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Alfalfa stem tissues: rate and extent of cell-wall thinning during ruminal degradation

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Abstract

Alfalfa stem internodes of advanced maturity were used to examine the variability among tissues for rate and extent of cell-wall degradation by rumen microorganisms. Thin sections (100 μm) were incubated with rumen fluid *in vitro* for 0, 2, 4, 8, 16, 24, 48, 72, and 96 h. The degradation of tissue cell walls was evaluated for each fermentation time interval against a nondegraded mirror control section by light microscopy. Cell-wall thickness of alfalfa stem tissues was measured using scanning electron microscopy for both control and fermented sections. Rate and extent of cell-wall degradation were calculated from these measurements. Non-lignified epidermis, collenchyma, chlorenchyma, cambium and primary xylem parenchyma were rapidly and completely degraded within the first 8 h of fermentation. Rates of degradation ranged from 0.04 μm h^{-1} for thin-walled (0.29 μm) primary xylem parenchyma tissue to 0.11 μm h^{-1} for thick-walled (0.90 μm) collenchyma tissue. The non-lignified secondary wall (1.70 μm) of the primary phloem fibres required 24 h for complete degradation. Cell walls of some lignified tissues (e.g. pith parenchyma and secondary xylem fibres) were only partially degradable (9.1 to 65.5 %) even after 96 h of fermentation. The primary and secondary xylem vessels appeared to be completely nondegradable. The observed rates of cell-wall degradation for nonlignified alfalfa stem tissues were two to five times faster than previously estimated for nonlignified grass mesophyll tissue. However, extent of degradation for the lignified tissues of alfalfa stems was less than reported for lignified grass stem sclerenchyma. These differences in cell-wall degradation characteristics among tissues within alfalfa and compared to grasses are probably related to cell-wall lignification and polysaccharide composition of individual tissues.
Keywords: alfalfa, cell wall, degradation, *Medicago sativa* L., stem tissue.

Introduction

When contrasted with grasses of similar maturity, forage legumes are considered to have more rapidly degradable cell walls, but the potential extent of wall degradabili-

ty is generally less (Smith *et al.*, 1972). Pectin is the most rapidly degradable cell-wall polysaccharide and pectin is more abundant in legumes than grasses (Hatfield, 1993; Hatfield & Weimer, 1995). Xylans are very slowly degraded from legume cell walls, but xylan concentration of legumes is less than that of grasses. Beyond these differences in chemical composition of cell walls, legumes and grasses differ in anatomical organization of their tissues. Grasses have vascular bundles distributed throughout the ground parenchyma of stem cross-sections, whereas the vascular tissues in legumes form a discrete and continuous ring around the stem that expands through cambial activity (Wilson, 1993). In addition, cell-wall thickness varies more among legume tissues than grasses (Jung & Engels, unpublished). While data are limited on chemical composition of cell walls from different tissue types, histological staining for pectin and lignin indicate that tissues may differ more dramatically in their cell-wall composition in legumes than grasses (Engels & Schuurmans, 1992; Engels & Jung, 1998).

Non-lignified, thin mesophyll walls in grasses have a cell-wall thickness of ~0.15 μm (Wilson, 1990) and isolated grass mesophyll tissue was completely degraded by rumen microorganisms in ~8 h (Chesson *et al.*, 1986). Based upon these data, Wilson & Mertens (1995) calculated a potential rate of cell-wall degradation of ~0.02 μm h^{-1}. Because many of the mesophyll cells had probably been ruptured during isolation, Wilson & Mertens (1995) concluded that the rumen microorganisms would have had access to both the internal and external surfaces of the isolated mesophyll cells so that the actual rate of wall degradation would be slower and that the 0.02 μm h^{-1} rate estimate may be the maximal possible rate of cell-wall degradation. Using this estimate for rate of cell-wall degradation, it was calculated that less than 40% of thick grass sclerenchyma walls would be degraded in 48 h (Wilson & Mertens, 1995). Of course lignification of sclerenchyma and other tissues is expected to reduce degradability even further.

