Alfalfa Stem Tissues: Cell Wall Deposition, Composition, and Degradability

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ABSTRACT
Declining cell wall degradability of alfalfa (Medicago sativa L.) stems with maturation limits the nutritional value of alfalfa for ruminants. This study characterized changes in cell wall concentration, composition, and degradability by rumen microbes resulting from alfalfa stem tissue proliferation and development during maturation. The seventh internode from the shoot base of three alfalfa clones was sampled after 12, 17, 21, 31, and 87 d of regrowth in 1996 and 21 and 31 d in 1997. Cross sections were examined by light microscopy for tissue development, and after 48-h in vitro degradation. Cell wall concentration and composition of the internodes were determined by the Uppsala dietary fiber method, and cell wall degradability by rumen microbes was measured after 12 and 96 h. All stem tissues were pectin-rich and nonlignified at the two youngest maturities in 1996, except for primary xylem vessels which had lignified and thickened walls, and the internode was actively elongating. Primary xylem was the only tissue not degraded from immature stems. The 21-d-old internodes had completed elongation and began secondary xylem proliferation. Secondary xylem lignified immediately, and lignification of primary phloem and pith parenchyma began when elongation ended. As tissues lignified, their cell walls became undegradable. Maturation increased stem proportion consisting of undegradable secondary xylem, and cell wall polysaccharide composition shifted from predominantly pectin toward cellulose. Degradability of pectin remained high regardless of maturity stage, but cellulose and hemicellulose degradabilities declined as secondary xylem proliferated. Degradability of alfalfa stems would be improved if the amount of lignified secondary xylem was reduced.

While alfalfa is a high-quality forage crop for ruminant livestock, digestibility of alfalfa declines severely with maturity (Nordkvist and Aman, 1986; Buxton and Brasche, 1991). This decline in forage quality results from the combination of two separate events during maturation. First, as legumes mature the leaf:stem ratio declines because stem material accumulates at a faster rate than leaves, and lower leaves senesce and die (Aman and Nordkvist, 1983; Nordkvist and Aman, 1986). This is important to forage quality because stems contain more cell wall material than leaves, and cell walls are less digestible by ruminants than are cell soluble components (Buxton and Brasche, 1991; Jung et al., 1997). Secondly, as stems mature they accumulate higher concentrations of cell wall material and the degradability of these cell walls by ruminants decreases (Buxton and Brasche, 1991). Improving the forage quality of alfalfa will require alterations in one or both of these maturation events, but given the large contribution of stems to alfalfa yield, changing the impact of maturation on stem quality may have the largest potential benefit.

The tools of molecular biology offer the potential of altering the development of alfalfa stems in very precise ways to improve forage quality. It has long been assumed that lignin limits cell wall degradability because lignin concentration of stems increases during maturation and degradability declines (Jung and Deetz, 1993). Shifting the composition of lignin toward a lower syringyl:guaiacyl monolignol ratio is another possible route to improving alfalfa stem quality because this type of lignin has been associated with more degradable cell walls (Cherney, 1990). Therefore, modifying lignin biosynthesis is a popular strategy for improving the cell wall degradability of alfalfa and other forages (Bernard Vailhe et al., 1996; Baucher et al., 1999). Another strategy for potentially improving the quality of alfalfa stems might be to increase pectin content of the cell wall because this polysaccharide is rapidly and extensively degraded in the rumen (Chesson and Monro, 1982; Hatfield and Weimer, 1995).

While these cell wall traits may represent reasonable targets for improving alfalfa stem quality, nontissue-specific genetic transformations of lignin biosynthesis to reduce plant lignin content have generally resulted in agronomically nonviable plants (Piquemal et al., 1998; Tamagnone et al., 1998). This negative result is not surprising, given the diversity of functions (mechanical support, water transport, disease resistance) ascribed to lignin (Higuchi, 1990). While almost every tissue in grasses will lignify to some extent (Engels and Schuurmans, 1992), lignification of alfalfa stems occurs in a limited number of tissues (Wilson, 1993; Vallet et al., 1996; Engels and Jung, 1998). Similarly, cell wall polysaccharide content of alfalfa stem tissues vary. There is limited information concerning differences among forage tissues in cell wall concentration, composition, and development (Chesson et al., 1997; Grabber et al., 1991; Hatfield et al., 1999). Successful implementation of molecular biology methods to improve alfalfa quality will require a more refined understanding of cell wall structure and degradability for the diversity of tissues that comprise the stem.

Our objectives were to describe the developmental pattern of cell walls in the various tissues that comprise the alfalfa stem, characterize the shifts in cell wall concentration and composition resulting from differences in tissue proliferation and development during maturation, and explain the pattern of declining cell wall degradability associated with plant maturation based on differences in proliferation and development of stem tissues. A single, specific internode of developing alfalfa stems was chosen as a model for stem tissue development. Three alfalfa genotypes were included to provide variation in cell wall development and/or degradability.


We combined microscopic examination of individual tissues with bulk chemical analysis of whole internodes to address these objectives. A description of the development and lignification of alfalfa stem tissues based on microscopic examination from this study has been published (Engels and Jung, 1998).

