid. Bar, 0.1 mm. Tissues present are epidermis, smaller vascular bundles on the outer region and larger vascular bundles on the inner region of the stem cross section. Below the epidermis tissue is the heavily lignified sclerenchyma ring. Larger vascular bundles are surrounded by parenchyma (b) Control stem incubated for 72 h in situ. Bar, 0.1 mm. After a long incubation the parenchyma started to disintegrate and larger vascular bundles have disappeared.

outer cell wall. Ruminal digestion of the same tissue for 72 h (Fig. 7(b)) reveals that the outermost layer, probably cutin, was not digested, but underneath this region ruminal microbes degraded some cell wall material. Below the degraded region (Fig. 7(b)), ruminococci-like bacteria attached to the cell wall, but the epidermal cell wall towards the interior was not digested. Epidermis of fungal-decayed tissue (not presented) showed the presence of fungal hyphae near the cell wall of the outermost dark region. When the same tissue was incubated in the rumen for 72 h, bacteria deep in the outermost dark layer of the epidermis cell were observed, leaving the innermost layer undigested and much of the cell wall intact.

Mesophyll tissue in the control undigested leaf tissue was electron transparent under

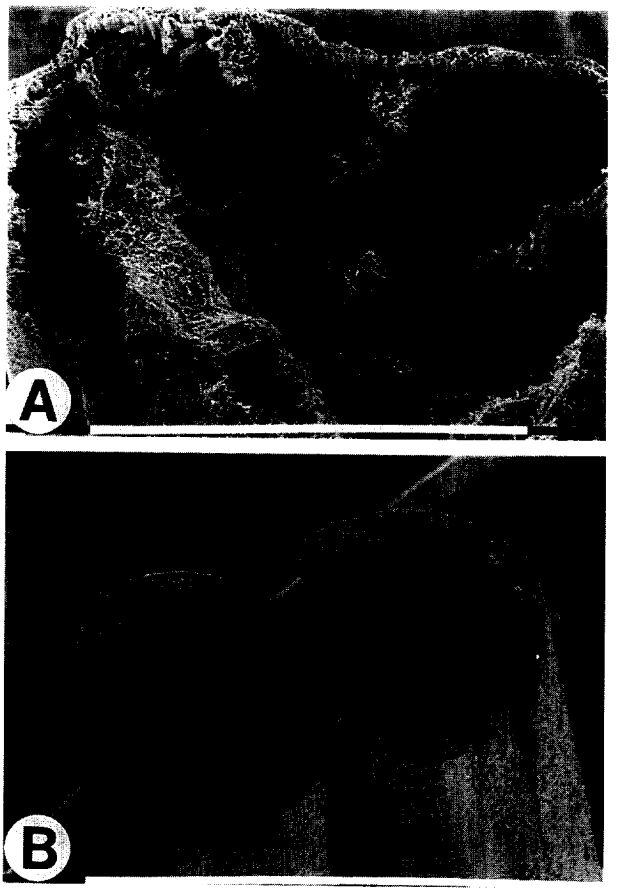

Fig. 6. Scanning electron micrographs of sections of stem after 30 d of SSF with *Pleurotus sajor-caju*. (a) Fungal treated stem not incubated with rumen fluid. Bar, 1 mm. Larger vascular bundles are collapsed because of the partial degradation of parenchyma tissue. Phloem tissues are degraded by the fungus. Fungal mycelium also is present in and around stem tissues. (b) Fungal treated stem incubated for 96 h in situ. Bar, 1 mm. Larger vascular bundles and the remaining mesophyll tissue were eroded away. Even after long hours of incubation the epidermis and part of the sclerenchyma ring still resisted ruminal digestion.

the TEM (Fig. 8(a)). Ruminal microorganisms were unable to degrade mesophyll cells even at extended incubation times (Fig. 8(b)). Mesophyll tissue decayed by Cs shows little evidence of removal or change in the cell wall (not presented). Slight losses of some middle lamella areas were also evident. Ruminal incubation of mesophyll of leaf tissue decayed by Cs for 24 h (Fig. 8(c)) shows partial degradation of the wall by ruminal bacteria; much of the cell wall is still present though it appears thinner than the undigested control tissue (Fig. 8(a)). After prolonged ruminal incubation for 72 h, mesophyll tissue of fungal-treated leaf was extensively degraded (not presented).

