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Phytochemistry 63 (2003) 543–549
PHYTOCHEMISTRY
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Maize stem tissues: ferulate deposition in developing internode cell walls

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Received 27 November 2002; received in revised form 30 January 2003

Abstract

It has been hypothesized that ferulates are only deposited in the primary cell wall of grasses. To test this hypothesis, the fourth elongating, above-ground internode of maize (*Zea mays l.*) was sampled from three maize hybrids throughout development. Cell wall composition was determined by the Uppsala Dietary Fibre method. Ester- and ether-linked ferulates were determined by HPLC analysis of ferulic acid released from the internodes by low and high temperature alkaline treatments. Internode length increased from 9 to 152 mm over 96 days of growth, with elongation being complete in the first 12 days. More than half of the cell wall material in the maize internodes accumulated after elongation had ended. Deposition of cell wall material appeared to reach its maximum extent 40 days after sampling began, well before physiological maturity of the maize plants. Galactose and arabinose began to accumulate early in cell wall development which was presumed to be associated with primary wall growth during internode elongation. The major secondary wall constituents (analyzed as glucose, xylose, and Klason lignin) did not begin to accumulate rapidly until shortly before internode elongation ended. Ferulate ester deposition began before ferulate ethers were observed in the cell wall, but both forms of ferulate continued to accumulate in secondary cell walls, long after internode elongation had ceased. These data clearly show that contrary to the hypothesis, ferulate deposition was not restricted to the primary wall and that active lignin/polysaccharide cross-linking mediated by ferulates occurs in the secondary wall.
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Keywords: *Zea mays*; *Poaceae*; Maize; Development; Cell wall; Ferulates

1. Introduction

Ruminant livestock depend upon forages as their primary feed resource (Galyean and Goetsch, 1993). Forages generally have high cell wall concentrations and which are typically of limited digestibility. As forages mature, cell wall concentration increases and the walls become more lignified (Jung and Deetz, 1993). While lignin is regarded as the primary limitation to cell wall degradation by rumen microorganisms cross-linking of lignin to arabinoxylan by ferulates in grasses has also been implicated in limiting cell wall degradability (Grabber et al., 1998; Casler and Jung, 1999).

It has been demonstrated that ferulate esters act as initiation or nucleation sites where lignin deposition begins in grasses (Ralph et al., 1995). Ferulate molecules connect lignin to arabinoxylans through ether bonds and other linkages, and form dimeric structures that cross-link arabinoxylan chains to each other and to lignin (Ralph et al., 1994b; 1998). It has been proposed that development of ferulate cross-linking structures between arabinoxylan and lignin is the mechanism whereby grass cells end their elongation process and shift from primary to secondary wall development (MacAdam et al., 1992a,b).

Prolonged degradation of lignified grass cell walls by rumen microorganisms results in a thin cell wall residue that appears to correspond with the original primary cell wall layer (Engels, 1989). Jung and Deetz (1993) suggested that grass primary cell walls remain undegraded because this is the site of lignin/arabinoxylan cross-linkage by ferulates, which confers non-degradability to the primary wall. It was hypothesized that because the secondary cell wall of lignified grass tissues

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doi:10.1016/S0031-9422(03)00221-8

is degradable, ferulate cross-links are only present in primary walls and ferulate esters are not deposited in the secondary wall of grasses.

The internodes of a grass stem provide a developmental profile from top to bottom, with each lower internode on the stem being older than the internode above it. Morrison et al. (1998) examined the cell wall concentration of ferulate esters and ethers in a series of maize (*Zea mays l.*) internodes to determine if the ferulate concentrations observed supported the hypothesis that ferulates are only deposited in elongating internodes that are actively undergoing primary wall growth and not in older internodes that have significant secondary wall development. The pattern of ferulate concentration observed by these authors did generally correspond to expectations of the hypothesis; however, potential differences in patterns of cell wall development due to internode position on the stem complicated interpretation of these data. In order to more definitively test the hypothesis that ferulate esters are only deposited in grass primary walls during cell elongation, a study of stem internode development in maize was undertaken utilizing a single internode position. By sampling a specific internode throughout development, from early elongation through physiological maturity of the maize plant, the deposition pattern of ferulates and other cell wall components could be described. Three maize hybrids were included in the research to account for possible genotypic variation and the study was replicated across years to account for potential growth environment effects on cell wall development.