Legumes such as alfalfa (*Medicago sativa* L.) have a wide diversity of tissue types; some of which are thin-walled and others thick-walled, and some walls are lignified and other tissues do not lignify (Engels & Jung, 1998). It is assumed that non-lignified, pectin-rich tissues such as collenchyma would be more rapidly and completely degraded than lignified, xylan-rich tissues such as secondary xylem fibres. However, there are no data available on rates of cell-wall degradation for the diversity of tissues found in alfalfa stems. We compared the rate and extent of cell-wall degradation for the cell types which comprise alfalfa stem tissues using microscopy to measure wall thickness and rates of cell-wall thinning during *in vitro* degradation by rumen microbes.

Materials and methods

Alfalfa samples were collected as part of a larger study of cell-wall development of alfalfa stem tissues (Engels & Jung, 1998). Briefly, field-grown stem shoots which had initiated from the residual nodes of previously cut stems (24 June 1996 and 1997) were harvested after 31 and 30 d of growth, respectively, at St. Paul, MN,

USA from an alfalfa clone designated as 718. Stem shoots were immediately preserved in 50% (vol/vol) ethanol:water. The seventh internode, counting from the base of the stem shoots, was removed from two random stems collected in each year for use in the present study.

A series of 100-μm thick mirror section pairs (Engels, 1989) were prepared from each stem internode. One mirror section from each pair was mounted on a microscope slide using double-sided tape to serve as the non-degraded control section. The other member of each mirror section pair was similarly mounted on another microscope slide for *in vitro* ruminal fermentation. The sections were incubated for 2, 4, 8, 16, 24, 48, 72, or 96 h with rumen fluid (Engels & Brice, 1985). Section pairs taken from each stem piece were randomized among the incubation time periods. Slides were removed from the fermentation vessels after incubation for the specified times, gently washed in tap water, and stored in 50% (vol/vol) glycerol:water until examined.

Using light microscopy (LM), each pair of control and degraded mirror sections was examined. The following tissues were examined for degree of removal during ruminal fermentation for each incubation interval: epidermis, collenchyma, chlorenchyma, primary phloem fibres, cambium (including secondary phloem), secondary xylem vessels and fibre, primary xylem vessels and parenchyma, and pith parenchyma. Scanning electron microscopy (SEM; [1]Camscan S2, Electronen Optik, Cambridge, UK) was used to determine cell-wall thickness of the individual alfalfa stem tissues in selected non-degraded sections. Selected sections were examined by SEM after degradation to measure the thickness of residual cell walls after different times of incubation. Cell-wall thickness was measured for 10 cells of each tissue type in non-degraded and degraded sections from two stems in both 1996 and 1997. For the samples collected in 1996, sections were prepared for SEM by air drying prior to coating with palladium-gold. In an attempt to reduce deformation and shrinkage of tissues during SEM preparation, the 1997 samples were prepared by critical-point drying using liquid CO_2 followed by palladium-gold coating (Engels & Brice, 1985). Rate of cell-wall degradation was calculated for completely degradable tissues as the thickness of cell wall removed in the fermentation interval required for complete degradation of the tissue, as assessed by LM. For partially degradable tissues, SEM cell-wall thickness measurements of sections degraded for 24, 48, and 96 h were used to calculate the amount of cell-wall thinning which had occurred when maximal extent of degradation had been reached.

Results

Alfalfa stem tissues differed in both their rate and extent of degradation. Because the degradation patterns for specific tissues were similar for stems harvested in both

[1] Mention of a trade name, proprietary products, or specific equipment does not constitute a guarantee of the product by the USDA, the University of Minnesota, or Wageningen University; and does not imply its approval to the exclusion of other products that may also be suitable.

years, data for most tissues have been averaged across years. Only degradation of pith parenchyma tissue varied between years. Pith parenchyma in stems harvested in 1996 consisted of a mixture of minimally and completely degraded cells. In contrast, in 1997 the pith parenchyma consisted only of minimally degraded cells.