Sclerenchyma of control (Fig. 9(a)) undigested leaf tissue by TEM shows the

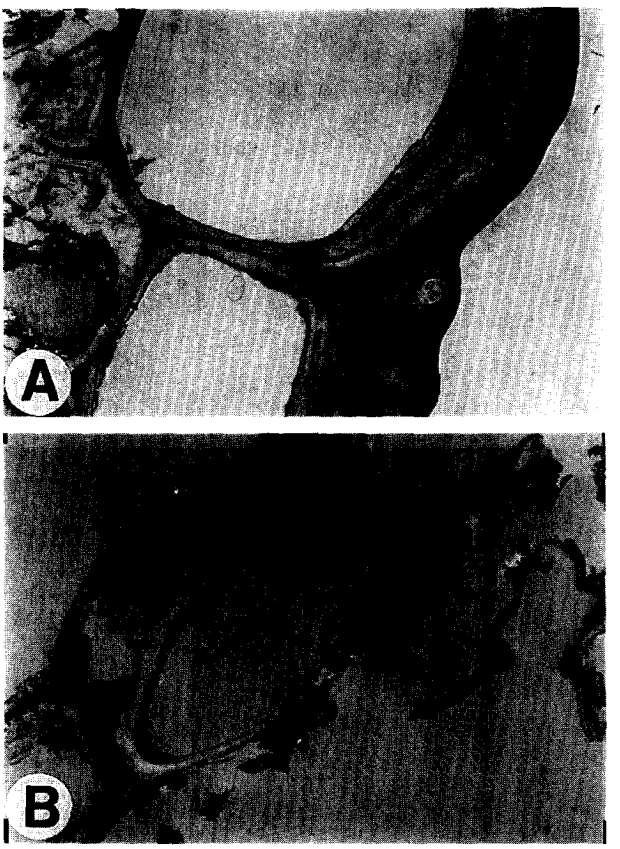

Fig. 7. Transmission electron micrographs of the control epidermal tissue in rice leaf blade. (a) Control epidermis cell not incubated with rumen fluid. ×1300. Presence of thicker outer cell wall region and the thinner inner cell wall which adjacent to the mesophyll tissue. (b) Control epidermis cell incubated for 72 h in situ. ×1300. The ruminal bacteria have tunnelled into the outer cell wall region, which is heavily concentrated with silica. Outermost layer was not digested but the middle layer was digested and bacteria attached to the cell wall.

thickness of the cell wall and bridging of the epidermal and vascular bundle sheath cells by sclerenchyma tissue. Sclerenchyma of leaf decayed by Cs (Fig. 9(b)) shows the presence of fungal hyphae in the lumen of cells; fungal penetration through cell wall; removal of wall material and loss of considerable amounts of the secondary layer of the cell walls. Ruminal digestion of the same tissue for 24 h. (Fig. 9(c)) reveals the formation of a bacterial tunnel in the middle lamella of the tissue and erosion of the secondary layer along the way. Another site shows preferential loss of the middle lamella by bacteria within the site and swelling of the secondary wall. Further ruminal digestion for 72 h of sclerenchyma fungal treated leaf (not presented) shows the penetration of ruminal bacteria, mostly ruminococci-like bacteria, through the middle lamella; degradation of cell wall layers in some sites is evident.