2. Results and discussion

2.1. Internode growth

Maize hybrid (genotype), year (growth environment), and their interactions with harvest (maturity) had small, but significant, effects on internode and cell wall development. Because all three hybrids and both years showed similar patterns of cell wall development across harvests, only data for harvest effects will be presented. Data are presented in the figures by day of the year which corresponded to the mean harvest dates across the two years of sampling.

The length of the fourth internode increased from 9 to 152 mm during the course of development (Fig. 1). Elongation of this internode appeared to be essentially complete by harvest five (indicated by the arrow on all figures), although internodes from harvest eight and nine were slightly longer ($P<0.05$) than from harvest five. Similarly, increase in cross-sectional area of the fourth internode was virtually complete by harvest six, with only harvest 10 being slightly larger ($P<0.05$) in area (Fig. 1). It appeared that addition of cells by meristem activity and growth of cell size in the fourth internode was concluded between harvests five and six. Scobbie et al. (1993) showed at the time of elongation cessation in maize internodes that cells near the top of the internode had already shifted to extensive secondary wall deposition, but at the base of the internode cell walls appeared to still be more primary in nature. Based on these observations it was concluded that in the current study, cell wall development shifted around harvests five and six from a mixture of primary and secondary wall deposition to only secondary wall development after the end of elongation of the fourth internode.

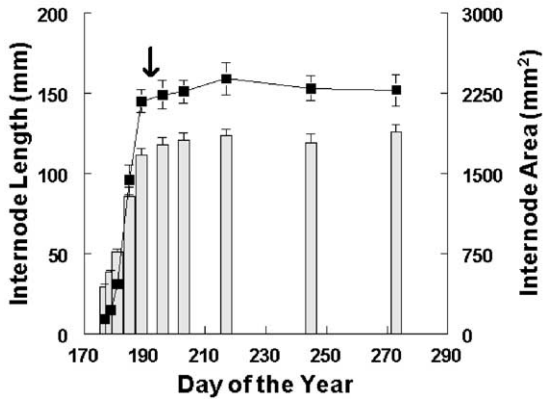

Fig. 1. Length (line) and cross-sectional area (bar) of the fourth elongated, above-ground internode of maize sampled throughout development. Data are averaged across three hybrids and two years. The arrow indicates the point during development when it is assumed that growth of all cells in the internode had reached completion and all cell wall development had shifted to secondary wall deposition. Error 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.

Accumulation of organic matter in the fourth internode was non-significant ($P>0.05$) over the first three harvest intervals, but then increased consistently until harvest nine, with a drop ($P<0.05$) in internode organic matter content at the last harvest (Fig. 2). This same deposition pattern was observed for cell wall accumulation in the fourth internode. The drop in organic matter accumulation at the last harvest was assumed to result from translocation of sucrose from the internode to the ear for grain development, and due to stem tissue senescence. The reason for reduction in cell wall content of the internode at harvest 10 is not known. At least half of the cell wall mass was deposited after elongation had ended, indicating that the majority of cell wall material in a maize stem internode must be secondary wall.

