Cell Wall Fractionation of Alfalfa Stem in Relation to Internode Development: Biochemistry Aspect

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In addition to morphological factors (height of the plant, proportion of stems), alfalfa quality is related to several biochemical factors, especially the content and monomeric composition of the cell wall components. This work was aimed at studying internode development in relation to the chemical composition and fractionation of cell walls. Analyses were performed on the fourth apical internodes (elongating), the two following ones (onset of cambial activity), and bottom internodes (mature). Deposition of guaiacyl type lignin occurred in the pectin rich cell walls of apical internodes. The onset of cambial activity corresponded to cell wall accumulation of syringyl−guaiacyl lignin, xylans and/or glucuronoxylans, and cellulose. Such events were related to the thickening of secondary cell walls which proportion increased in mature internodes. Chemical fractionation of the cell walls allowed the release of a high content of water soluble pectins from apical internodes, whereas alkalis were more efficient in extracting heteroxylans from bottom internodes. A partial release of lignin from the cell walls also occurred during the extraction steps. Lignin solubilization was enhanced by the cleavage of labile-ether lignin structures and appeared closely related to the extent of internode differentiation along the stem. Data gained from chemical fractionations evidenced that lignin−xylan−pectic complexes accumulated progressively in secondary wall rich tissues.

Keywords: *Cell wall; lignin; structural polysaccharides; growth; length; internode; Medicago sativa*

INTRODUCTION

Alfalfa is a valuable forage crop because of its high protein content and acceptability by cattle. However, the fiber fraction of alfalfa is often poorly digested. The feeding value of alfalfa leaves remains high throughout the growing season in contrast to its stems whose nutritive value declines during plant maturation (Kühbauch and Voigtländer, 1981). The depressed digestibility of alfalfa cell walls has been ascribed to increased maturity and decreased leaf:stem ratio (Nordvisk and Aman, 1986; Lemaire and Allirand, 1993; Kühbauch and Voigtländer, 1981). The feeding value of stems declines during plant maturation due to cell wall component deposition (lignin and polysaccharides) (Buxton and Hornstein, 1986).

Plant cell walls consist mostly of pectin, hemicellulose, cellulose, and lignin. Cell wall feeding quality is mainly related to the cross-links existing between polysaccharides and lignin (Jung, 1989). These are dependent on the structural composition of each wall polymer. The structural polysaccharides are only partly digested in the rumen; lignin is considered to be the main impediment to cell wall degradability, presumably by reducing access to the carbohydrates by rumen bacteria. However, results from several forage grass species have failed to identify lignin content as a primary predictor of forage digestibility when the effects of maturity are eliminated (Jung and Casler, 1991; Jung and Vogel, 1986); lignin interactions with polysaccharides may be a major factor (Chesson, 1993). In addition, spatial distribution of lignified tissues across the stem and the shape of the cells affect the accessibility of noncellulosic polysaccharides (Wilson and Mertens, 1995; Wilson, 1993; Kühbauch and Bestajovsky, 1989).

Correlation between dicot cell wall structural modification and maturation were obtained with plants characterized at different stages of development. However, only a few studies are available describing the variation of cell wall carbohydrate composition along the stem (Hatfield, 1992). In addition, most investigations on alfalfa feeding value have focused on the restricted accessibility of the noncellulosic polysaccharides along with lignin deposition while looking mainly at lignin concentration rather than composition.

The objective of this study was then to determine quantitative and qualitative variations of both structural carbohydrate and lignin within alfalfa stem regions which differ in their maturity. For convenience, internode was defined as the stem segment located between two leaf bases. The physiological stage of the selected internodes (from the apical part to the basal part of the stem) was determined by histochemical evaluation of tissue differentiation across the internodes and measurement of the length of the corresponding internodes. Four kinds of internodes were thus selected starting at the apex and going to the base of the stem. The corresponding cell walls were investigated for the content and composition of their main components, noncellulosic polysaccharides and lignin. The chemical

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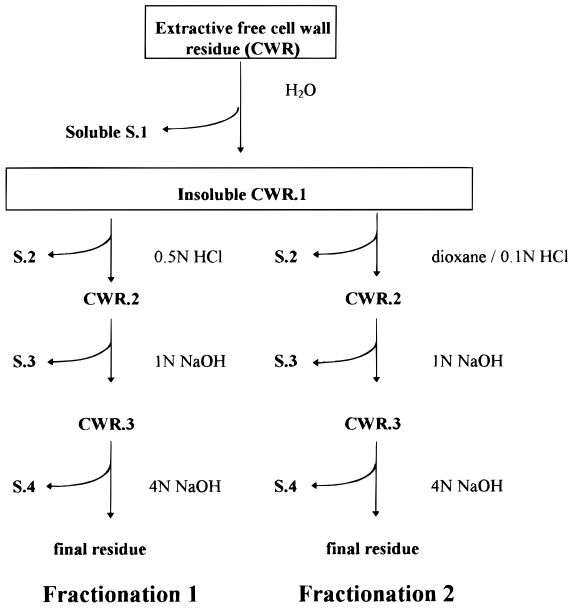

Figure 1. Schedule of the procedure used in the fractionation of alfalfa cell wall components.

fractionation of the cell walls was performed to investigate changes in the wall material extractibility among the distinct stem regions. Cell wall bound phenolic acids are found at very low levels as compared to monocot cell walls (Jung, 1989) and were not studied in this study.

MATERIALS AND METHODS

Sample Collection. Alfalfa (*Medicago sativa* L.) cultivar Europe was sown at Lusignan, France, in 1993 and collected during spring 1995. The plots (1 × 5 m) were set out in five blocks.

Twenty plants were selected from each block for internode length measurements. Dynamics of internode length was followed in the upper part of the stem; this region initially included five internodes. These ones and the newly formed internodes were then measured at 2 or 3 day intervals over a period of 2 weeks until bud formation. At this stage, plants were harvested; internodes were numbered from the apex to the base prior to measurement of the length and diameter. Three stems were further used for microscopic observation; each internode was thus stored in a water/ethanol mixture (4:6) at 4 °C.

For investigation of the cell wall components, the set of plants corresponding to the main part of the crop was also harvested at bud formation with a cut at 6 cm above ground level. Plant material collected from the blocks were combined and categorized according to the stem length into 10 cm height classes. The leaves were discarded, and stems belonging to the two longest classes (that represent the major proportion of the crop stems) were divided into internode groups. The first group containing four internodes was named IN 1−4. The following internodes were separated every two internodes (IN 5−6, IN 7−8, IN 9−10, IN 12−13) in order to supply enough material for chemical study. Samples were then freeze-dried and ground with a rotative knive mill. The ground materials were refluxed with water/ethanol (2:8) and freeze-dried to produce a cell wall residue (CWR).

Cell Wall Fractionation. CWRs were subjected to sequential extraction in order to remove pectic polysaccharides, noncellulosic polysaccharides, and lignin fractions. The procedure consisted of four steps conducted in duplicate, as depicted in Figure 1.

Fractionation without Mild Delignification Step (Fractionation 1). The first step consisted of water extraction on 600 mg of CWR with 100 mL of distilled water with continuous stirring in a water bath (80 °C) for 1 h. This step is known to remove pectic polysaccharides (Fry, 1988). The water insoluble residue (CWR_1) was recovered by filtration on a Buchner funnel equipped with a glass fiber filter (1.0 μm retention) and washed with water. CWR_1 was subsequently treated with 50 mL of diluted hydrochloric acid (0.5 N) with continuous stirring in a water bath (85 °C) for 1 h. The resulting residue, CWR_2 was recovered as previously described. Extractions with water and diluted acid were repeated twice.

The following steps consisted of 1 M NaOH and 4 M NaOH extractions in order to extract the noncellulosic polysaccharides (Fry, 1988; Titgemeyer et al., 1992). CWR_2 was suspended in 100 mL of 1 M NaOH containing 3 mg/mL $NaBH_4$. The bottle was flushed with nitrogen, capped, and stirred overnight at room temperature. After extraction, the residue CWR_3 was filtered on a Buchner funnel with a glass fiber filter and washed with water until the filtrate was neutral. Extraction of CWR_3 was then performed with 4 M NaOH as above. This last step gave rise to the CWR_4, which was freeze-dried prior to analysis of individual neutral sugars and lignin. In each case, the alkali extracts and the wash were combined and dialyzed against running water for 48 h.