Figure 1 illustrates the degradation of collenchyma and primary phloem fibre tissues with increasing time of fermentation by rumen microorganisms. After only 4 h of fermentation, collenchyma exhibited extensive degradation (Figure 1b). By 8 h, this tissue was completely degraded (Figure 1c). Epidermis, chlorenchyma, and cambium tissue behaved similarly. In contrast, 24 h of fermentation was needed for complete degradation of the secondary wall of primary phloem fibres (Figure 1d). However, the lignified ring structures of the primary wall of primary phloem fibres remained after 24 h of fermentation. The thick, nonlignified regions of the primary wall of this tissue had been removed by microbial activity. Removal of these thick primary walls during degradation resulted in the fragmentation of the primary phloem fibre bundles into smaller tissue aggregates. In some cases individual prima-

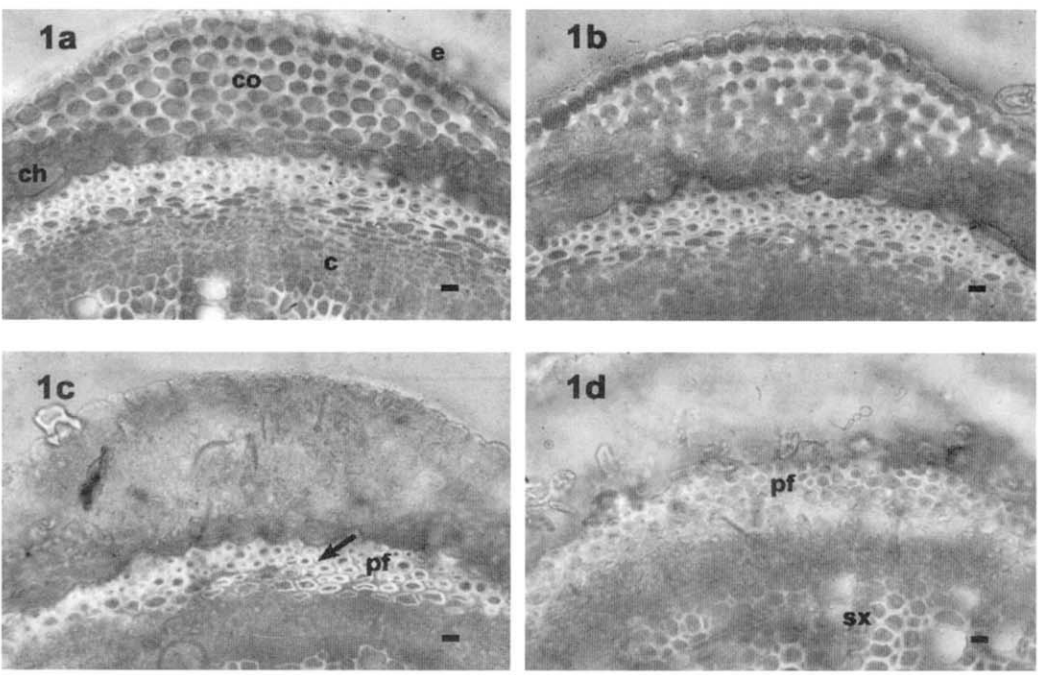

Figure 1. The pattern of alfalfa stem tissue degradation after 0, 4, 8, and 24 h of *in vitro* incubation with rumen microorganisms is shown. Epidermis (e), collenchyma (co), chlorenchyma (ch), and cambium (c) tissues are identified in the nondegraded control section (1a). After 4 h of degradation (1b) these four non-lignified tissues showed clear signs of partial degradation. The epidermis, collenchyma, chlorenchyma, and cambium tissues were completely degraded within the first 8 h of fermentation (1c). Even after 24 h of fermentation (1d), the lignified ring structure of primary phloem fibres (pf) and the secondary xylem (sx) remained undegraded. However, the thick nonlignified secondary wall of primary phloem fibres (indicated by an arrow in 1c) was degraded during the 8- to 24-h fermentation interval. (bar = 10 μm).