Unfortunately there are no other directly comparable data in the literature concerning the quantitative deposition of secondary cell walls in grasses. Obel et al. (2002) described the accumulation of primary walls in wheat (*Triticum aestivum* L.) coleoptiles from days 2 to 7 of seedling growth when coleoptile length increased

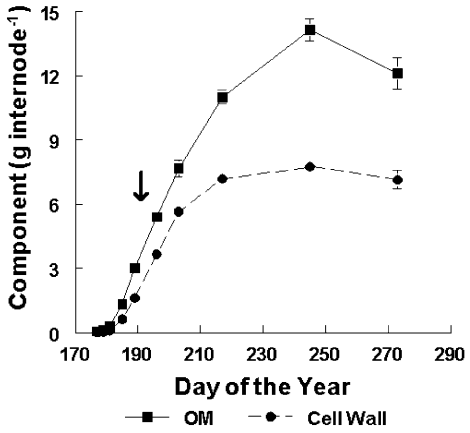

Fig. 2. Accumulation of organic matter (OM) and cell wall material in the fourth elongated, above-ground internode of maize throughout development. Data are averaged across three hybrids and two years. The arrow and error bars are described in the caption to Fig. 1.

from 4 to 20 mm. They found that coleoptile fresh weight increased from 0.4 mg to almost 90 mg over this five day period and that cell wall concentration increased from 27 to 38 g kg^{-1} fresh weight. However, little secondary wall had accumulated during this growth period as the lower one-third of the coleoptiles were still elongating. Recently, MacAdam and Grabber (2002) reported that cell wall accumulation (expressed as mg mm^{-1} of leaf length) reached a maximum 46 h after leaf elongation ceased in tall fescue (*Festuca arundinacea* Schreb.).

2.2. Monosaccharide deposition

Fig. 3 illustrates the deposition pattern for the three major cell wall components found in maize vegetative tissues. Not unexpectedly glucose, xylose, and Klason lignin all reflected the same deposition pattern as total cell wall, including the drop in amount for harvest 10 internodes. Glucose was the wall component laid down in the greatest amount, presumably as cellulose. Similar amounts of xylose and lignin were deposited, but substantially less than glucose accumulation. The other cell wall polysaccharide constituents were deposited in much lower quantities. Uronic acids and mannose followed the same deposition patterns as the major wall components (Fig. 4), but arabinose and galactose deviated from this pattern by not exhibiting the significant drop in abundance at harvest 10. Fucose was never found in more than trace amounts in fourth internode cell walls and rhamnose only reached a maximum abundance of 12 mg $internode^{-1}$.

Because arabinose and galactose are present in higher concentrations in primary than secondary maize cell walls (data not shown), the accumulation pattern observed for these two monosaccharides suggests some meristem activity with development of new cells, and their primary walls, or that loss of secondary wall material occurred between harvests nine and 10. The re-initiation of meristem activity would be unlikely. A loss of some secondary wall mass because of fungal attack on senescent tissue may have occurred.

2.3. Ferulate deposition

Deposition of ferulates in the fourth maize internode followed the same pattern as observed for the major cell wall components with an increase until harvest nine and a significant decrease at harvest 10 (Fig. 5). It should be noted that the total ferulate amounts presented are an under-estimate of total ferulate in maize. Diferulates were not included in the total shown because the analy-

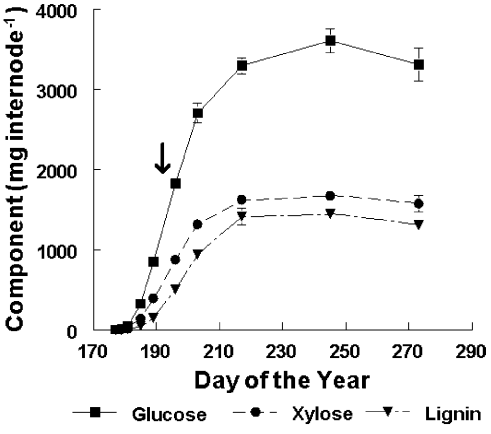

Fig. 3. Patterns of cell wall glucose, xylose, and Klason lignin accumulation in the fourth elongated, above-ground internode of maize throughout development. Data are averaged across three hybrids and two years. Monosaccharide data are presented on an anhydro-sugar basis. The arrow and error bars are described in the caption to Fig. 1.