The four filtrate volumes (S.1, S.2, S.3, S.4) were reduced to 10 mL under vacuum evaporation and stored at 4 °C. The four filtrates were analyzed for total sugars, total uronic acids, and individual neutral sugars.

Fractionation with Mild Delignification Step (Fractionation 2). This fractionation was similar to the previous one except that dioxane/0.1 N HCl was used instead of the 0.5 N HCl in order to extract part of the lignin (Monties, 1988). This experiment did not aim at a total lignin solubilization as is usually allowed by chlorite reagent. The fractionation was applied to two kinds of internodes (IN 5−6 and IN 12−13) and compared to the procedure run without delignification.

The residue obtained after each extraction was freeze-dried. Part of the residue was kept for chemical analysis, whereas the major part was submitted to the next treatment. The residues and filtrates were treated as in the former fractionation performed without delignification. The carbohydrate fractions released by the treatments were analyzed as above.

Lignin Analysis. Lignin content was determined by spectrophotometric procedure using acetyl bromide reagent (Iiyama and Wallis, 1990) and spruce milled wood lignin (containing 5% neutral sugar as determined by Kurek et al. (1990)) as reference.

Lignin monomeric composition was determined by thioacidolysis. The reaction was performed in duplicate for 4 h at 100 °C on 12 mg of CWR, in a 10 mL mixture of dioxane:ethanethiol (9:1, v/v) containing 0.2 M boron trifluoride etherate (Lapierre et al., 1986). Monomeric products, recovered from the mixture by dichloromethane extraction, were analyzed by capillary column gas chromatography as trimethylsilyl derivatives. Separation was performed on a SPB1 column (30 m × 0.3 mm; 0.25 μm film thickness) using a temperature gradient of 170−280 °C at 2 °C/min. Helium was used as the carrier gas at 0.5 mL/min. Detection was flame ionization. Amounts were calculated with reference to the internal standard (tetracosane).

Sugar Analysis. The total sugar analysis was performed on filtrates according to the phenol sulfuric acid method (Saha and Brewer, 1994) using glucose as standard. Total uronics were analyzed using the *m*-hydroxydiphenyl method (Blumenkrantz and Asboe-Hansen, 1973) and galacturonic acid as standard.

Neutral sugar composition of CWR before and after extraction was determined according to Blakeney et al. (1983). Isolated polysaccharides were hydrolyzed with 2 M sulfuric acid at 100 °C for 2 h. Neutral sugars were quantified as their alditol acetates by gas chromatography using the procedure of Englyst and Cummings (1984) using inositol as an internal standard. Alditol acetates were separated on a SP-2380 column (30 m × 0.25 mm internal diameter; 0.20 μm film; Supelco) using a temperature gradient of 230−250 °C at 2 °C/min. The carrier gas was helium (0.8 mL/min). Detection was flame ionization.

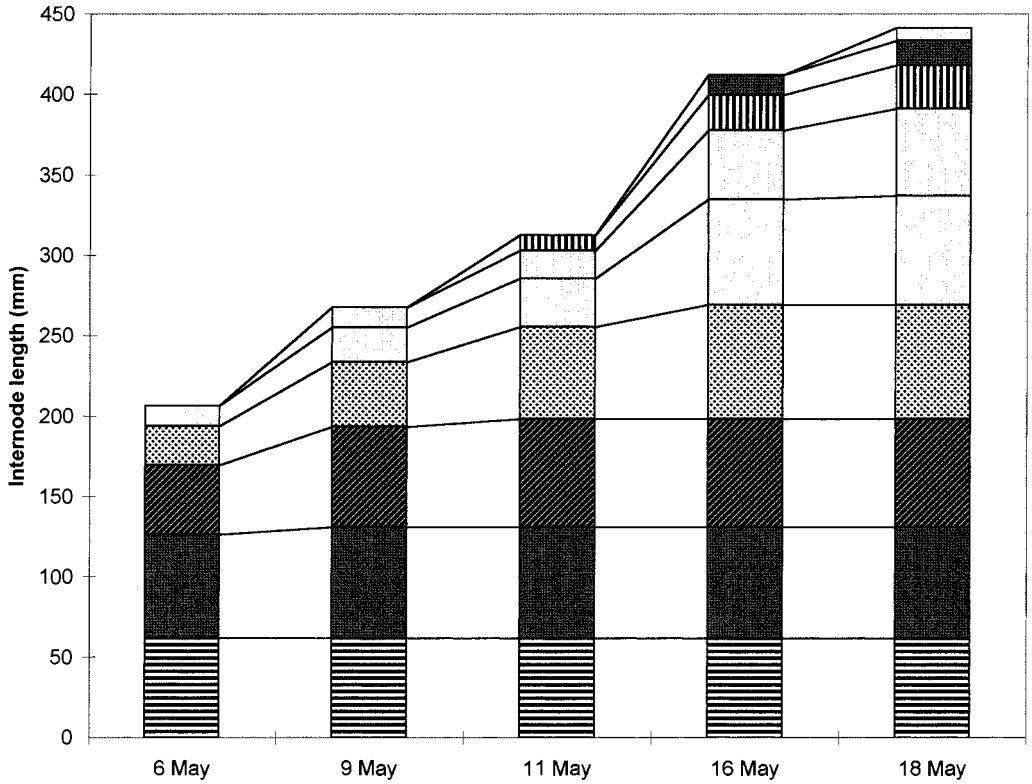

Figure 2. Internode length of alfalfa growing stem region. Each bar corresponds to one internode; the first one is located at the top of the stem.

Microscopy. Internode sections (60 μm) were obtained with a freezing microtome and stained with acidic phloroglucinol which produces a reddish color with lignins containing cinnamaldehyde groups (Adler et al., 1948). The samples were incubated 5 min in 2% phloroglucinol ethanolic solution. The sections were mounted in 6 N HCl solution prior to observation.

RESULTS

Morphological Development of Stems and Tissue Differentiation. The dynamic of the growing stem region, as determined over a period of 2 weeks on 20 plants, was described in Figure 2 by the length of internodes. Four internodes were newly formed within the 2 weeks of measurements. The length of the fourth apical internodes increased but kept constant from the fifth internode and lower. This result was observed at any stage of measurement. The most basal internode had already reached its maximum length at the beginning of the experiment but was shorter than the above internodes.

Morphological traits were described for the whole stem prior to cell wall chemical investigations. Length and diameter are reported in Figure 3. The length of internodes increased from 1 to 4, and they were thus called elongating internodes. The diameter of these internodes increased quickly. Microscopic observation showed the occurrence of primary tissue only, primary xylem being lignified in contrast to sclerenchyma fibers (Figure 3). From internodes 5 to 14, the internode length approached a steady state (or near constant length), indicating that these internodes had just stopped their elongation growth. The internode 5 represented the stage of the onset of cambial activity which generated an incomplete ring of lignified secondary xylem elements. Internode lengthening stopped but internode diameter still increased due to cambial activity which produced a large circle of secondary xylem. Mature internodes 9−12 were shorter than fully elongated internodes 5−8. Mature internodes were characterized by a complete and thick ring of vascular tissues which had thick secondary walls.

On the basis of morphological data, chemical analysis was performed on some selected internodes corresponding to the following stages: IN 1−4 were elongating internodes, IN 5−6 had just stopped elongation growth, and IN 9−10 and IN 12−13 corresponded to the mature part of the stem.

Cell Wall Composition. The cell wall residue recovered from hot ethanol extraction included mainly structural polysaccharides and lignin. As internode age increased, the content of cell walls increased dramatically (53−76% for IN 1−4 to IN 12−13), as reported in Table 1. For internodes 1−4, the total (polysaccharides−lignin) accounted for about 60% of the CWR. The remaining 40% might originate from the occurrence of metabolically active protein in such young tissues. The total nitrogen of CWR represented almost 5% of the ethanol residues (data not shown) and thus could bring out 30% protein (N $\times$ 6.25). The highest amounts of lignin and total sugars (nearly 90%) were observed in the cell walls of internodes 12−13. Accordingly, less protein contamination in these CWRs was evidenced as the nitrogen proportion represented 1.2% of the ethanol residue of internodes 12−13, i.e., nearly 7% protein.