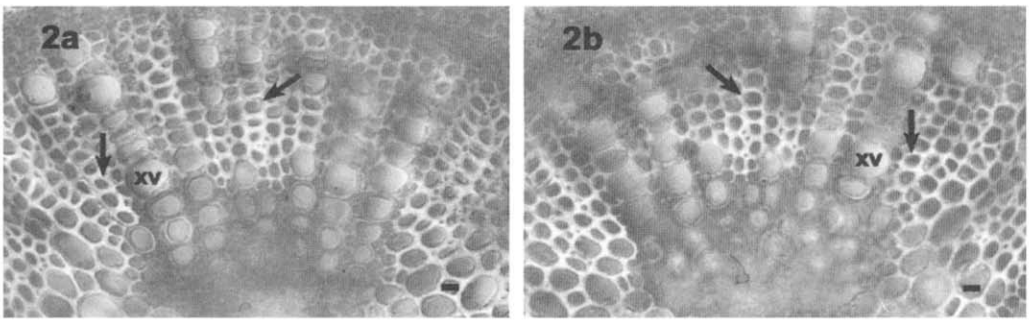

Figure 2. The degradation of secondary xylem tissues in mirror sections is shown after 0 and 24 h (2a and 2b, respectively) of *in vitro* incubation with rumen microorganisms. Using light microscopy, xylem vessels (xv) appeared to be completely undegraded (2b). The additional secondary wall of xylem fibres (indicated by arrows in 2a and 2b) was degraded in 24 h whereas the lignified primary/secondary wall appeared undegraded. (bar = 10 μm).

ry phloem fibre cells became completely separated from the bundles due to degradation of adjoining cells and these individual cells fell over on the slides because they lacked support from adjoining cells (not shown). The apparent non-degradability of xylem fibre is shown in Figures 2a & b. Unlike other tissues, some pith parenchyma cells were completely degradable while other cells of the same tissue appeared non-degradable (Figures 3a & b).

Table 1 summarises the degradation patterns in 100-μm sections for the various tissues that comprise the alfalfa stem, based on examination by LM. Some tissues (epidermis, collenchyma, chlorenchyma, cambium, and primary xylem parenchyma) were always completely degraded within the first 8 h of fermentation. In contrast, primary and secondary xylem vessels appeared to be completely non-degradable, even after 96 h of fermentation. Primary phloem and secondary xylem fibres exhibited differential degradation among the various layers of their cell walls. The secondary wall of primary phloem fibre was always completely degradable, but this

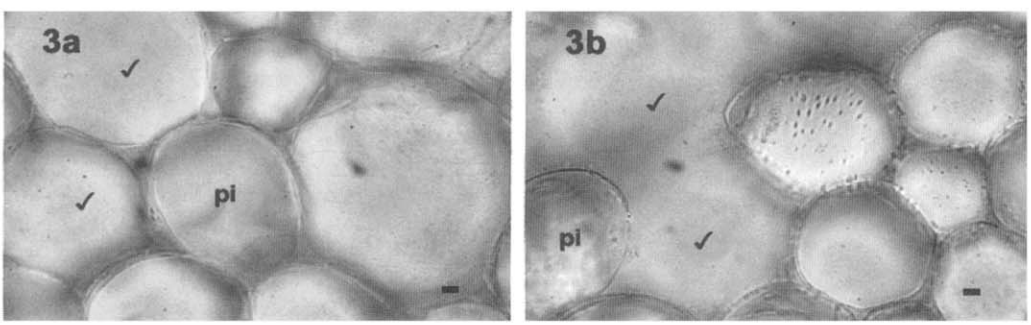

Figure 3. Degradability of pith parenchyma tissue (pi) after 0 and 24 h (3a and 3b, respectively) of *in vitro* incubation with rumen microorganisms is shown using mirror sections. Some pith parenchyma cells were completely degraded (indicated by check marks) while other parenchyma cells remained intact after 24 h of fermentation. (bar = 10 μm).

Table 1. Summary of cell-wall degradation observed by light microscopy in alfalfa stem tissues fermented *in vitro* for various time intervals with rumen microorganisms.