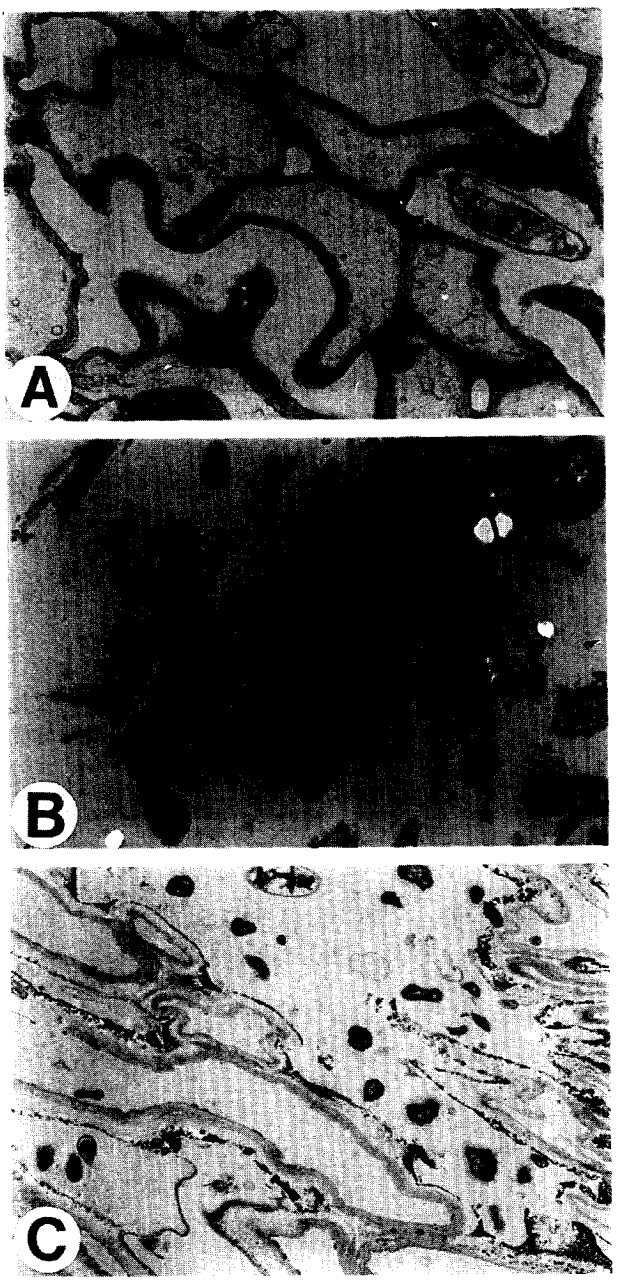

Fig. 8. Transmission electron micrographs of the mesophyll tissue in rice leaf blade. (a) Control mesophyll tissue not incubated with rumen fluid. ×1300. (b) Incubated for 72 h in situ. Presence of few bacteria near the cell wall is evident and part of the cell wall is randomly digested. ×1300. (c) Mesophyll tissue after 30 d of SSF with *Cyathus stercoreus* and incubated for 24 h in situ. ×1300. Tissue is distorted and partially digested and the cell wall is thinner compared to the undigested control tissue.

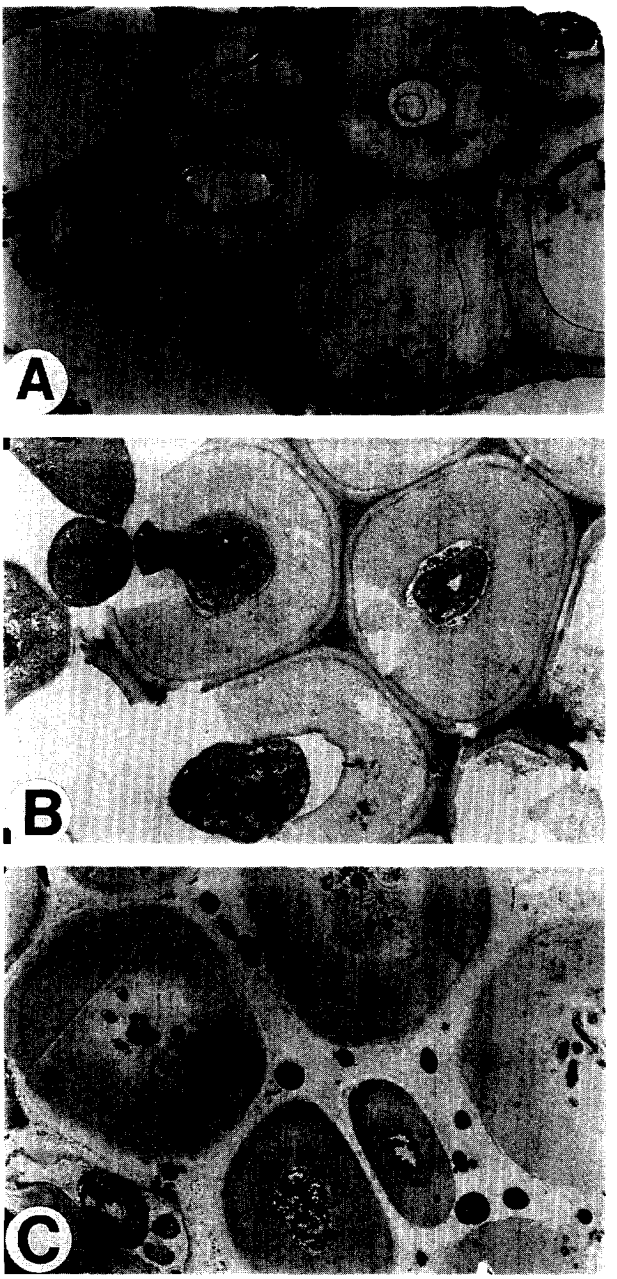

Fig. 9. Transmission electron micrographs of the sclerenchyma tissue in rice leaf blade. (a) Control sclerenchyma tissue not incubated with rumen fluid. ×1300. Secondary layers are thick and middle lamellae distinct. (b) Sclerenchyma tissue after 30 d of SSF with *Cyathus stercoreus*. ×1300. Fungal hyphae are in the lumen of cell and have removed considerable amounts of the secondary layer. (c) Sclerenchyma tissue after 30 d of SSF with *Cyathus stercoreus* and incubated for 24 h in situ. ×1300. Rumen bacteria are in the region between cells, and formation of bacterial tunnels in the middle lamella of the tissue occur.