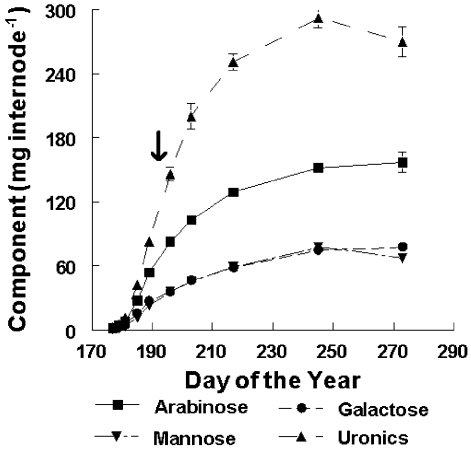

Fig. 4. Deposition of the minor cell wall polysaccharide components (arabinose, galactose, mannose, and uronic acids) in the fourth elongated, above-ground internode of maize throughout development. Data are averaged across three hybrids and two years. Monosaccharide data are presented on an anhydro-sugar basis. The arrow and error bars are described in the caption to Fig. 1.

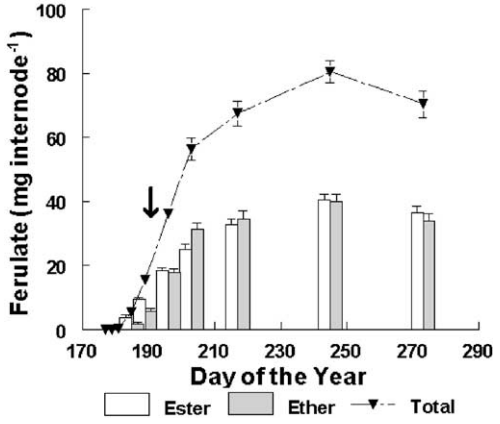

Fig. 5. Accumulation of ester- and ether-linked, and total ferulates (measured as ferulic acid release) in the fourth elongated, above-ground internode of maize throughout development. Data are averaged across three hybrids and two years. The arrow and error bars are described in the caption to Fig. 1. The bar for etherified ferulate at the third harvest overlaps the bar for the esterified ferulate at the fourth harvest because the ferulate bar pairs for each harvest date are centered under the symbol for total ferulates.

tical method utilized does not recover these compounds (presumably retained on the solid-phase extraction column used to purify ferulic and *p*-coumaric acids). The ether-linked ferulate concentrations presented account for only a portion of the total lignin/arabinoxylan cross-linking in maize cell walls because only ether-linked ferulate is recovered as an identifiable product by the high-temperature alkaline treatment used for cleaving ferulate cross-links of lignin to arabinoxylan whereas other known cross-linking structures are not recovered by this method (Ralph et al., 1992). Grabber et al. (1995) estimated that only 40% of the ferulate which cross-links lignin to arabinoxylan is in ether-linkages that can be quantified as ferulic acid when released by high temperature alkaline treatment.

While both ferulate esters and ethers were present in only trace amounts in the three earliest harvests, esterified ferulate increased much more rapidly than etherified ferulate at harvests four and five. This earlier deposition of ester-linked ferulates must occur because all ferulate is apparently esterified to arabinoxylan and the ether linkage to lignin is formed subsequent to deposition of feruloylarabinoxylan in the wall. However, by the sixth harvest the amount of etherified ferulate equaled the ester-only linkage form of ferulate. Again this would be expected because, by harvest six, virtually all internode cells should have shifted to secondary wall development and lignification.