MATERIALS AND METHODS

Plant Material

On the basis of a single determination of in vitro dry matter digestibility of the lower stem, three alfalfa plants (designated 143, 403, and 718) were selected from a spaced plant nursery containing several hundred plants to provide a potential range in digestibility for use in the current study (D.R. Buxton, 1995, personal communication). After clonal propagation and inoculation in the greenhouse, cuttings were transplanted to the field on 16 May 1996 at the University of Minnesota St. Paul Campus. The field layout was a randomized complete block design with three replications of each alfalfa clone. Plots consisted of 60 plants per row with a 15-cm spacing within the rows and 90 cm between rows. The soil was a Waukegan silt loam (fine-silty over sandy-skeletal, mixed, mesic Typic Hapludolls). The plots were fertilized according to soil test screen (Jung et al., 2000). The plots were fertilized just prior to planting, and a fertilizer solution was applied at planting and after each harvest at the rate of 7 kg ha⁻¹ N, 6 kg ha⁻¹ P, and 5 kg ha⁻¹ K. Plots were subdivided into five segments, and one random segment of each plot was designated for use at each sampling date. Plants were sprayed with insecticides to control insect damage as needed. Both growing seasons were warmer than normal, and 1996 and 1997 were drier and wetter than normal, respectively (Table 1).

The alfalfa plants were cut 24 June 1996 to a 4-cm stubble height. Growth of new stem shoots from the remaining nodes on previously cut stems was monitored to determine when sampling was to begin. The sampling protocol was designed to collect the seventh internode of shoots, counting from the base, initiating from the remaining nodes on previously cut stems across a range of maturity stages. Five maturity stage samples were collected. The harvest dates were 6, 11, 15, and 25 July and 19 September 1996. These sampling dates represented 12, 17, 21, 31, and 87 d of regrowth after the alfalfa plants were initially cut 24 June 1996. The first sample was collected when Internode 7 was just visible on the majority of new shoots. The second sample was taken when Internode 7 was approximately half the length of Internode 5 on the same shoot. The third sampling occurred when Internode 7 reached the same length as Internode 5. The fourth sample was taken 10 d after the third sampling date and Internode 7 was at least as long as Internode 5 on the same shoot. Any shoots harvested from the plots but not meeting these collection criteria upon more careful examination in the laboratory immediately after harvest were discarded. The fifth sample was taken just prior to the first predicted frost for St. Paul, MN. The following year, the primary spring growth was removed from the plots on 23 June 1997. Samples of Internode 7 from stem shoots initiating from nodes of previously cut stems were taken on 14 and 24 July 1997, 21 and 31 d following the 23 June 1997 harvest. These samples corresponded in shoot development to the third and fourth sampling maturity stages of the previous year.

The number of stem shoots collected from individual plots at each sampling date ranged from 24 to 98, with more stems being collected at the earlier maturities in an attempt to obtain sufficient internodes for subsequent analyses. For every sampling date, 10 fresh stem shoots from each plot were immediately placed in 50% ethanol:water (vol/vol) for preservation until microscopic examinations were conducted. Internode 7 was excised from the remaining shoots. These internodes were then counted, frozen, and lyophilized for cell wall chemical and in vitro degradability analyses. After weighing, the lyophilized internode samples were ground in a shaking ballmill for 15 min. This grinding treatment results in particle size reduction such that 98% of alfalfa stem samples pass through a 106 μm screen (Jung et al., 2000).

Chemical Analysis

Concentration and composition of cell walls in the internodes was determined on the dried samples using the Upsala dietary fiber method (Theander et al., 1995). Briefly, after removal of simple sugars and starch using enzymes and 80% ethanol:water (vol/vol) the cell wall residues were hydrolyzed with sulfuric acid in a two-stage procedure. Klason lignin was measured gravimetrically as the ash-free, nonhydrolyzed residue and the neutral sugar components of the cell wall polysaccharides were determined as alditol-acetate derivatives by gas chromatography. Uronic acid polysaccharide components were measured colorimetrically in an aliquot from the first step of the acid-hydrolysis procedure using galacturonic acid as the calibration standard (Ahmed and Labavitch, 1977). Total cell wall concentration was calculated as the sum of Klason lignin, glucose, xylose, arabinose, galactose, mannose, rhamnose, fucose, and uronic acid residues. On the basis of the known general composition of alfalfa polysaccharides (Hatfield, 1991, 1993), cellulose concentration was estimated as the glucose residue content, hemicellulose as the sum of xylose, mannose, and fucose residues, and pectin as the sum of uronics, arabinose, galactose, and rhamnose residues. All data were corrected to an organic matter basis by determining 100 °C dry matter content overnight and subsequent ashing at 450 °C for 6 h.

Composition of the lignin in the alfalfa internodes was determined by pyrolysis-GC-MS analysis (Ralph and Hatfield, 1991). The syringyl:guaiacyl ratio of the monolignol components in lignin was calculated using data normalized to the guaiacol content of each sample (Jung and Buxton, 1994).

In Vitro Degradability

Degradability of the cell walls in the alfalfa internodes was determined using rumen fluid in vitro incubations. For the dried samples, the donor animal was a fistulated lactating Holstein cow fed a total mixed ration containing alfalfa hay, maize (Zea mays L.) silage, and a concentrate supplement. Rumen fluid was collected 12 h post-feeding. A 20% rumen fluid:McDougall's buffer (vol/vol) mixture was added to screw-
cap centrifuge tubes containing 100 mg of sample and were incubated at 39 °C for 12 and 96 h (Jung and Buxton, 1994). At the end of the incubation periods, the entire contents of the in vitro tubes were frozen and subsequently lyophilized. The fermentation residues were analyzed for cell wall components using the Uppsala dietary fiber method as described previously. Empty centrifuge tubes were inoculated, incubated, and analyzed to correct for cell wall material contributed by the rumen fluid.