Thioacidolysis allows the release of thioethylated derivatives of the mono- and dimethoxylated cinnamyl alcohols (as guaiacyl and syringyl residues, respectively) specifically involved in the labile-ether-linked lignin structure (Lapierre et al., 1986). The yields of ether-linked monomeric products recovered by thioacidolysis increased during aging; strong differences were obtained between the apical internodes (IN 1−4) and those

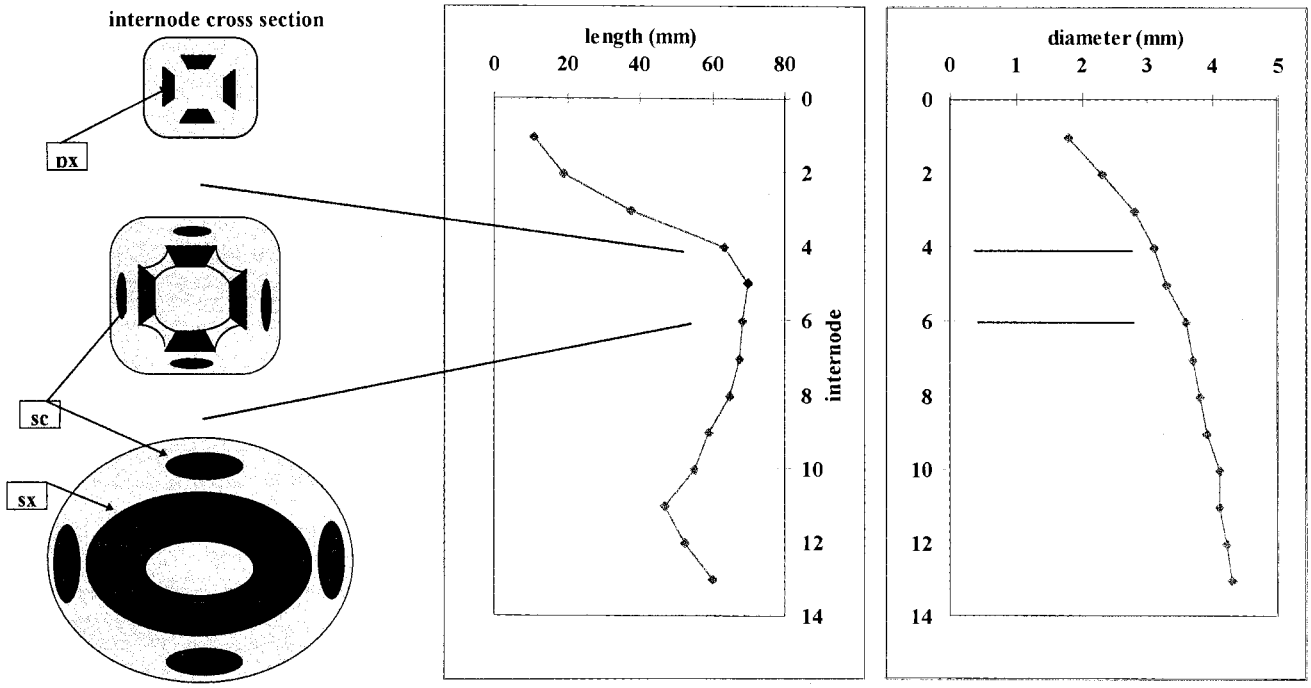

Figure 3. Morphological and schematic representation of tissular development of alfalfa internodes (black area represents lignified tissues: sc, sclerenchyma; px, primary xylem; sx, secondary xylem).

Table 1. Cell Wall Content and Composition of Alfalfa Internodes

	internodes			
	IN 1−4	IN 5−6	IN 9−10	IN 12−13
cell wall content[a]	53.0	60.1	73.4	76.4
acetyl bromide lignin[b]	9.5	12.6	19.6	21.6
guaiacyl[c]	186	359	465	497
syringyl[c]	41	141	260	272
total S+G[c]	227	500	725	769
ratio S/G	0.22	0.39	0.56	0.55
total sugar[b]	49.7	59.8	60.7	65.5
arabinose[b]	7.16	6.13	2.46	2.69
xylose[b]	4.87	7.34	11.3	11.62
mannose[b]	1.78	1.73	1.55	1.4
galactose[b]	3.54	3.48	1.87	1.64
glucose[b]	24.33	28.67	37.98	44.42
uronic acids[b]	20.9	16.7	11.6	11.2

[a] Cell wall content was determined after ethanol treatment of dry alfalfa internodes and express as percent of dry matter. [b] Lignin content, total sugar content, and individual sugar content expressed as percent of total cell wall. [c] Yields of lignin monomer products recovered by thioacidolysis, expressed in micromoles per gram of lignin.

having an active cambium (IN 12−13). Higher yields of recovered products (S + G) mainly reflected an increase of syringyl units from lignin of mature internodes compared to younger ones, as shown by the great increase in S/G molar ratio along the stem internodes.

Structural polysaccharides were enriched in xylose from apical to basal internodes, whereas arabinose and galactose residues represented a lower proportion of the noncellulosic polysaccharides (Table 1). Uronic acids that are representative of the pectic polysaccharides corresponded to a declining proportion of the total structural sugars from apical toward basal internodes. However, total sugars were recovered in the highest amount in bottom internodes. The highest content of arabinose, galactose, and uronic acids were found in

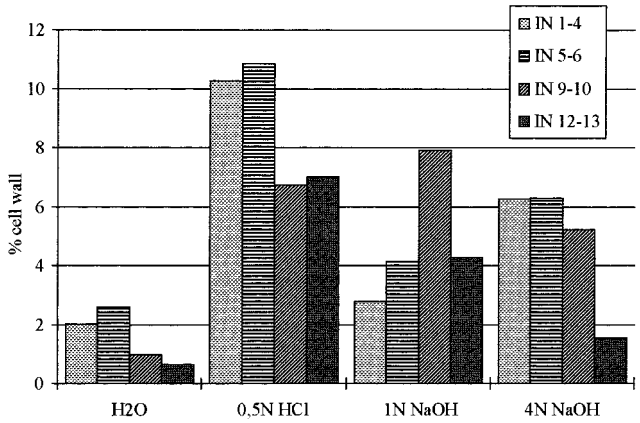

Figure 4. Neutral sugar content of the soluble fractions recovered from alfalfa internodes with H_2O, 0.5 N HCl, 1 N NaOH, and 4 N NaOH (expressed in percent of initial cell wall residue).

apical internode polysaccharides, whereas xylose and glucose were the major sugar of older internode polysaccharides.

Fractionation of Alfalfa Cell Walls. Results of fractionation 1 are reported in Figures 4 and 5. Various amounts of total sugar were released after sequential treatments of internode cell walls. Diluted hydrochloric acid was the most efficient in the removal of neutral sugars whatever the stage of internode differentiation was (Figure 4). Large quantities of neutral sugars were also removed by 1 N NaOH from the cell walls of bottom internodes (IN 9−10, IN 12−13). Different types of polysaccharides were extracted according to chemical treatments of cell walls (Figure 5). Water extracted mainly polysaccharides having arabinose and uronic residues. Sugar released by HCl also contained a large amount of arabinose and uronic acids but was enriched in xylose residue, indicating a concomitant removal of xylans to pectic materials by acidic conditions. Likewise, the xylose proportion of the cell wall HCl soluble