If the hypothesis that ferulates are only deposited in primary walls were correct, then no further accumulation of ferulates should have been observed after the fifth or sixth harvest. But in fact the amount of ferulate in the fourth internode approximately doubled after internode elongation ended (Fig. 5). These data indicate that ferulate esters are deposited in the secondary wall of maize, and presumably all grasses. Recent data for expanding tall fescue leaves reached the same conclusion (MacAdam and Grabber, 2002). The possibility remains that ferulates continue to be laid down in the primary wall region while secondary wall deposition is occurring, rather than in the secondary wall itself, but this seems highly unlikely. Such a scenario would require that feruloylarabinoxylan polymers traverse the thickening and lignifying secondary wall to reach the original primary wall layer. However, the demonstration that ferulates exist in secondary walls of grasses does present a difficulty in explaining how lignin/polysaccharide cross-linking can limit cell wall degradation in the primary wall but not the secondary wall (Engels, 1989; Jung and Deetz, 1993; Casler and Jung, 1999). It is possible that the generally higher concentration of lignin in the primary wall region and/or a greater frequency of ferulate cross-links provide greater resistance to degradation than exists in the secondary wall.

The earlier observation by Morrison et al. (1998) that ferulate concentration in the cell wall follows an increasing then decreasing pattern for maize internodes sampled from the top (immature internodes) to the bottom (mature internodes) of a maize stem was most likely due to a greater deposition rate for lignin and the other major wall components than the deposition rate for ferulates, not because ferulates were only deposited in the primary wall. The same cell wall concentration pattern for ferulates as seen by Morrison et al. (1998) was observed across harvests in the current study (data not shown), providing support for the above interpretation.

The accumulation of *p*-coumarate esters followed the same pattern as observed for most other cell wall constituents (Fig. 6). However, unlike the rest of the cell wall which approximately doubled in amount after internode elongation ended, *p*-coumarate esters

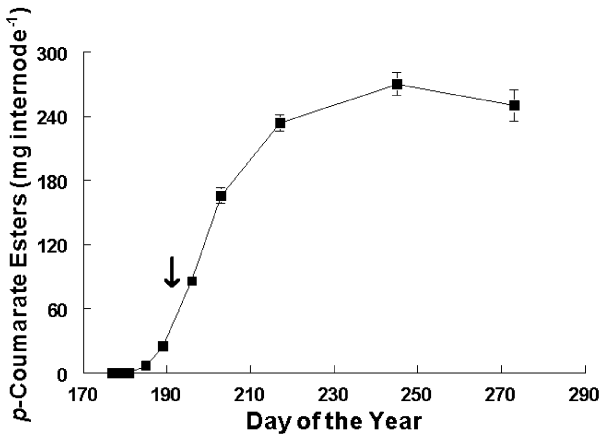

Fig. 6. Accumulation of *p*-coumarate esters in the fourth elongated, above-ground internode of maize throughout development. Data are averaged across three hybrids and two years. The arrow and error bars are described in the caption to Fig. 1.

increased 10-fold between the fifth and ninth harvests. This massive increase occurred because *p*-coumarate esters appear to be incorporated into lignin largely as sinapyl *p*-coumarate (a sinapyl alcohol-*p*-coumarate conjugate) and the majority of syringyl-type lignin is deposited late in the lignification process (Ralph et al., 1994a).

Unlike the current study where deposition of ferulate and *p*-coumarate followed the same pattern as cell wall accumulation in the maize internode, in tall fescue leaves deposition of ferulate and *p*-coumarate appeared to end before cell wall accumulation was complete (MacAdam and Grabber, 2002). The hydroxycinnamates reached maximum concentration per unit of leaf length 33–34 h after leaf elongation ended, whereas cell wall accumulation continued until 46 h after cessation of leaf elongation. Whether this difference in accumulation patterns is a plant part effect or a species difference is unknown.