Thin sections prepared from the ethanol preserved internode samples (see following section for details) from 1996 were fermented with rumen fluid using the method of Engels and Brice (1985). Rumen fluid was collected ~12 h after feeding from a fistulated Holstein steer fed maize stover and a small amount of concentrate supplement. Sections were mounted on microscopic slides with double-sided tape and placed in a 250-mL fermentation vessel fitted with a Bunsen valve, including 0.5 g of ground maize stover to maintain a normal fermentation. The fermentation vessel was inoculated with a 20% rumen fluid:bicarbonate buffer (vol/vol) mixture and incubated at 39 °C for 48 h. At the conclusion of the fermentation the slides were gently washed with tap water and placed in 50% ethanol for preservation until they were examined under the microscope.

**Microscopic Analysis**

Two randomly chosen stem shoots from each of the ethanol-preserved samples were used for measurement of physical dimensions and microscopic analysis. The length of the stem shoots and number of internodes were determined. Length of Internode 7 was measured and its diameter was determined with calipers. After excising Internode 7 from the shoots, 15 serial sections 100 μm in thickness were made from the middle of each internode piece. Three randomly chosen sections from each internode of each sample were then mounted on a slide with double-sided tape for examination by light microscopy. Replicate slides with three random sections from each internode of each sample in 1996 were mounted on slides for the in vitro fermentations described above.

Nonfermented control sections were first examined under a microscope with normal light. The diameter of the sections was measured using a calibrated ocular eyepiece and the approximate width of the xylem tissue ring was similarly determined. The individual tissues comprising the stem cross section (epidermis, collenchyma, chlorenchyma, phloem, cambium, xylem, and parenchyma) were examined to estimate the degree of cell wall thickening with advancing maturity across sampling dates. Polarized light was used to visualize thickened secondary cell walls rich in cellulose microfibrils. After examination with normal and polarized light, sections were stained with phloroglucinol (Jensen, 1962) to determine which tissues were lignified and when during development the lignification occurred. Additional replicate sections from each sample were stained with ruthenium red (Jensen, 1962) to visualize tissues with pectin-rich cell walls.

The fermented internode cross sections were examined under normal light to estimate the degree of cell wall degradation that had taken place during the 48-h in vitro incubation with rumen microbes. Degradation was estimated using the following categories: 0, undegraded; 1, <50% of the walls degraded for thin-walled tissues or partial thinning of thick walls; 2, >50% of the walls degraded, but not complete degradation; 3, complete degradation of the wall. For each individual tissue, an average extent of degradation score was assigned based on examination of the three replicate sections. Degradation scores were averaged for the two internodes for every sample. After this scoring, the sections were stained with phloroglucinol to determine if the residual cell walls of the nondegraded tissues were lignified.

**Statistical Analysis**

All chemical and degradability analyses were done in duplicate and the results averaged. The data for 1996 were subjected to an analysis of variance as a randomized complete block with three replications and maturity stage (sampling date) as a subunit in a split-plot design. Alfalfa clone was the main unit and it was tested for significance with the clone × replicate interaction term. Data for the third and fourth maturity stages from 1996 and 1997 were combined into an analysis of variance using a randomized complete block design with three replications in a split-split-plot. Year was the main unit, alfalfa clone the subunit, and maturity stage the subsubunit. Each unit of the split-split-plot design was tested for significance with the appropriate error term. Year was considered a fixed effect because the perennial nature of alfalfa required use of sequential years. Clone and maturity were also treated as fixed effects because the experimental alfalfa clones and maturity stages were selected for specific reasons. The P < 0.05 probability level was used to determine significance. For those model parameters that had a significant F-test, means were compared using the least-significant difference method. All statistical analyses were done using PC-SAS (SAS Institute, 1985).

**RESULTS AND DISCUSSION**

**Plant Morphological Development**

The alfalfa stem shoot samples were all in a vegetative stage of development at the first three sampling dates in 1996. By the fourth sampling, all shoots had open flowers. Flowers, seed pods, and extensive branching were present on shoots collected at the final sampling date in 1996. Stem shoots collected in 1997 were similar to the third and fourth maturity stage samples from 1996 for the absence or presence of flowers. No noticeable differences were observed among the three alfalfa clones for time of flowering. Figure 1 illustrates the mean shoot length and number of internodes for the five maturity stages.
stages in 1996. For both traits, all maturity stages were different and clone × maturity stage interactions were also significant; however, the differences among clones were small and switched among maturity stages (data not shown). The most noticeable differences were that by the last sampling date, Clone 403 stem shoots were 20 and 42% longer than shoots of Clones 143 and 718, respectively, and Clone 403 had three more internodes than the other two clones.