Tissue	Fermentation time (h)							
	2	4	8	16	24	48	72	96
Epidermis	None	Partial	Complete					
Collenchyma	None	Partial	Complete					
Chlorenchyma	None	Partial	Complete					
Primary phloem fibre								
1° wall[a]	None	None	Partial	Partial	Partial	Partial	Partial	Partial
2° wall[b]	None	Partial	Partial	Partial	Complete			
Cambium	None	Partial	Complete					
Xylem vessels	None	None	None	None	None	None	None	None
Secondary xylem fibre								
1° and 2° wall	None	None	None	None	None	None	None	None
Additional 2° wall	None	Partial	Partial	Partial	Partial	Partial	Partial	Partial
Primary xylem								
Vessels	None	None	None	None	None	None	None	None
Parenchyma	None	Partial	Complete					
Pith parenchyma	None	Partial	Variable[c]					

[a] Primary (1°).
[b] Secondary (2°).
[c] Pith parenchyma in some stems was completely degraded, but the same tissue was apparently non-degraded in other stems.

degradation required 24 h to reach completion. In contrast, the primary wall of primary phloem fibres was only partially degradable even after extended fermentation times. The primary and secondary wall of xylem fibre appeared non-degradable when viewed by LM, whereas the additional secondary wall layer of xylem fibres was extensively, but not completely, degraded. The pith parenchyma was unique in that some cells (in 1996 samples only) were completely degradable within 8 h of fermentation, whereas other cells appeared non-degraded after 96 h. The degradable pith parenchyma cells were always located in the center of the stem cross-section.

Cell-wall thickness of individual alfalfa stem tissues or cell-wall layers are presented in Tables 2 and 3. Some tissues were relatively thin-walled (e.g., primary xylem parenchyma) while other tissues had very thick walls (e.g., secondary walls of primary phloem fibre and additional secondary wall layer of xylem fibre). All tissues were quite variable in wall thickness, as illustrated by the observed standard deviations and ranges in wall thickness. A note of caution must be expressed relative to these measurements of cell-wall thickness based on SEM preparations. A comparison of tissues by LM (Figures 1, 2, & 3) with the same tissues in SEM preparations (Figure 4) indicated that several tissues appeared to have distorted shapes (less symmetrical, flattened) and may have shrunk as a result of the dehydration processes used for SEM. The level of tissue alteration was similar for air-drying and critical-point drying methods.