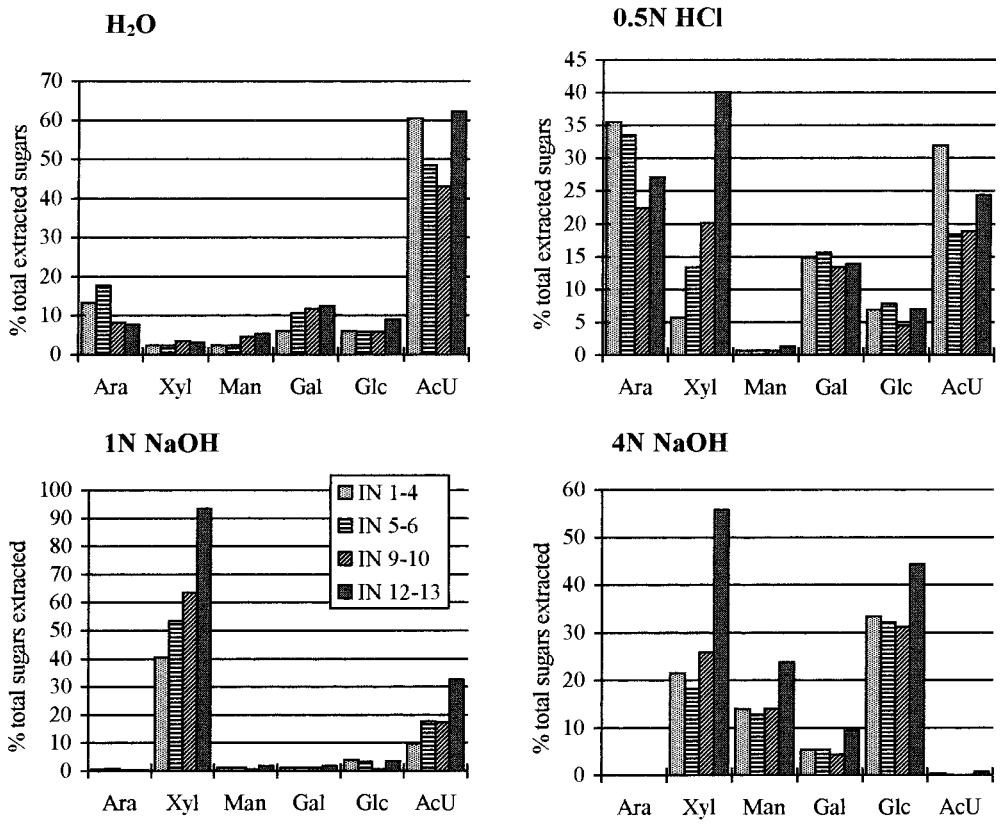

Figure 5. Neutral sugar composition and uronic acids content of the soluble fractions recovered with H_2O, 0.5 N HCl, 1 N NaOH, and 4 N NaOH from alfalfa internodes. Sugar contents are expressed in percent of total sugar present in the fraction (Ara, arabinose; Xyl, xylose; Man, mannose; Gal, galactose; Glu, glucose; AcU, uronic acids).

Table 2. Individual Sugar Composition and Lignin Content of the Final Residues Recovered from Chemical Fractionation 1 of Internode Cell Walls (as Percent of Final Residue; nd = Nondetected)

	sugar composition					
internode	Ara	Xyl	Man	Gal	Glc	lignin content
IN 1−4	0.39 ± 0.09	0.87 ± 0.09	0.48 ± 0.04	nd	83.7 ± 1.3	11.8 ± 0.6
IN 5−6	0.32± 0.03	0.94 ± 0.06	0.68 ± 0.01	nd	75.4 ± 2.0	18.9 ± 0.4
IN 9−10	0.28 ± 0.01	1.54 ± 0.12	0.42 ± 0.02	nd	66.3 ± 2.8	26.3 ± 0.7

fraction increased from the apex to the bottom of the stem. Alkali-released polysaccharides included mainly xylose, glucose, and fewer residues of mannose. Arabinose and uronic groups represented only minor proportions in these last fractions.

The final residue issued from the sequential treatment of the various internode cell walls was composed essentially of glucose (Table 2). However, as the internodes got older, from the top to the base of the stem, the remaining glucose represented a lower proportion of the original cell walls. This trend was concomitant with the largest proportion of lignin in the final residue issued from the oldest cell walls. In addition, the final residue contained xylose whose content showed a 2-fold increase from top to basal internodes.

Fractionation of Alfalfa Cell Walls Including Delignification. Two kinds of internodes, IN 5−6 that corresponded to the onset of cambial activity and IN 12−13 where a significant amount of secondary xylem was differentiated, were fractionated using dioxane/HCl (fractionation 2). This fractionation was run in parallel to the procedure performed without partial delignification (fractionation 1). The yields of cell wall material remaining after each step is reported in Figure 6. All residual fractions contained less lignin than initial cell wall residues. The fractionation 2 including dioxane/HCl allowed a larger proportion of lignin to be extracted than fractionation 1. The greatest release of lignin was obtained in the case of the mature internode (IN 12−13) (62.5% loss versus 33.6% without dioxane). In younger internodes (IN 5−6), the extent of lignin loss reached a total of 41.8% of the initial amount of the cell walls; when dioxane was used, this proportion represented 52.5%.

Dioxane/HCl removed more material than HCl alone, and this trend was more evident on old internodes. In addition, the dioxane step allowed the largest removal of lignin as compared to other steps involved in fractionation 2. In contrast, the first alkali extraction step of fractionation 1 (1 N NaOH) was the most efficient in removing lignin (Figure 6).

According to the chemical extraction, the monomeric composition of residual lignin was modified (Figure 7). As expected, the residue from dioxane/HCl extraction gave rise to lower recovery yields of lignin monomers by thioacidolysis than HCl residue in both types of internode. The subsequent 1 N NaOH extraction led to a residue which was enriched in labile-ether bonds as compared to HCl or HCl/dioxane residues. This behavior was found whatever the internode or fractionation studied but was emphasized in the case of basal internodes or after the dioxane step. Using both kinds

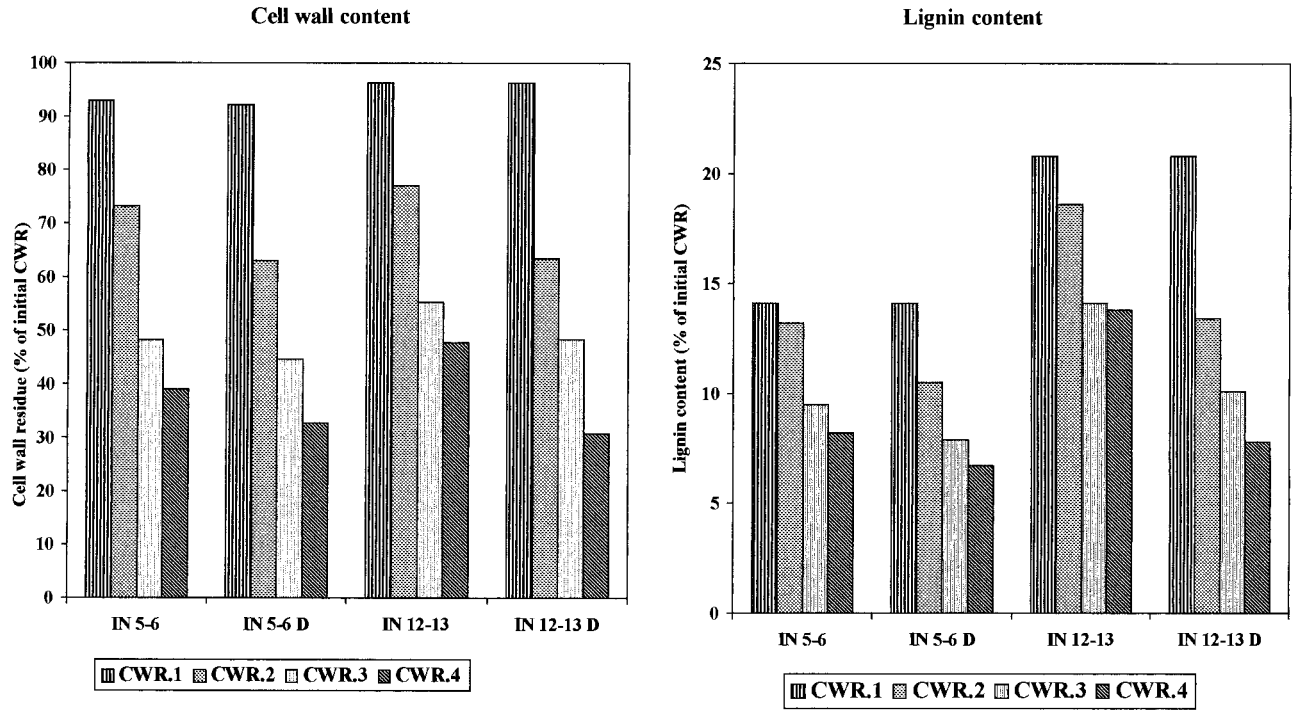

Figure 6. Yields of cell wall residue and lignin content at each step of the fractionation with (D) or without delignification performed on internodes 5−6 (IN 5−6, IN 5−6D) and on internodes 12−13 (IN 12−13, IN 12−13D). CWRs 1−4 were recovered from the following: H_2O, 0.5 N HCl or 0.1 N HCl/dioxane, 1 N NaOH, and 4 N NaOH extractions, respectively. Results are expressed as a percent of initial CWR.