Engels and Jung (1998) reported that the length of Internode 7 in these three alfalfa clones increased rapidly between the first and third sampling dates, with no change between the third and fourth samples, followed by a slight decline in length at the final maturity stage. The reason for the shrinkage is unknown. The third sampling date appeared to correspond to the point in maturation where internode elongation ceased and development shifted to only radial growth due to cambial activity (Engels and Jung, 1998). The diameter of Internode 7 increased rapidly during the first three maturity stages as a result of cell growth and differentiation, and was different among the three alfalfa clones (Fig. 2). Internode diameter continued to increase slowly in the fourth and fifth samples due mainly to the addition of xylem tissues. The proportion of the internode cross-sectional diameter accounted for by xylem increased sharply with sampling date (Fig. 3). Alfalfa Clone 403 had a consistently larger diameter for Internode 7 at all maturity stages (Fig. 2), but xylem tissue in Clone 403 constituted less than the stem cross section at the second, third, and fourth maturity stages than observed for the other clones. However, Clone 403 had the largest percentage xylem tissue at the final sampling date (Fig. 3). Clone 143 had fewer vascular bundles than were present in the other two clones (18.1, 21.5, and 21.5 for Clones 143, 403, and 718, respectively). A major difference between stem shoots in 1996 and 1997 was observed for pith parenchyma tissue. All stems at the older maturity stage in 1997 had a hollow pith region where the parenchyma tissue had been disrupted. In the 1996 samples, no hollow stem shoots were observed at any maturity stage. Whether growth environment or the fact that the plants were a year older in 1997 accounted for this difference in pith development is unknown.

As Internode 7 increased in physical size, its mass rose quickly during the first four maturity stages (Fig. 4). No differences among the alfalfa clones in weight of Internode 7 were observed in 1996; however, in the combined 1996 and 1997 data set for the third and fourth maturity stages it was found that while the clones did not differ in weight of Internode 7 at the vegetative stage, all three clones were different in internode weight at the flowering stage (41, 63, and 49 mg internode−1 for Clones 143, 403, and 718, respectively). Also, Internode 7 was consistently heavier in 1997 than 1996 for both maturity stages.

**Tissue and Cell Wall Development**

Stem tissue development in Internode 7 did not differ among the three alfalfa clones when examined by light
Fig. 5. Micrographs of alfalfa stem cross sections from Internode 7 showing anatomical development during internode maturation from Sampling Dates 1 through 5 (a through e) and corresponding residues remaining after 48-h in vitro degradation by rumen microbes (f through j). For each maturity stage, the control and degraded micrograph pairs are from serial sections taken from the same alfalfa shoot. Bar = 100 μm, c = cambium, ch = chlorenchyma, co = collenchyma, cl = waxy cuticle, e = epidermis, pp = primary phloem, pf = phloem fiber, pi = pith parenchyma, pxv = primary xylem vessels, xf = xylem fiber, sxv = secondary xylem vessels, x = xylem.

Microscopy, and was described in detail by Engels and Jung (1998). The general pattern of alfalfa stem tissue development is illustrated in Fig. 5. Cell wall development was most pronounced in collenchyma, primary phloem, and primary xylem vessels at the first sampling date, while other tissues such as epidermis, chloren-
Fig. 7. Mean Klason lignin concentration of the cell wall and lignin composition (syringyl/guaiacyl ratio) of Internode 7 of three alfalfa clones harvested at different stages of maturity. Bars represent one standard error of the mean. Absence of error bars indicates that the standard error interval was smaller than the size of the data symbol. CW = cell wall.

Fig. 6. Mean cellulose (cell), hemicellulose (hemi), and pectin concentrations in the cell walls of Internode 7 of three alfalfa clones harvested at different stages of maturity. Bars represent one standard error of the mean. Absence of error bars indicates that the standard error interval was smaller than the size of the data symbol.

Chyma, and pith parenchyma had only thin primary walls (Fig. 5a). Only the primary xylem vessels were lignified. The primary walls of collenchyma and primary phloem underwent extensive thickening by the second sampling date (Fig. 5b). When stem elongation had ceased by the third sampling date, cambial activity had resulted in the formation of secondary xylem vessels and xylem fiber (Fig. 5c). These tissues lignified almost immediately. Xylem tissues continued to proliferate through cambial activity, and secondary wall thickening was extensive at the fourth sampling date (Fig. 5d). Phloem fiber (mature primary phloem tissue) and pith parenchyma tissues also began to lignify by this stage of development. By the final sampling date, phloem fiber and xylem tissues had thick secondary cell walls and both tissues, along with pith parenchyma, were heavily lignified (Fig. 5e).

The concentration of cell wall material in Internode 7 increased very rapidly across the first four maturity stages and then stabilized (Fig. 4). The increase in cell wall concentration during internode development was the result of both primary and secondary wall development. The primary cell walls of most stem tissues (chlorenchyma, cambium, secondary phloem, xylem fibers and vessels, and parenchyma) were thin walled and did not thicken during maturation (Engels and Jung, 1998). Epidermis, collenchyma, and primary phloem tissues were different in that they developed thick primary walls. Thickening of primary walls in these three tissues began very early in the development of Internode 7 and was essentially complete by the third sampling date when elongation ended and deposition of secondary xylem tissues began (Engels and Jung, 1998). Secondary xylem tissues and primary phloem developed very thick secondary cell walls, while pith parenchyma underwent a minor amount of wall thickening (Engels and Jung, 1998).