Based on measurements of enlarged LM and SEM photographs, we found that

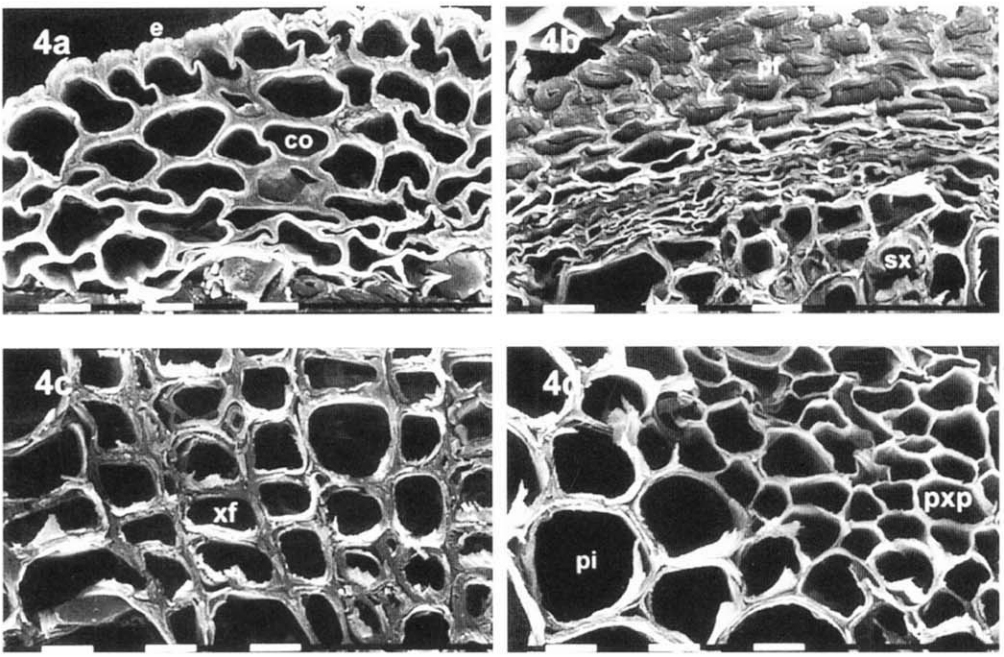

Figure 4. Preparation of alfalfa stem sections for scanning electron microscopy (SEM) resulted in very marked distortion of cell shapes due to the drying process compared to the shape of these same tissues in Figure 1. The non-lignified tissues (epidermis, e; collenchyma, co; cambium, c; and primary xylem parenchyma, pxp) were particularly distorted in their shapes (4a, 4b, and 4d). Primary phloem fibre (pf), which is partially lignified, also suffered from shape distortion during drying (4b). In contrast, the lignified secondary xylem fibre (xf, 4c) and pith parenchyma (pi, 4d) tissues retained their physical shape during SEM preparation. (white bars = 10 μm).

shrinkage occurred in all tissues we examined (collenchyma, primary and secondary walls of primary phloem fibres, secondary xylem fibre, and pith parenchyma). For the limited number of sections examined, it did not appear that any tissue was more or less susceptible to shrinkage during SEM preparation. Observed shrinkage ranged from 23 to 61% and variability in these measurements was extremely large. It was apparent that lignified tissues (secondary xylem vessels and fibre, and pith parenchyma) retained their shape better during shrinkage than did non-lignified tissues (collenchyma, thick primary walls of primary phloem fibre). Because of the higher resolution of SEM, we decided to use the SEM preparations for measurements of cell-wall thickness as these measurements were more precise than those from LM. Also, by using the SEM preparations in which water had been removed the differences in wall thickness observed among tissues may more accurately reflect differences in cell-wall mass rather than differences in thickness due to swelling by water.

Complete removal of collenchyma, cambium, and primary xylem parenchyma tissues was verified by examination of SEM preparations (Figures 5a & c). Also, complete degradation of the secondary wall layer of primary phloem fibre was clearly