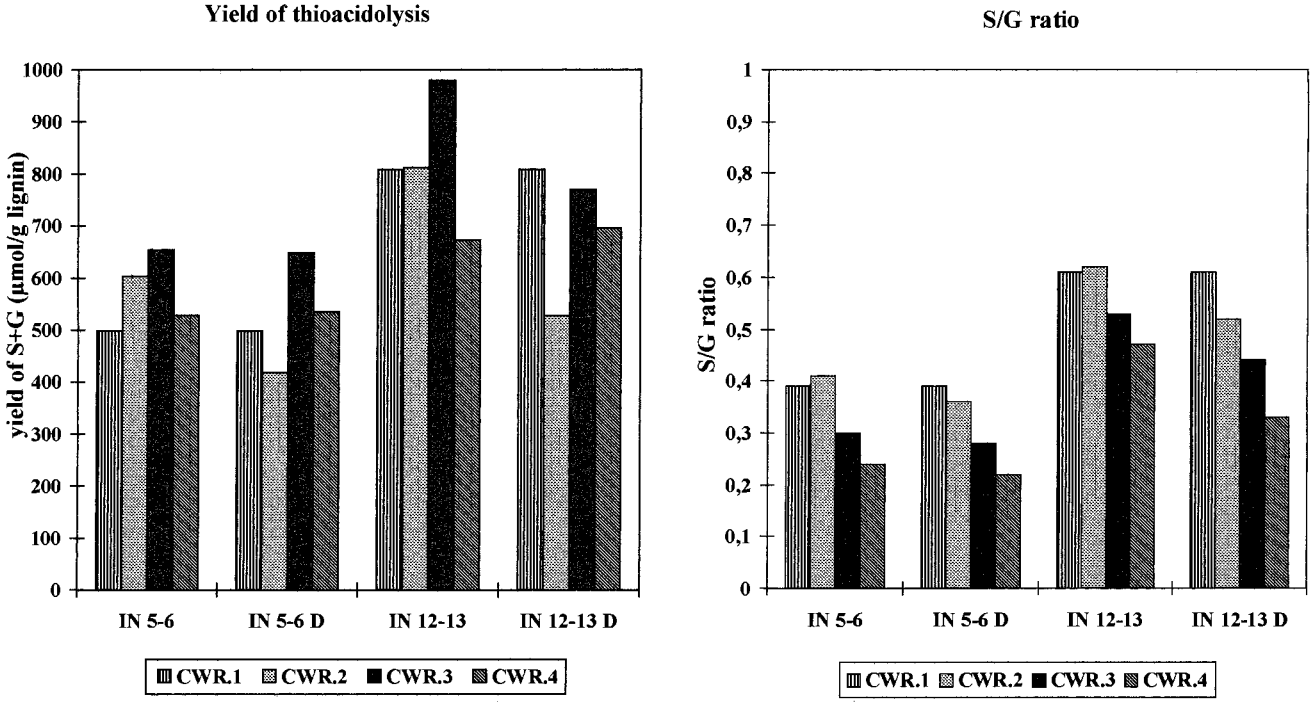

Figure 7. Monomer composition of the lignin of cell wall residues obtained after each step of fractionation with (D) or without the delignification step performed on internodes 5−6 (IN 5−6, IN 5−6D) and on internodes 12−13 (IN 12−13, IN 12−13D): thioacidolysis yields of lignin monomeric products (as micromoles per gram of lignin) and syringyl/guaiacyl molar ratio. CWRs 1−4 were recovered from water, 0.5 N HCl or 0.1 N HCl/dioxane, 1 N NaOH, and 4 N NaOH extractions, respectively.

of fractionation, the molar ratio S/G tended to decrease, especially when dioxane/HCl was applied to the cell walls.

The largest total amount of sugar was removed from the cell wall residue of internodes 12−13 when fractionation 2 (including dioxane/HCl) was used. In the case of internodes 5−6, the extent of total sugar removal and of uronic acids was similar in both fractionation procedures (Figure 8). However, the pattern of uronic acids release was distinct; the largest amounts were found in 1 N NaOH and 0.5 N HCl in the case of fractionation 2 and fractionation 1, respectively. Fractionation 2 improved the total removal of uronic acids from internodes 12−13 cell walls as compared to fractionation 1 (7.1% versus 5.5% initial CWR), but the release followed the same patterns in both fractionation

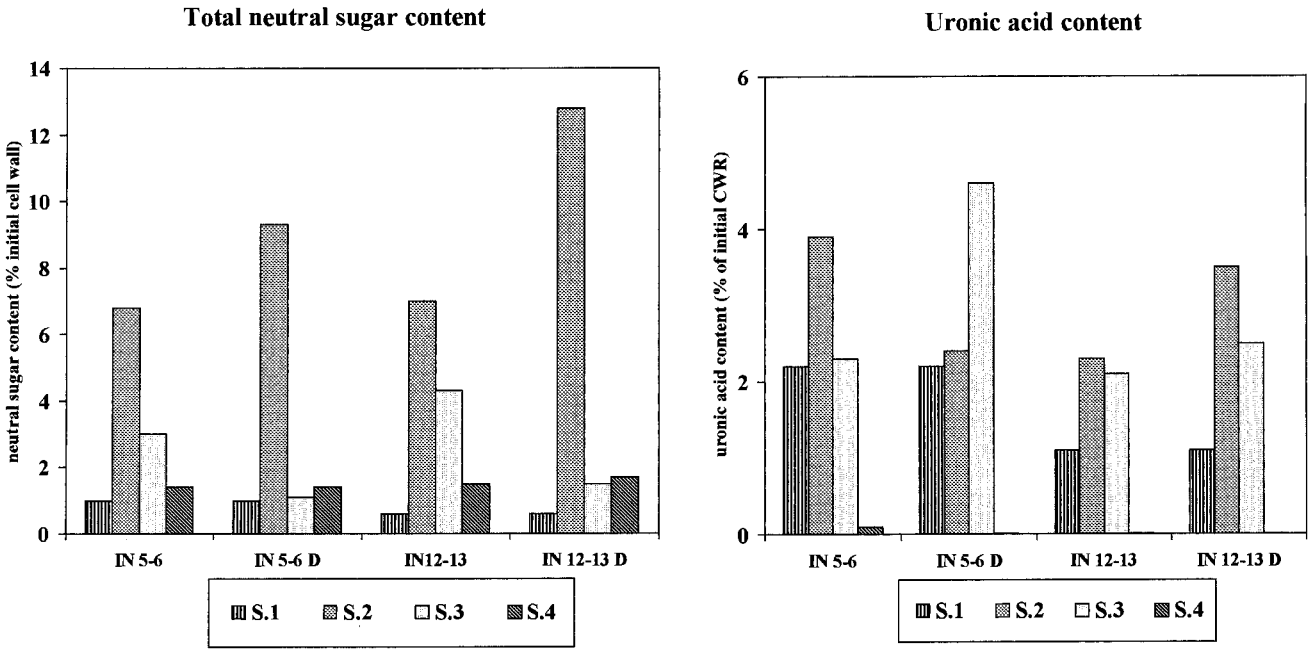

Figure 8. Total sugar content and uronic acids content of the fractions solubilized during fractionation with (D) or without the delignification step performed on internodes 5−6 (IN 5−6, IN 5−6D) and on internodes 12−13 (IN 12−13, IN 12−13D). The soluble fractions were recovered with H_2O (S.1), HCl or HCl/dioxane (S.2), 1 N NaOH (S.3), and 4 N NaOH (S.4). Results expressed as a percent of initial CWR.