Associated with the increase in cell wall concentration during maturation of the internode, pectin concentration declined sharply and cellulose, hemicellulose, and lignin concentrations increased (Fig. 6, 7). No significant changes in cell wall composition were found between the first and second sampling dates when the internode was still rapidly elongating. The greatest changes in cell wall composition occurred between the second and third sampling dates when internode development shifted from elongation to radial growth plus secondary cell wall thickening. Tissues that developed thickened secondary walls were the only alfalfa internode tissues to deposit lignin as the cells matured, but the pattern of lignification was different in each tissue (Engels and Jung, 1998). Primary xylem vessels were all lignified at the youngest maturity stage sampled. This is presumably because primary xylem vessels are the first water-conducting tissue that develops in the internode. Xylem tissues deposited lignin in both primary and secondary wall regions, and deposition of lignin and thickening of the secondary wall appeared to be almost simultaneous events in xylem tissue. Some xylem fibers developed an additional secondary wall layer by the last sampling date that was only slightly lignified, as determined by visual appraisal of phloroglucinol staining. Lignin was also deposited in pith parenchyma cell walls, beginning with cells closest to the xylem tissue and progressing toward the center of the internode with advancing maturity. In both pith parenchyma and xylem tissues, lignification began in the original primary wall and progressed into the secondary wall (Engels and Jung, 1998). Phloem fiber (mature primary phloem tissue) had a unique lignification pattern in that lignification also began in the primary wall, but for those individual cells with thickened primary walls, lignification occurred at the lumen edge of the primary wall and did not progress throughout the thick primary wall (Engels and Jung, 1998). In phloem fiber, the thick secondary wall never deposited lignin during maturation, contrary to the conclusion of Vallet et al. (1996). The final result was that phloem fiber developed lignified ring structures that only included a portion of the primary wall and did not include secondary wall.

Composition of the hemicellulose and pectin polysac-
Table 2. Composition of the hemicellulose and pectin polysaccharide fractions in Internode 7 as influenced by the main effects for sample maturity and alfalfa clone.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Hemicellulose components†</th>
<th>Pectin components‡</th>
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<tr>
<td></td>
<td>Xyl</td>
<td>Man</td>
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<tr>
<td>Clone</td>
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</tr>
<tr>
<td>First</td>
<td>520</td>
<td>426</td>
</tr>
<tr>
<td>Second</td>
<td>640</td>
<td>371</td>
</tr>
<tr>
<td>Third</td>
<td>781</td>
<td>198</td>
</tr>
<tr>
<td>Fourth</td>
<td>811</td>
<td>178</td>
</tr>
<tr>
<td>Fifth</td>
<td>804</td>
<td>187</td>
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<td>SE</td>
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<td>718</td>
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<td>244</td>
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<td>SE</td>
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† Xyl, xylose; Man, mannose; Fuc, fucose.
‡ UA, uronic acids; Ara, arabinose; Gal, galactose; Rha, rhamnose.
§ Means within the same column and main effect not sharing the same letter are different (P < 0.05).

Tissues changed during maturation (Table 2). The proportion of xylose in hemicellulose increased by >50% between the first and fourth sampling dates as mannose and fucose components declined. The effect of maturation on pectin composition was not as pronounced with only a small increase in the proportion of uronics and a decrease in galactose content. The proportion of pectin uronics was probably due to the inclusion of 4-O-methyleuronic acid in the pectin fraction when this acidic sugar is actually a part of alfalfa hemicellulose, but our analytical procedures could not differentiate between galacturonic and glucuronic acids. Significant differences were determined among the alfalfa clones for composition of hemicellulose and pectin (Table 2). While the clone × maturity interactions were generally significant for composition of hemicellulose and pectin, the differences were small in magnitude and maturity trends were similar among the clones (data not shown).

Lignin composition also changed with maturity (Fig. 7). The syringyl:guaiacyl monolignol ratio indicated that at the time elongation ended around the third sampling date, the lignin being deposited shifted to a more syringyl-rich form of lignin. This change in the type of lignin being deposited in the cell walls continued to shift the syringyl:guaiacyl ratio as the internode matured further. All three clones were different in lignin composition for the 2 yr data set (0.64, 0.73, and 0.87 syringyl:guaiacyl ratios for Clone 143, 403, and 718, respectively).

Microscopic observations showed that xylem was the predominant tissue type that proliferated through cambial activity after elongation of alfalfa internodes ended and that xylem tissues had thin primary cell walls and thick secondary walls (Engels and Jung, 1998). Histochemical staining indicated the presence of a small amount of pectin in the primary walls and high concentrations of lignin in both primary and secondary walls of secondary xylem tissues. Because secondary xylem was the only alfalfa stem tissue that proliferated after elongation ended, it can be inferred from our data that the cell walls of secondary xylem contained more xylan and less mannan, and have a more syringyl-rich lignin than the other tissues in the alfalfa stem. We estimate that alfalfa secondary xylem has a cell wall concentration of =750 g kg⁻¹ organic matter with a composition of 400 g cellulose, 200 g hemicellulose, 200 g pectin, and 200 g lignin kg⁻¹ cell wall. These estimates are based on the fact that cell wall concentration and composition of Internode 7 did not change between the fourth and fifth sampling dates, even though secondary xylem proliferation increased the xylem proportion of the internode cross sections by 50% (Fig. 3). However, it must be noted that a portion of the cellulose, and presumably other polysaccharides, deposited in cell walls formed during internode maturation derive from the development of the thick, nonlignified secondary wall in the phloem fiber, although this tissue did not proliferate through the addition of new cells after internode elongation ceased.