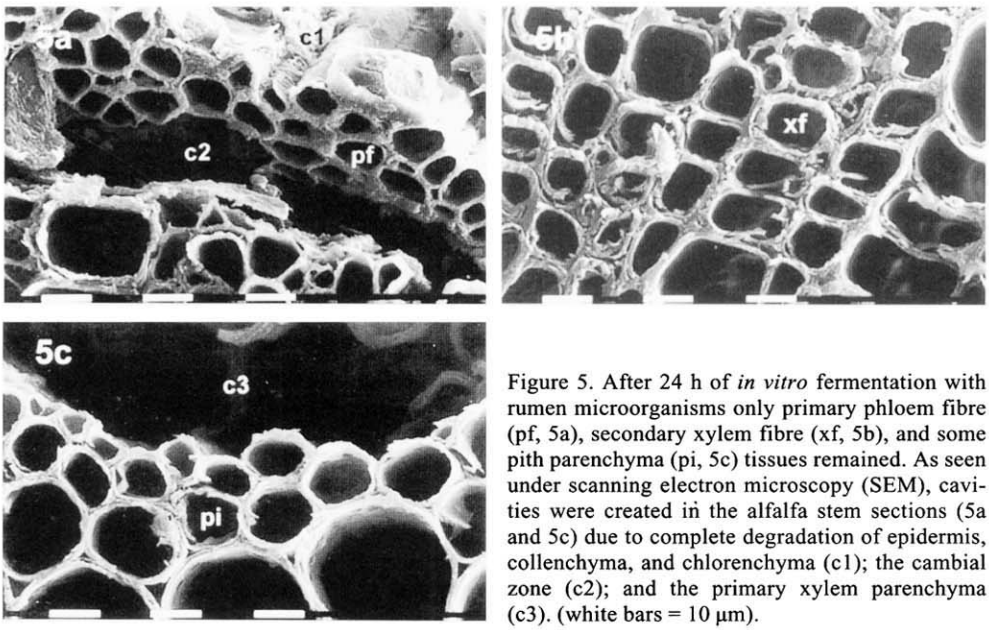

Figure 5. After 24 h of *in vitro* fermentation with rumen microorganisms only primary phloem fibre (pf, 5a), secondary xylem fibre (xf, 5b), and some pith parenchyma (pi, 5c) tissues remained. As seen under scanning electron microscopy (SEM), cavities were created in the alfalfa stem sections (5a and 5c) due to complete degradation of epidermis, collenchyma, and chlorenchyma (c1); the cambial zone (c2); and the primary xylem parenchyma (c3). (white bars = 10 μm).

observed (Figure 5a). However, under SEM the apparently non-degradable tissues, as judged by LM, did appear altered. We estimated mean rate of cell-wall degradation for completely degradable tissues and wall layers by dividing the average wall thickness by the hours of fermentation required for complete degradation of the tissue. The estimated mean rates of cell-wall degradation ranged from 0.04 μm h^{-1} for primary xylem parenchyma to 0.11 μm h^{-1} for collenchyma and pith parenchyma (Table 2). The very thick secondary wall of primary phloem fibre had a mean rate of degradation (0.07 μm h^{-1}) almost twice that observed for thin-walled primary xylem parenchyma.

Measurement of cell-wall thickness in SEM preparations of the tissues that appeared non-degradable by LM indicated that some degradation had occurred (Table

Table 2. Cell-wall thickness and mean rate of *in vitro* ruminal degradation for some completely degradable alfalfa stem tissues as determined by scanning electron microscopy.

Tissue	Cell-Wall Thickness (μm)				Degradation rate (μm h^{-1})
	Mean	Standard Deviation	Minimum	Maximum	
Collenchyma	0.90	0.29	0.29	1.57	0.11
Primary phloem fibre – 2° wall	1.70	0.64	0.58	4.39	0.07
Primary xylem parenchyma	0.29	0.07	0.14	0.43	0.04
Pith parenchyma[a]	0.88	0.20	0.57	1.18	0.11

[a] Estimate is for pith parenchyma cells which were completely degradable in some stems, 1996 samples only.

Table 3. Cell-wall thickness and mean extent of wall removal by *in vitro* ruminal fermentation for some partially degradable alfalfa stem tissues as determined by scanning electron microscopy.

Tissue	Cell-wall thickness (μm)				Degradation extent (%)
	Mean	Standard Deviation	Minimum	Maximum	
Primary phloem fibre					
– 1° wall	0.58	0.21	0.29	1.14	25.9
Secondary xylem fibre					
– 1° and 2° walls	0.79	0.30	0.29	1.76	9.1
– additional 2° wall	1.39	0.46	0.57	2.35	65.5

3). The values given in Table 3 for extent of degradation are means obtained from alfalfa stem sections fermented for 24, 48, and 96 h. We averaged the data because no consistent progressive thinning of cell walls was observed as fermentation time increased from 24 to 96 h and variability among calculated extents of degradation was large. We detected some marginal degradation of the secondary wall of xylem fibres. In contrast, the additional secondary wall of xylem fibres was extensively, and in some cases completely, degraded. The observed partial degradation of the primary wall portion of the primary phloem fibres resulted from the non-degradability of the lignified ring structure on the lumen edge of the primary wall coupled with the extensive, if not complete, degradability of the thick, non-lignified primary wall region present between some primary phloem fibre cells. As for the secondary xylem fibre, primary phloem fibre degradation reached a maximum within 24 h of fermentation.

Discussion

Engels & Jung (1998) described cell-wall development of alfalfa stem tissues. From that work it was apparent that stem tissues of alfalfa are very diverse. These authors grouped alfalfa stem tissues into four categories based on their cell-wall development: (1) tissues that retain thin, non-lignified walls, (2) tissues which have minimal wall thickening and then lignify, (3) tissues which deposit thick walls that do not lignify, and (4) tissues that develop thick cell walls that lignify. In the current study we found that this diversity in cell-wall development among alfalfa stem tissues leads to a similar diversity in degradation characteristics (Table 1). Non-lignified walls were completely degradable, regardless of their thickness, in all tissues examined. In contrast, tissues that lignified exhibited differential patterns of degradation depending on the distribution of lignin in their wall structures.