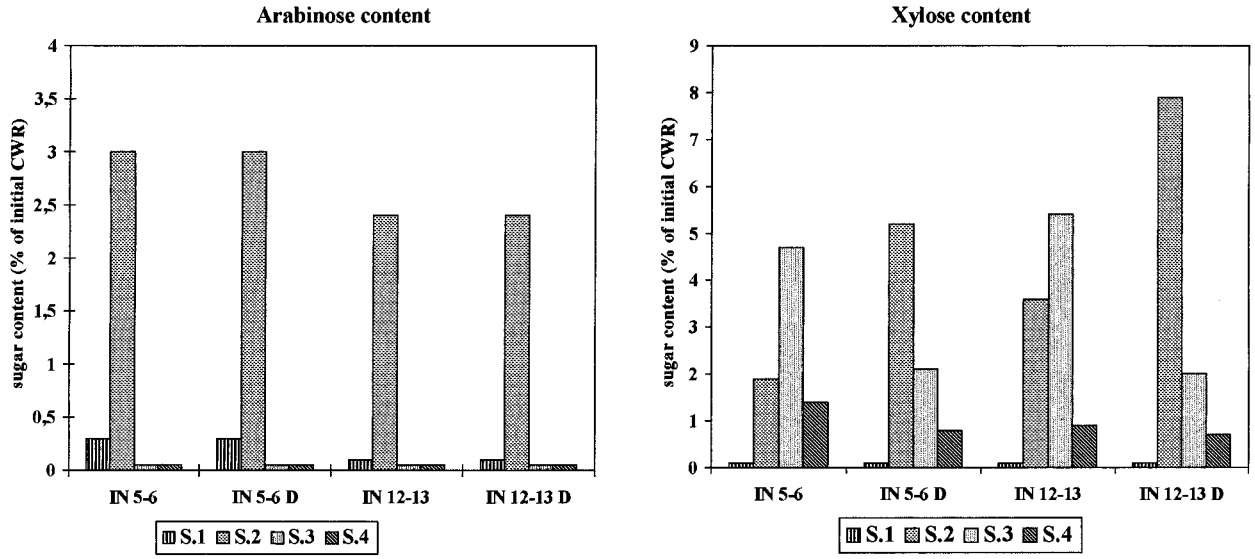

Figure 9. Arabinose and xylose contents of the fractions solubilized during fractionation with (D) or without the delignification step performed on internodes 5−6 (IN 5−6, IN 5−6D) and on internodes 12−13 (IN 12−13, IN 12−13D). The soluble fractions were recovered with H_2O (S.1), 0.5 N HCl or 0.1 N HCl/dioxane (S.2), 1 N NaOH (S.3), and 4 N NaOH (S.4). Results expressed as a percent of initial CWR.

procedures. The total amounts of the main neutral sugars recovered from the fractionated polysaccharides were almost similar in both chemical schemes. The arabinose content of soluble fractions represented a total of 3.3% and 2.5% of CWR prepared from internodes 5−6 and 12−13, respectively. The total amount of xylose of released polysaccharides did not vary significantly according to the fractionation (7% versus 8% in the case of IN 5−6; 11% versus 10% in the case of IN 12−13). In addition, the profiles of arabinose and of other main sugar solubilizations were not modified by the dioxane treatment except for xylose (Figure 9). In this last case, the largest amount of xylose was recovered after sulfuric acid hydrolysis of the soluble dioxane fraction in fractionation 2, whereas 1 M NaOH was the most efficient in removal of xylose containing polysaccharides when the delignification step is omitted (fractionation 1).

DISCUSSION

Cell Wall Structural Changes along with Internode Maturation. Morphological and microscopic data have shown that alfalfa stems could be divided in two main regions on the basis of the stage of tissue differentiation. In the first four apical internodes, the *elongation stage* took place as intensive cell multiplication and elongation occurred. The size of these internodes (length, diameter) increased very quickly. Internodes were constituted from primary tissues derived from the apical meristem; primary xylem only was lignified, and primary walls were abundant (Esau, 1977;

Vallet et al., 1996). The high levels of arabinose, galactose, and uronic acids in the upper internode walls (Titgemeyer et al., 1992) could presumably originate from pectins or branched arabinoxylans (Darvill et al., 1980; Carpita and Gibeault, 1993), which are main components of the primary cell wall (Esau, 1977). A substantial amount of xylose containing polysaccharide indicated the occurrence of xyloglucan, a typical component of the primary cell wall. Apart from structural polysaccharides, a large amount of protein was determined in CWR of internodes 1−4. However, 80% ethanol is not optimal for protein removal as some protein precipitation may have occurred during the preparation of CWR. The cell wall could thus be contaminated with cytoplasmic protein (Coimbra et al., 1996). In young tissues, cell walls may also contain relatively large amounts of proteins (Keller, 1993) that might be implicated in wall interactions. During the elongation stage, lignin deposition corresponded mainly to condensed guaiacyl lignins, giving rise to low thioacidolysis yields. Such lignins were assumed to be from the primary wall composed of pectins (arabinose, galactose, and uronic acids).

The second stage was a *thickening stage* of growth where internodes had stopped elongation growth. The secondary growth originated from cambial activity and was responsible of the differentiation of vascular tissues which causes the thickening of the stem (Esau, 1977). This stage began at internode 5; cambium started to differentiate and to produce phloem and lignified secondary xylem (Esau, 1977), leading to increased stem diameter owing to the large proportion of thick secondary walls. Sclerenchyma fibers underwent progressive cell wall thickening and lignifying (Vallet et al., 1996). The observed decrease of the length of the most basal internodes could be explained by the fact that this stem region had actually grown and elongated in the field under a period of cooler temperatures (weather data not shown) than the last formed internodes. The polymers deposited during this phase differed from previous ones in content and monomer composition. Xylose and glucose, as the main neutral sugars of basal internodes, could belong to cellulose, xylans, and glucuronoxylans. The type of lignin deposited after cambium differentiation changed, i.e., higher frequency of both labile-ether links and syringyl units as shown by thioacidolysis results. The patterns of lignin deposition in both stages are consistent with the model of Terashima et al. (1993).

Cell Wall Component Interaction as a Function of Tissular Maturation. The chemical scheme of cell wall fractionation led to heterogeneous fractions of solubilized polysaccharides. Indeed, we performed a sequential cell wall fractionation in order to highlight changes in the structural carbohydrates network rather than isolating homogeneous polysaccharide groups. Data gained from the fractionation procedure thus showed evidence of a gradient in the chemical solubilization of cell wall components from the top to the bottom part of the stem in agreement with the previous study on distinct stem regions of alfalfa (Hatfield, 1992). In our study, the extent of carbohydrate interaction along with tissue differentiation was further considered with regard to lignin modification (composition, content, and solubility) in contrast to previous investigations (Hatfield, 1992).

Water extracted polysaccharides may be pectin such as polygalacturonic acid, arabinogalactans, and arabinogalacturonans and some noncellulosic polysaccharides such as xylans (Souty et al., 1981; Renard et al., 1990). Hydrochloric acid is also efficient in releasing xylan or arabinoxylan polysaccharides by cleavage of arabinose side chains on xylans. Likewise, the highest content of xylose was found in the HCl soluble fractions from the xylan rich and mature internodes. The noncellulosic polysaccharides released by the above reagents may be covalently linked to the pectins (Hatfield, 1992; Renard et al., 1997) as pectin−xylan complexes. The amount of recovered material from internodes 1−4 and 5−6 might be underestimated in these cases; cell wall residue included a significant proportion of materials other than lignin or polysaccharides, presumably proteins. The extracted noncellulosic polysaccharides were probably linear glucuronoxylans in the 1 N NaOH fraction and xyloglucomannans in 4 N NaOH (Hatfield, 1992; Titgemeyer et al., 1992). The low level of arabinose in alkali extracts may reflect the loss of some of the hemicelluloses following HCl extraction (Weightman et al., 1994). Lignin and xylose contents of the final residue were the highest in the case of the highly lignified cell walls (internodes 12−13), indicating that stronger interactions between cell wall components might occur in the highly lignified tissues.

Lignin−Polysaccharide Interaction. Fractionation including acidolysis in dioxane was aimed at comparing the removal of ether-linked lignin structures on chemical solubility of polysaccharides in the light of the extent of tissue differentiation. For that purpose, dioxane/HCl was preferred to chlorite delignification owing to its specificity in cleaving the alkyl−aryl ether linkages of the lignin polymeric network (Adler et al., 1968; Pepper et al., 1959). This allowed a milder and more selective disorganization of cell walls and polysaccharides. Furthermore, possible side reactions such as the condensation of lignin moieties were minimized by the mean of a short action of the reagent (30 min). Additionally, our preliminary experiments indicated a loss of significant amounts of sugars (xylose) using chlorite delignification (data not shown) in agreement with previous studies (Wise et al., 1946; Morrison, 1975); further alkali extraction polysaccharide of such delignified cell walls was not enhanced (data not shown).