Tissue and Cell Wall Degradability

As Internode 7 of alfalfa stems became more mature, the amount of tissue remaining undegraded after a 48-h in vitro incubation with rumen microorganisms increased markedly (Fig. 5). Only the lignified, spiral ring structure of primary xylem vessels (Engels and Jung, 1998) could be identified as a relatively intact tissue after degradation of the youngest sample of Internode 7 (Fig. 5f). Primary xylem was the only lignified tissue at the first sampling date and all other stem tissues appeared to have been completely degraded by the rumen microbes. The same result was seen for the second sampling date (Fig. 5g), although a few individual internode sections from the second sampling date contained some lignified secondary xylem and this tissue also remained as a fermentation residue after incubation with rumen microbes (data not shown). Tissues with only nonlignified, primary walls (collenchyma, chlorenchyma, secondary phloem, cambium, and primary xylem parenchyma) remained completely degradable at all stages of stem development and maturation (Fig. 5). In contrast, the xylem, phloem fiber, and pith parenchyma tissues all became less degradable during internode maturation. Waxy cuticle, secondary xylem vessels, and xylem fiber residues remained after degradation of the third sampling date internodes (Fig. 5h). A thin-walled residue of the phloem fiber, xylem tissues, and some pith parenchyma on the periphery remained after degradation of internodes from the fourth sampling date (Fig. 5i). By the last sampling date, all pith parenchyma cells remained undegraded (Fig. 5j). The effect of maturity on degradability of these tissues is quantified in Table 3.

A critical turning point for the degradation of some alfalfa stem tissues was observed at the third sampling date when elongation of Internode 7 had ended. When elongation of the internode ended, secondary xylem
tissue proliferation and lignification of internode tissues began. It was at this point in development that secondary xylem vessels and xylem fibers could be identified in the degradation residue (Fig. 5h). Only the xylem cells immediately adjacent to the cambium and most recently formed were degradable because xylem tissue lignified almost immediately upon formation (Engels and Jung, 1998). Progressively more xylem tissue was formed and remained undegraded after the 48-h in vitro incubation with rumen microbes as maturity progressed beyond the end of elongation (Fig. 5d, e, i, j). Jung and Engels (2001) found that only marginal thinning of xylem cell walls occurred after even 96 h of degradation of alfalfa internodes harvested after 31 d of regrowth. However, some individual xylem fiber cells deposit an additional secondary wall layer on the lumen side of the cell, which is only minimally lignified (Engels and Jung, 1998). This additional secondary wall was partially degradable (Table 3).

Primary phloem was completely degradable in most Internode 7 sections from the first through the third sampling dates. By the fourth sampling date, primary phloem had begun to mature into phloem fiber by deposition of a secondary cell wall and lignification of its primary wall (Engels and Jung, 1998). Lignification of phloem fiber coincided with the appearance of a thin ring structure in the in vitro degradation residues (Fig. 5d, i). This residue was the unique lignified ring structure that developed in the primary wall of alfalfa phloem fiber tissue (Engels and Jung, 1998). Nonlignified portions of the primary wall from phloem fiber remained completely degradable with further maturation. Also, the secondary wall of phloem fiber, which did not lignify (Engels and Jung, 1998), remained completely degradable at both the fourth and fifth sampling dates (Fig. 5d, e, i, j).

Pith parenchyma underwent limited cell wall thickening during internode maturation and ultimately became lignified in 1996 (Fig. 5). These changes in cell wall development of pith parenchyma were associated with a progressive decline in degradability of the tissue, from completely degradable to entirely undegradable (Fig. 5). The decrease in pith parenchyma degradability began at the end of elongation and was first observed along the outer edge of the pith, next to xylem fibers (data not shown). This change from degradable to undegradable pith parenchyma cells progressed inward to the center of the pith region as maturity of the internode advanced. This pattern for changing pith parenchyma degradability mirrored the pattern for lignin deposition in this tissue (Engels and Jung, 1998).

Reduced degradability of alfalfa stem tissues was always associated with the deposition of lignin in cell walls of these tissues, except for the epidermis which did not lignify but did exhibit a decline in degradability. The epidermis was completely degradable in the youngest internode samples, but a thin sheet of residue from the epidermis remained from the third sampling date onwards (Fig. 5h). It appeared that the only part of the epidermal tissue remaining after in vitro degradation was the waxy cuticle layer on the outside surface of the epidermis. Apparently the entire polysaccharide cell wall matrix was degraded.

On the basis of visual appraisal of degradation extent, differences were detected among the three alfalfa clones for degradability of the primary wall of phloem fiber and pith parenchyma (Table 3). Clone 718 was the least degradable genotype, while Clone 403 was intermediate between Clones 143 and 718 in degradability of these tissues. The least degradable tissues (xylem vessels and fiber) did not appear to differ in degradability among the clones.