Based on their analysis of data for grass cell walls, Wilson & Mertens (1995) concluded that the maximal rate of wall degradation was ~0.02 μm h^{-1}. While this estimated rate may be correct for grass cell walls, some alfalfa cell walls were clearly capable of being degraded more rapidly (Table 2). The slowest mean rate of degrada-

tion we observed was for thin-walled, non-lignified primary xylem parenchyma and even this tissue was degraded at approximately twice the rate calculated by Wilson & Mertens (1995) for thin-walled, non-lignified grass mesophyll walls. Mean degradation rate estimates for thick, nonlignified walls in alfalfa tissues were much greater (3– to almost 4–fold) than the estimate for grass mesophyll. We observed no correlation of cell-wall thickness with mean rate of degradation for non-lignified tissues or wall structures. Polysaccharide composition may influence rate of wall degradation because the thick, pectin-rich collenchyma walls were degraded about 57% faster than the thick, pectin-poor secondary wall of primary phloem fibres (Engels & Jung, 1998). The secondary wall of primary phloem fibres is apparently rich in cellulose (Jung & Engels, unpublished). However, when maximal rate of degradation was calculated using the maximum rather than average wall thickness for each tissue, collenchyma was only degraded marginally faster than secondary wall of primary phloem fibre (0.20 and 0.18 $\mu m\ h^{-1}$, respectively). Why the thinner, non-lignified walls of primary xylem parenchyma were degraded more slowly (both mean and maximal rates of degradation) than these thick-walled tissues is unclear as primary xylem parenchyma also has pectin-rich walls (Engels & Jung, 1998). Perhaps the differences in rate of degradation relate to factors such as polysaccharide branching, cross-linkage, and/or degree of hydration. From our measurements of tissue shrinkage and deformation it was clear that non-lignified tissues contain significant amounts of water that impact linear dimensions of these tissues. Also, because we do not know the actual mass per unit of linear measurement for individual wall types, similar estimates for rates of degradation based on linear measurements may not represent similar rates of mass removal by degradation.

Alfalfa stem tissues with lignified wall structures were incompletely degraded (Table 1). For all lignified tissues, maximum extent of degradation was reached within the first 24 h of fermentation. In the case of the thick primary and secondary wall of xylem fibres, less than 10% of the wall was degradable (Table 3). In contrast, thick and lignified sclerenchyma tissue in grasses is extensively degraded leaving only a thin, lignified primary wall residue after fermentation for 48 h with rumen microorganisms (Engels & Schuurmans, 1992). There are numerous literature reports involving ground forages that indicate grass cell walls are more extensively degraded than legume cell walls with extended fermentation times (72 to 96 h) (Smith *et al.*, 1972; Buxton & Brasche, 1991). However, accessibility limitations (Wilson & Mertens, 1995) may account for the impact of long fermentation times on extent of grass cell-wall degradation (Smith *et al.*, 1972; Buxton & Brasche, 1991). For alfalfa and maize stems that had been ball-milled to remove all accessibility barriers, increasing fermentation time from 16 to 96 h only increased alfalfa cell-wall polysaccharide degradability by 18% whereas maize wall degradation more than doubled (Jung *et al.*, 2000). The virtually complete degradation of cell walls in ground alfalfa stems after only 16 h of fermentation is in agreement with our current observation that wall degradation reached its maximum within 24 h of fermentation for thin sections from alfalfa stems. It appears that degradation of lignified cell walls is more extensive in grasses than it is in legumes, but that the structure of these grass walls results in a slow rate of degradation. Structural analysis and degradation studies on

the cell walls of individual tissue types will be needed to provide an explanation for these differences in degradation characteristics of grass and legumes cell walls.

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