Regarding the change in lignin composition (proportion of labile-ether linkages) and cell wall differentiation along the stem, dioxane/HCl was shown to be more efficient on mature internodes having a large proportion of secondary cell walls with a higher content of syringyl lignin and of alkyl−aryl ether linkages. Indeed, this procedure was previously shown to efficiently delignified secondary walls (Monties et al., 1981), whereas primary tissues (vessels, sclerenchyma fibers) containing mainly guaiacyl lignin and a low proportion of ether bonds were poorly delignified (Vallet et al., 1997). Therefore, as the internode got mature along the stem, lignin tended to be less extractable by the various aqueous reagents but was more specifically removed by treatment involving dioxane.

Fractionation 1 was aimed at sequential extraction of the main cell wall polysaccharides. However, some lignin was lost, particularly when 1 N NaOH was used. Alkali extraction of grass lignins is well-reported (Scalbert and Monties, 1986; Qian et al., 1992) in contrast to legume lignins (Canale et al., 1990, 1991). In our study, the partial extraction of lignins by alkali was actually after two steps (H_2O, HCl) allowing removal

of pectins and a fraction of hemicelluloses. Diluted hydrochloric acid may be responsible for some lignin loss as acid soluble lignin−polysaccharide complexes (Musha and Goring, 1974; Sawai et al., 1984). However, a similar proportion of acid soluble lignin could be estimated in both kinds of internodes. Moreover, the largest extent of lignin loss occurred at the first alkali extraction. Acid treatment may thus disrupt the association between hemicellulose and/or pectins, thereby unmasking lignin−polysaccharide complexes toward the alkali reagent. The mechanism of alkali upon lignin structure differed from the dioxane/HCl delignification. As expected, noncondensed lignin structures involving syringyl units are preferentially degraded by acidolysis (Pepper et al., 1959). In contrast, alkali removed the condensed lignin type, leaving a residue with lignin enriched in noncondensed bonds. This trend was particularly noticeable in the case of mature internodes or when dioxane/HCl is used at first. The cell wall modifications induced by acidolysis might thus render some condensed lignin structure that is more alkali-soluble.

Data gained from both fractionations indicated that the removal of xylans was concomitant with the loss of lignin. Dioxane/HCl in fractionation 2 and 1 N NaOH in fractionation 1 were the most efficient in removing lignin and xylans. Xylose may be associated with lignin forming lignin−polysaccharide complexes. Association of phenolics with xylans can proceed through esterified acetyl groups (Morris and Bacon, 1977; Tignemeyer et al., 1992) and also occurrence of direct connection of xylose residue to lignin (Watanabe et al., 1989) as in Gymnosperm. In addition, xylans might form linear chains interacting with cellulose (McNeil et al., 1975; Reis et al., 1994). Such phenolic−xylan interactions could be responsible for the restricted digestibility of xylose (Nordkvist and Aman, 1986; Buxton and Brasche, 1991).

The higher solubilization of pectic materials (as uronic acids) in the fractionation of CWR including incomplete delignification was consistent with the data obtained by Hatfield (1992) using a complete delignification by chlorite. However, we found that the proportion of the enhanced dioxane soluble pectin fraction, while accounting for 12% and 40% in internodes 5−6 and 12−13, respectively, varied according to the stem regions. In the most mature tissues, pectins are solubilized along with ether-labile lignin structures, whereas in internodes 5−6, HCl/dioxane appeared to be less efficient than HCl itself in the uronic acid removal but rendered a larger fraction of pectin material soluble in alkali.

Pectic carbohydrates could be covalently cross-linked to lignin as suggested by Minor (1982). Our data support this proposal in the case of mature cell walls and further agree with Hatfield (1992). Waldron and Selvendran (1992) also reported increasing occurrence of pectic−xylan complexes during maturation of asparagus. The glucuronic residue of xylans may thus be involved in lignin polysaccharide cross-linkage apart from acetyl ester linkages involving xylose moieties.

CONCLUSION

A gradient in the chemical composition of cell walls along the stem was evidenced in connection with the growth phase of internodes. Altogether, the data showed a close relation between the extent and the type of tissue differentiation of a stem region and between cell wall composition and interaction. The four stem regions studied for their morphology, cytology, and cell wall composition and structure gave indications of the main events occurring in one alfalfa stem. Young (i.e., short) stems contain a large proportion of young internodes (elongation stage), meanwhile the proportion of old internodes increases in older (i.e., taller) stems. Chemical analysis of the stems showed a decrease from the top to the bottom of pectic polysacccharides and an increase of xylan, heteroxylans, cellulose, and syringyl lignin contents. These features were indicative of the prominent proportion of secondary walls in bottom mature internodes. Sequential fractionation of cell walls evidenced a weaker extractibility of the main noncellulosic sugars as the internode was located down in the stem, i.e., highly lignified. When dioxane/HCl delignification was included, the labile ether lignin structure were removed with xylans.

A similar study has been performed on plants grown under controlled environment and harvested at various sampling dates. The same pattern in internode morphology and structural composition was demonstrated (to be published). Combined morphological and biochemical data might thus help in plant breeding programs aimed at the digestibility improvement of alfalfa.

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LITERATURE CITED

Adler, E.; Björkqvist, K. J.; Häggroth, S. Über die ursache der Farbreaktionen des holzes. *Acta Chem. Scand.* **1948**, *2*, 93−94.

Adler, E.; Miksche, G. E.; Johansson, B. Über die benzylarylätherbindung im lignin. *Holzforschung* **1968**, *22*, 171−174.

Blakeney, A. B.; Harris, P. J.; Henry, R. J.; Stone, B. A. A simple and rapid preparation of alditol acetates for monosaccharide analysis. *Carbohydr. Res.* **1983**, *113*, 291−299.

Blumenkrantz, N.; Asboe-Hansen, G. New method for quantitative determination of uronic acids. *Anal. Biochem.* **1973**, *54*, 484−489.

Buxton, D. R.; Brasche, M. R. Digestibility of structural carbohydrates in cold-season grass and legume forages. *Crop Sci.* **1991**, *31*, 1338−1345.

Buxton, D. R.; Hornstein, J. S. Cell-wall concentration and components in stratified canopies of alfalfa, birdsfoot trefoil, and red clover. *Crop Sci.* **1986**, *26*, 180−184.

Canale, C. J.; Abrams, S. M.; Varga, G. A.; Muller, L. D. Alkali-treated orchadgrass and alfalfa: Composition and *in situ* digestion of dry matter and cell wall components. *J. Dairy Sci.* **1990**, *73*, 2404−2412.

Canale, C. J.; Varga, G. A.; Abrams, S. M. *In situ* disappearance of cell wall monosaccharides in alkali-treated orchadgrass and alfalfa. *J. Dairy Sci.* **1991**, *74*, 1018−1025.

Carpita, N. C.; Gibeault D. M. Structural models of primary cell walls in flowering plants: Consistency of molecular structure with the physical properties of the walls during growth. *Plant J.* **1993**, *3*, 1−30.

Chesson, A. Mechanistic models of forage cell wall degradation. In *Forage cell wall structure and digestibility*; Jung, H. G., Buxton, D. R., Hatfield, R. D., Ralph, J., Eds.; ASA, CSSA, SSSA: Madison, WI, 1993; pp 347−376.

Coimbra, M. A.; Delgadillo, I.; Waldron, K. W.; Selvendran R. R. Isolation and analysis of cell wall polymers from olive pulp. *Modern Methods Plant Anal.* **1996**, *17*, 19−44.

Darvill, J. E.; McNeil, M.; Darvill, A. G.; Albersheim, P. The structure of plant cell walls. XI. Glucuronoarabinoxylan, a second hemicellulose in the primary cell walls of suspension-cultured sycamore cells. *Plant Physiol.* **1980**, *66*, 1135−1139.