Because of insufficient amounts of sample, the in vitro degradability of cell wall polysaccharides of the dried internodes from the youngest maturity stage sampled in 1996 could not be determined. Figure 8 illustrates the patterns for cellulose, hemicellulose, and pectin degradation across the remaining four maturity stages. After 12-h incubations, pectin was almost completely degraded from the cell walls of internodes collected on the second sampling date (Fig. 8a). The pectin degraded during the 12-h incubations probably originated mainly from the cell walls of epidermis, collenchyma, and chlorenchyma because these tissues were previously shown to be completely degraded after only 8 h of microbial activity (Jung and Engels, 2001). Degradability of the pectin declined with advancing maturity, but remained >600 kg ha⁻¹ for even the most mature internode cell walls. The small decline in pectin degradation observed with maturation was partially due to inclusion of 4-O-methylglucuronic acid residues from alfalfa hemicellulose in the pectin fraction. Lengthening the time of fermentation to 96 h had no effect on extent of pectin degradability (Fig. 8b), indicating that the potentially degradable pectin in the cell walls of alfalfa internodes was very rapidly degraded. Degradability of cellulose

<table>
<thead>
<tr>
<th>Sample date</th>
<th>pf-f</th>
<th>ssv</th>
<th>xf</th>
<th>xf+</th>
<th>pi</th>
</tr>
</thead>
<tbody>
<tr>
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<td>nd</td>
<td>3.00a</td>
<td>nd</td>
<td>3.00a</td>
</tr>
<tr>
<td>Second</td>
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<td>nd</td>
<td>2.78a</td>
<td>nd</td>
<td>3.00a</td>
</tr>
<tr>
<td>Third</td>
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<td>1.67a</td>
<td>1.22b</td>
<td>nd</td>
<td>2.56a</td>
</tr>
<tr>
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<td>0.50b</td>
<td>0c</td>
<td>2.00a</td>
<td>1.44b</td>
</tr>
<tr>
<td>Fifth</td>
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<td>0c</td>
<td>0c</td>
<td>1.38b</td>
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</tr>
<tr>
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<td>0.14</td>
<td>0.21</td>
<td>0.12</td>
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</tr>
</tbody>
</table>

Table 3. Mean degradability scores for cell-wall tissues in 100 μm thin sections from Internode 7 of three alfalfa clones sampled in 1996 after 48-h in vitro fermentations within rumen microbes. Cross-sections were scored for extent of degradation using the following scale: 0, undegraded; 1, <50% of the walls degraded for thin-walled tissues or partial thinning of thick walls; 2, >50% of the walls degraded, but not complete degradation, for thin-walled tissues or extensive thinning of thick walls; and 3, complete degradation of the wall.

<table>
<thead>
<tr>
<th>Clone</th>
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<th>ssv</th>
<th>xf</th>
<th>xf+</th>
<th>pi</th>
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<td>0.07</td>
<td>0.14</td>
<td>0.16</td>
<td>0.05</td>
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</table>

| † pf-f, primary wall of phloem fiber; ssv, secondary xylem vessels; xf, xylem fiber; xf+, additional secondary wall of xylem fiber; pi, pith parenchyma. |
| ‡ Means within the same column and main effect not sharing the same letter are different (P < 0.05). |
| § Cell-wall structure not detected (nd). |
and hemicellulose were the same after 12 h of fermentation, except for the most mature samples (Fig. 8a). Increasing the incubations to 96 h in length resulted in marked increases in cellulose and hemicellulose degradation (Fig. 8b), unlike the lack of response for pectin degradation to longer incubation times. Also, extended fermentations caused greater degradabilities for cellulose than hemicellulose at all maturity stages, except the second sampling date. At this youngest maturity stage, the degradabilities of cellulose and hemicellulose were not different and almost as great as observed for pectin.

Clonal differences were detected after both 12- and 96-h incubations. Degradability of the total cell wall polysaccharides was least for alfalfa Clone 718 after 12 h of fermentation with rumen microbes (Fig. 8c). The other two clones did not differ from each other in overall 12-h cell wall polysaccharide degradation. The lower 12-h degradability for total cell wall polysaccharides of Clone 718 was due to lower degradabilities of the cellulose and pectin fractions (data not shown). The clone × maturity interaction was significant for the 96-h incubations (Fig. 8d). No differences in cell wall polysaccharide degradability were observed among clones for the second and third sampling dates, Clone 143 was the most degradable at the fourth maturity level, and Clones 143 and 718 were different in degradability for the most mature internode cell walls. These data indicating Clones 143 and 718 had high and low cell wall polysaccharide degradabilities, respectively, were in general agreement with the microscopic assessment of alfalfa clonal differences in cell wall degradation (Table 3).

Isolated alfalfa pectins were very rapidly degraded by rumen microbes in vitro (Hatfield and Weimer, 1995). The pectic fraction of both alfalfa and red clover (Trifolium pratense L.) was also rapidly degraded from ground forage samples (Chesson and Monro, 1982; Hatfield and Weimer, 1995). Using thin sections, Jung and Engels (2001) demonstrated that pectin-rich tissues in alfalfa (chlorenchyma, collenchyma, epidermis, and the primary wall of phloem fiber) were completely degraded in vitro within 8 h by rumen microbes. Therefore, it was not surprising that increasing time available for microbial activity from 12 to 96 h did not alter the extent of pectin degradation in the current study. As shown in Fig. 5, the pectin-rich alfalfa tissues were completely
degradable from all maturity stages of Internode 7. The data from the first two sampling dates of our experiment, when only primary xylem vessels were lignified while all other tissues consisted only of nonlignified primary walls, indicated that primary cell walls in alfalfa contained approximately twice as much pectin as did thick secondary, lignified xylem cell walls (Fig. 6). It appears that most pectin in alfalfa is located in tissues that do not lignify during maturation, and therefore remains highly degradable. The rapid and high degradability of pectin, even from mature alfalfa internodes, is a reflection of both the intrinsically high degradability of pectin and its distinctive distribution pattern among alfalfa stem tissues.