4. Discussion

The various botanical parts of plants affect the nutritional value of forages to ruminant animals. Leaf is generally more digestible than stem in forages because of higher amounts of protein and cell solubles and lower cell wall contents compared to stem. Rice straw is different in that its stem fraction has been shown to be similar to leaf (Walli et al., 1988) or higher than leaf (Bainton et al., 1987) in degradability. In the present study, we also have found that stem and leaf blade of rice straw are equally digested in vitro. The low digestibility of rice leaf blade has been attributed to the higher concentration of silica in leaf compared to stem. The largest proportion of silica absorbed by the plant generally accumulates in the aerial parts of the plant (Yoshida et al., 1959). Rice leaf blade, in this study, had four percentage units higher silica than stem. Silica has been shown to inhibit digestibility by three percentage units for every one unit increase in silica content (Van Soest and Jones, 1968).

Solid state fermentation of crop residues with WRF often results in the improvement of IVDMD (Reid, 1989). Overall the improvement in the quality of crop residues by WRF is dependent upon fungal species selected, substrates, environmental factors and their interactions (Rolz et al., 1986). During colonization on substrates, fungi first convert easily digestible polysaccharides into low molecular weight sugars. This is called primary metabolism. Once the low molecular weight sugars are depleted, the fungus starts to starve and then begins to degrade structural carbohydrates and lignin. This is called secondary metabolism (Eriksson, 1988). During this phase, complex carbohydrates of the cell wall are converted into low molecular weight sugars (cell solubles) by fungal extracellular enzymes as shown in Table 2. Similar results were reported by Rolz et al. (1986) and Agosin et al. (1987). However, no relationship was found between cell solubles and IVDMD of leaf ($r = -0.28$; $P > .1$) suggesting that the contribution of cell solubles towards the improvement of digestibility is insignificant (Rolz et al., 1986; Han et al., 1975).

We have found that silica in rice leaf was negatively correlated ($r = -0.54$; $P < .07$) with IVDMD. Although, in the regression model for the prediction of IVDMD of fungal-decayed rice leaf, silica and lignin were not included due to their low r value, an additive effect should not be overlooked. In rice stem, where Cs and Pc failed to grow, lignin had a negative effect on digestibility. When twelve species of WRF were grown on lemon grass and citronella bagasse, Rolz et al. (1986) found a significant negative correlation of loss of lignin with IVDMD. In contrast to their findings, data herein showed that hemicellulose and cellulose of fungal-decayed rice leaf explained almost 98% of the variation in IVDMD. Agosin et al. (1986) reported increased degradation of cell wall cellulose in situ, when wheat straw was decayed by WRF. In the present study, all three fungi selectively degraded hemicellulose and the remaining hemicellulose showed a negative relationship with IVDMD. It is possible that the remaining hemicellulose is the more recalcitrant fraction of the hemicellulose that is left.

Within botanical parts, rice leaf decayed by Pc resulted in lower IVDMD compared to control leaf. The lower digestibility was primarily due to the extensive degradation of the structural carbohydrates, hemicellulose and cellulose, by Pc. The remaining structural carbohydrates are probably the more recalcitrant to rumen microbial digestion.

Therefore it is conceivable that intact cellulose is comprised of two fractions; easily digestible and a more recalcitrant fraction. Cellulose in plants is composed of amorphous and crystalline structures (Han, 1978), the latter more resistant to microbial degradation. In rice straw, 43% of the total cellulose is in the crystalline form (Han, 1978). Wood et al. (1988) reported that enzymes secreted by aerobic and anaerobic fungi are capable of solubilizing crystalline cellulose, and it is possible that our fungi acted through that mechanism. Information from Wood et al. (1988) and data herein suggest that in fungal decayed material, where part of the lignin was already removed by the fungi, the limitation in the digestion of structural carbohydrates may due to other factors such as the crystallinity of cellulose.