Englyst, H. N.; Cummings, J. H. Simplified method for the measurement of total non starch polysaccharides by gas−

liquid chromatography of constituent sugars as alditol acetates. *Analyst* **1984**, *109*, 937−942.
Esau, K. *Anatomy of seed plants*; John Wiley and Sons: New York, 1977.
Fry, S. C. *The Growing Plant Cell Wall: Chemical and Metabolic Analysis*; Longman Scientific and Technical: Harlow, Essex, 1988.
Hatfield, R. D. Carbohydrate composition of alfalfa cell walls isolated from stem sections differing in maturity. *J. Agric. Food Chem.* **1992**, *40*, 424−430.
Iiyama, K.; Wallis, A. F. A. Determination of lignin in herbaceous plants by an improved acetyl bromide procedure. *J. Sci. Food Agric.* **1990**, *51*, 145−161.
Jung, H. G. Forage lignins and their effects on fiber digestibility. *Agron. J.* **1989**, *81*, 33−38.
Jung, H. G.; Casler, M. D. Relationship of lignin and esterified phenolics to fermentation of smooth bromegrass fibre. *Anim. Feed Sci. Technol.* **1991**, *32*, 63−68.
Jung, H. G.; Vogel, K. P. Influence of lignin on digestibility of forage cell wall material. *J. Anim. Sci.* **1986**, *62*, 1703−1712.
Keller, B. Structural cell wall proteins. *Plant Physiol.* **1993**, *101*, 1127−1130.
Kühbauch, W.; Bestajovsky, J. Veirteilung von Lignin in den Stengeln von Lucerne und Rotklee. *Wirtschaftseigene Futter* **1989**, *35*, 19−28.
Kühbauch, W.; Voigtländer, G. Berechnung der Futterqualität bei Weissklee, Rotklee und Luzerne mit morphologischen Kritetrien und/oder aus Pflanzeninhaltsstofen. III. Mitteilung: Ertragsbildung und Futterqualität bei Rotklee und Luzerne unter besonderer Berücksichtigung der morphologischen Differenzierung. *Z. Acker- Pflanzenbau* **1981**, *150*, 339−348.
Kurek, B.; Monties B.; Odier E. Dispersion of lignin in water: Characterization of the phenomenon and influence of lignin enzymic degradability. *Holzforschung* **1990**, *44*, 407−414.
Lapierre, C.; Monties, B.; Rolando, R. Thioacidolysis of poplar lignins: Identification of monomeric syringyl products and characterization of guaiacyl-syringyl rich fractions. *Holzforschung* **1986**, *40*, 113−119.
Lemaire, G.; Allirand, J. M. Relation entre croissance et qualité de la luzerne: Interaction génotype-mode d'exploitation. *Fourrages* **1993**, *134*, 183−198.
McNeil, M.; Albersheim, P.; Taiz, L.; Jones, R. L. The structure of plant cell walls. VII. Barley aleurone cells. *Plant Physiol.* **1975**, *55*, 64−68.
Minor, J. L. Chemical linkage of pine polysaccharides to lignin. *J. Wood Chem. Technol.* **1982**, *2*, 1−16.
Monties, B. Preparation of dioxane lignin fractions by acidolysis. *Methods Enzymol.* **1988**, *161*, 31−35.
Monties, B.; Odier, E.; Janin, G.; Czaninski, Y. Ultrastructural evidence of bacterial and chemical delignification of poplar wood. *Holzforschung.* **1981**, *35*, 217−222.
Morris, E. J.; Bacon, J. S. D. The fate of acetyl groups and sugar components during the digestion of grass cell walls in sheep. *J. Agric. Sci.* **1977**, *89*, 327−340.
Morrison, I. AM. Delignification and hemicellulose extraction of cell walls of *Lolium perenne* and *Trifolium pratense*. *Phytochemistry* **1975**, *14*, 505−508.
Musha, Y.; Goring, D. A. I. Klason and acid-soluble lignin content of hardwoods. *Wood Sci.* **1974**, *7*, 133−134.
Nordkvist, E.; Aman, P. Changes during growth in anatomical and chemical composition and *in-vitro* degradability of lucerne. *J. Sci. Food Agric.* **1986**, *37*, 1−7.
Pepper, J. M.; Baylis, P. E. T.; Adler, E. The isolation and properties of lignins obtained by the acidolysis of spruce and aspen woods in dioxane-water medium. *Can. J. Chem.* **1959**, *37*, 1241−1248.
Qian, P.; Islam, A.; Sarkanan, K. V.; McCarthy, J. L. Certain characteristics of the dissolved alkali lignins of four angiosperm plant species. *Holzforschung* **1992**, *46*, 321−324.
Reis, D.; Vian, B.; Roland, J.-C. Cellulose-glucuronoxylanes and plant cell wall structure. *Micron* **1994**, *25*, 171−187.
Renard, C. M. G. C.; Voragen, A. G. J.; Thibault, J.-F.; Pilnik, W. Studies on apple protopectin: I−Extraction of insoluble pectin by chemical means. *Carbohydr. Polym.* **1990**, *12*, 9−25.
Renard, C. M. G. C.; Weightman, R. M.; Thibault, J. F. The xylose-rich pectins from pea hulls. *Int. J. Biol. Macromol.* **1997**, *21*, 155−162.
Saha, S. K.; Brewer, G. F. Determination of the concentrations of oligosaccharides complex type carbohydrates and glycoproteins using the phenol-sulfuric acid method. *Carbohydr. Res.* **1994**, *254*, 157−167.
Sawai, A.; Kondo, T.; Ara S. Relationship between acid-soluble lignin content and hemicellulose degradability. *Jpn. Grassl. Sci.* **1984**, *30*, 165−169.
Scalbert, A.; Monties, B. Comparison of wheat straw lignin preparations. II. Straw lignin solubilisation in alkali. *Holzforschung* **1986**, *40*, 249−254.
Souty, M.; Thibault, J.-F.; Navarro-Garcia, G.; Lopez-Roca, J.-M.; Breuils, L. Les substances pectiques de l'abricot (*Prunus armeniaca* L.) var. Rouge du Roussillon. *Sci. Aliments* **1981**, *1*, 67−80.
Terashima, N.; Fukushima, K.; He, L.-F.; Takabe, K. Comprehensive model of the lignified plant cell wall. In *Forage cell wall structure and digestibility*; Jung, H. G., Buxton, D. R., Hatfield, R. D., Ralph, J., Eds.; ASA, CSSA, SSSA: Madison, WI, 1993; pp 247−270.
Titgemeyer, E. C.; Bourquin, L. D.; Fahey, G. C. J. Disappearance of cell wall monomeric components from fractions chemically isolated from alfalfa leaves and stems following *In situ* ruminal digestion. *J. Sci. Food Agric.* **1992**, *58*, 451−463.
Vallet, C.; Chabbert, C.; Czaninski, Y.; Monties, B. Histochemistry of lignin deposition during sclerenchyma differentiation in alfalfa stems. *Ann. Bot. (London)* **1996**, *78*, 625−632.
Vallet, C.; Chabbert, C.; Czaninski, Y.; Lemaire, G.; Monties, B. Extractibility of structural carbohydrates and lignin deposition in maturing alfalfa internodes. *Int. J. Biol. Macromol.* **1997**, *21*, 201−206.
Waldron, K. W.; Selvendran, R. R. Cell wall changes in immature Asparagus stem tissue after excision. *Phytochemistry* **1992**, *31*, 1931−1940.
Watanabe, T.; Ohnishi, J.; Yamasaki, Y.; Kaizu, S.; Koshijima, T. Binding-site analysis of the ether linkages between lignin and hemicelluloses in lignin-carbohydrate complexes dy DDQ-oxidation. **1989**, *53*, 2233−2252.
Weightman, R. M.; Renard, C. M. G. C.; Thibault J. F. Structure and properties of the polysaccharides from pea hulls. Part 1: chemical extraction and fractionation of the polysaccharides. *Carbohydr. Polym.* **1994**, *24*, 139−148.
Wilson, J. R. Organization of forage plant tissues. In *Forage cell wall structure and digestibility*; Jung, H. G., Buxton, D. R., Hatfield, R. D., Ralph, J., Eds.; ASA, CSSA, SSSA: Madison, WI, 1993; pp 1−27.
Wilson, J. R.; Mertens, D. R. Cell wall accessibility and cell structure limitations to microbial digestion of forage. *Crop Sci.* **1995**, *35*, 251−259.
Wise, L. E.; Murphy, M.; D'Addieco, A. A. Chlorite holocellulose, its fractionation and bearing on summative wood analysis and on studies on the hemicelluloses. *Pap. Trade J.* **1946**, *122*, 35−42.

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