Degradation rate of isolated cellulose by rumen bacteria ranges from 0.05 to 0.08 h⁻¹ (Weimer, 1996). Hemicellulose degradation rates range across a wider interval (0.07 to 0.26 h⁻¹), presumably because of the diversity of structures (sugar residues and linkages) found among different hemicelluloses (Hespell and Cotta, 1995). These rates of cellulose and hemicellulose degradation are substantially lower than observed for isolated pectins (0.30 to 0.50 h⁻¹) from alfalfa and other sources (Hatfield and Weiner, 1995). Degradation rates of cell wall polysaccharides from forage cell walls are similar to those observed for the isolated polysaccharides (Mertens, 1993), and the slow degradation rates for cellulose and hemicellulose explain why increasing length of the in vitro incubation increased observed extent of degradation for these polysaccharides in the current study (Fig. 8). Cellulose and hemicellulose typically have much lower potential extents of degradation than pectin, even when time for degradation is not limited (Buxton, 1991). This indicates that because most of the alfalfa stem cellulose and hemicellulose is concentrated in lignified xylem tissues, lignification is responsible for their limited potential degradability. Obviously, the presence of lignin in alfalfa cell walls is critical to the potential degradation of cell wall polysaccharides, but unfortunately, lignin concentration alone does not explain variability of forage cell wall degradation in alfalfa (Jung and Deetz, 1993; Jung et al., 2000). It would appear that reducing lignification of secondary xylem tissues may increase cell wall polysaccharide degradability; however, the modification should probably be limited to xylem fiber because reduced lignification of xylem vessels caused vessel collapse in transgenic tobacco (Nicotiana tabacum L.) (Piquemal et al., 1998). The presence of lignin in primary xylem vessels very early in alfalfa development may signal its importance to water transport and normal plant development (Engels and Jung, 1998).

CONCLUSIONS

As alfalfa stem internodes mature, the internode tissues follow different developmental pathways. Some tissues (such as cambium and chlorenchyma) do not develop thickened walls, while a few tissues (epidermis, collenchyma, and primary phloem) develop thick primary walls. Cambium, chlorenchyma, epidermis, and collenchyma do not lignify during alfalfa maturation and always remain completely degradable by rumen microbes. The high degradability of pectin from alfalfa cell walls results from the fact that the majority of the stem pectin is located in these nonlignified tissues. The cellulose and hemicellulose in these nonlignified tissues is also completely degradable. As alfalfa internodes mature, there is a proliferation of cellulose- and hemicellulose-rich xylem tissues. These xylem tissues lignify almost immediately upon their formation and are almost completely undegradable. Because secondary xylem accounts for the bulk of biomass accumulation by stems, it is the proliferation of secondary xylem tissues through cambial activity that causes the decline in alfalfa stem degradability. Improving forage quality of alfalfa should target lignin deposition in secondary xylem tissues to increase the potential degradability of the cellulose and hemicellulose located in these tissues.

REFERENCES


Hatfield, R.D., J.R. Wilson, and D.R. Mertens. 1999. Composition of
Application of Canonical Discriminant Analysis for the Assessment of Genetic Variation in Tall Fescue

Ravi Vaylay and Edzard van Santen*

ABSTRACT

Tall fescue pastures are perennial in habit and are continually exposed to intensive natural selective forces. Therefore, the genetic composition of tall fescue cultivars changes with time and the selection is cumulative. The objective of this study was to investigate the genetic diversity of tall fescue cultivars and age groups within cultivars in response to natural selective forces using a multivariate statistical method, canonical discriminant analysis. Ungrazed survivors from four cultivars (GA-5 EF, GA-5 EI, Johnstone, and KY-31) were collected randomly from four 1-yr-old paddocks. These paddocks included among the age groups in GA-5 EF, GA-5 EI, and Johnstone. The age groups of KY-31 were stable in terms of genetic variation. Significant genetic diversity was observed among the age groups in GA-5 EF, GA-5 EI, and Johnstone.

A n insight into the magnitude of variability present in crop species is of utmost importance, as it provides the basis for effective selection. Phenotypic variation present in a population arises due to genotypic and environmental effects. Phenotypic variability is the observable variation present in a character in a population; it includes both genotypic and environmental components of variation and, as a result the magnitude of phenotypic variability, differs under different environmental conditions. Genotypic variation, on the other hand, is the component of variation that is due to the genotypic differences among individuals within a population or among populations within a species, and is the main concern of a plant breeder. The phenotype is based on quantitative characters and has a strong genotypic basis, though it often cannot be directly related to genotype (Loos, 1993). If phenotype observations are based on sufficiently large sample sizes and the traits measured show significant differences among populations, they can provide a reasonable representation of overall genetic performance (Humphreys, 1991).

Genetic variation may be measured in several ways. Considerable overlapping may occur in univariate analysis, since each variable is viewed separately. In canonical discriminant analysis, a multivariate statistical technique, all independent variables (traits) are considered simultaneously in the differentiation of cultivars. The resulting differentiation of populations is more distinct compared with univariate analysis. It extracts compo-

Abbreviations: $D^2$, Mahalanobis distance; NDF, neutral detergent fiber.