This study has demonstrated that the quality of rice leaf can be selectively improved compared to the stem by treating with Cs. In contrast, no improvement in the digestibility of stem decayed by Cs was found. The reason for the inhibition in digestibility of stem is not known, but we found an insignificant amount of hemicellulose and cellulose to be consumed during the 30 d of SSF. This was merely due to poor or no growth of the fungus on the rice stem fraction. This may suggest that longer than 30 d of SSF is required for Pc and Cs on rice stem to switch to secondary metabolism to degrade lignin and free structural carbohydrates. The other possibility is that these two fungi may produce some metabolites on rice stem that are inhibitory to rumen bacteria and reduces the digestibility of partially decayed material. A similar pattern of inhibition was also reported by other researchers as reviewed by Reid (1989). However, the digestibility of rice stem decayed by Ps was significantly improved compared to the control. A similar pattern of selection for botanical parts of barley straw by enzyme treatment was reported (Nakashima and Ørskov, 1989); enzyme was more active on leaf than on stem.

Scanning electron microscopy and transmission electron microscopy have been used to visualize and explain forage cell wall degradation by rumen microorganisms (Akin, 1989). Plant tissues may be classified into two groups based on their thickness of the cell wall (Wilson, 1991). Tissues with thick cell wall (sclerenchyma, parenchyma bundle sheath, vascular tissue and outer wall of the epidermis) are generally lower in digestibility compared to thin cell wall type (mesophyll, phloem, inner walls of the epidermis and stem pith parenchyma). In the present study, leaf decayed by Cs and Ps was higher in IVDMD compared to control material. Microscopic observation showed that fungal treatment enhanced digestion of the mesophyll tissue by ruminal microbes. Mesophyll tissue comprises a major proportion of the leaf cross sectional area (Wilson, 1991) and any improvement in digestibility of mesophyll cell wall, and therefore materials within the walls would significantly improve leaf digestibility, Presence of lignified tissues in leaf, such as sclerenchyma, gives physical strength to the leaf by holding the epidermis, mesophyll and vascular tissues together. These lignified tissues form a strong "I" girder structure. Data presented in this study showed that fungal treatment destroyed the girder structure and thus facilitated the digestion of adjoining tissues. The collapsing of the vascular bundles and the subsequent disappearance of parenchyma was also found in stem decayed by Ps. A similar pattern of improvement in degradation of lignified tissues was reported when chemically treated coastal bermudagrass leaf blade was degraded in the rumen (Spencer and Akin, 1980). Lignification of structural carbohydrates not only

inhibits ruminal microbial digestion of polysaccharides by forming a three dimensional matrix, but the presence of highly lignified tissues forms a physical barrier preventing the accessibility of the otherwise highly digestible tissues to the ruminal microorganisms.

In the early 1950s extensive studies were conducted on the role of silica in rice plants. Histological studies by Yoshida et al. (1959) showed that absorbed silica by rice plants are generally deposited in the cell walls, especially in the epidermis of the leaf. In a later study by the same authors, it was found that silica was localized in the epidermis, vascular bundle and sclerenchyma (Yoshida et al., 1962a). According to Yoshida et al. (1962a) the outer region of the epidermis consisted of several layers including the cuticle, silica layer, and outer cell membrane that was also filled with silica (Yoshida et al., 1962b). The data herein showed the presence of distinctive layers in the outer cell wall for the epidermis region of the control rice leaf. After ruminal digestion for 72 h, the microorganisms tunneled inside the outer cell wall of the epidermis and digested part of the cell wall. Ruminal bacterial attachment to the cell wall did not appear to be inhibited by the presence of silica in the epidermis.

Although mesophyll tissue of grasses are, in general, more easily digestible (Akin, 1984), SEM results in this study suggest that most of mesophyll tissue of control rice leaf resisted ruminal microbial digestion. After fungal treatment, most of the mesophyll tissue was digested by ruminal microorganisms and the thickness of the cell wall was substantially reduced as shown by TEM.

Lignified tissues can be divided into syringyl type and coniferaldehyde type tissues (Akin et al., 1987). According to this theory, syringyl type sclerenchyma of leaves is generally more easily attacked by ruminal microorganisms than the coniferaldehyde type, which is found in the xylem tissues. In our study, xylem and epidermis were the least digestible tissues when colonized by WRF. Evaluation of sclerenchyma by TEM showed fungal penetration of the cell and subsequent digestion of intercellular layers by ruminal microorganisms within 24 h.

Of all fungi evaluated, Cs and Ps treatment gave the greatest improvement in the IVDMD of rice leaf and leaf plus stem, respectively. Improvement in the digestibility of rice straw was primarily due to the selective improvement of leaf compared to stem for the Cs treatment. In this study, lack of a relationship between lignin and silica with IVDMD of fungal-decayed material may suggest that additional factors such as crystallinity may act as a digestion ceiling for further improvement in digestibility.

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