2.4. Kinetics of cell wall accumulation

The fractional rates of deposition for the cell wall components and the lag time before accumulation could be observed after the first harvest are shown in Table 1. Galactose had the shortest lag time (<2 days) of all cell wall components, with arabinose and mannose being the next components to begin accumulation (<4 days). This result appears reasonable because galactose is a major pectin component and pectin is thought to be associated with primary cell walls (Moore and Hatfield, 1994); however, the actual polysaccharide origin of galactose and other monosaccharides was not determined by the methods employed in this study. Uronic acids were another cell wall polysaccharide residue that accumulated early during internode development and may have been associated with pectin as galacturonic acid, although glucuronic acid is also a component of grass xylans. Galacturonic and glucuronic acids could not be differentiated by the method of analysis used. Rapid accumulation of glucose and xylose corresponded to when total cell wall rapidly accumulated.

Table 1
Fractional deposition rates and lag time before accumulation could be observed of cell wall constituents in the fourth elongating, aboveground internode of maize. Data are averaged over hybrids and years

Constituent	Fractional rate (d^{-1})	Lag (d)
Total cell wall	0.068^{bc}	7.9^{ef}
Klason lignin	0.082^{b}	11.9^{h}
Glucose	0.071^{bc}	7.4^{e}
Xylose	0.108^{a}	8.4^{f}
Arabinose	0.056^{cd}	3.7^{b}
Galactose	0.039^{e}	1.8^{a}
Mannose	0.036^{e}	3.3^{b}
Uronic acids	0.051^{cde}	4.8^{c}
Esterified ferulate	0.047^{de}	5.9^{d}
Etherified ferulate	0.090^{ab}	9.9^{g}
Esterified p-coumarate	0.057^{cd}	10.7^{g}
Standard error of the mean	0.008	0.3

^{abcdefgh} Means in the same column not sharing a superscript differ ($P<0.05$).

Ester-linked ferulate acid accumulation began approximately 6 d after the first harvest, which was before major cellulose and xylan accumulation, but after pectin accumulation had begun (Table 1). The ether-linked form of ferulates did not begin accumulating until almost 10 days after the first harvest, which was between the fourth and fifth harvest dates. This pattern of delay in accumulation of etherified ferulate compared to the esterified form supports the concept that ferulate esters become cross-linked to lignin after primary wall growth ceases and lignin polymerization begins. In the current data set, lignin accumulation actually appeared to lag behind ferulate ethers by 2 days (Table 1). This delay in lignin accumulation compared to ferulate cross-link formation may indicate a slow initial deposition rate of lignin, or it may result from low molecular weight lignins during early secondary wall development that were not precipitated as Klason lignin in the analytical procedure. Ester-linked *p*-coumarate also began to accumulate after a relatively long lag.

Fractional rates of deposition for the cell wall constituents varied widely, but large overlaps were observed (Table 1). Xylose and etherified ferulate had the largest fractional rates of deposition (0.108 and 0.90 $days^{-1}$, respectively), while galactose, mannose, and uronic acids were the slowest. No clear relationships were evident between fractional rates of deposition and the cell wall structures with which particular wall components are associated. Presumably the different tissues which comprise maize internodes differ in their relative rates of development and composition, and these tissue differences may account for the observed kinetics of deposition found for the entire internode. Unfortunately information at such a level of detail is not available.

3. Conclusions

Contrary to a previous hypothesis, ferulate esters were deposited in the secondary wall of maize internode tissues and these esters became incorporated into lignin/arabinoxylan cross-links similar to what has been observed in primary cell walls. However, as expected, ferulate ether accumulation during cell wall development began after ferulate ester deposition had started. The accumulation of primary wall components (pectic sugars) was observed before the accumulation of the major secondary wall constituents (glucose, xylose, and lignin) accelerated after internode elongation was

complete. An apparent loss of secondary wall material occurred very late in maize internode development for which no satisfactory explanation could be provided.

4. Experimental

4.1. Plant material

Three non-related maize hybrids (A632×A619; A679×FR481; and Mycogen 2677) adapted to local growing conditions were planted on the University of Minnesota St. Paul campus 14 May 1998 and 19 May 1999. In both years the field plot design was a randomized complete block with two replications for each maize hybrid. Plots consisted of eight rows with 44 seeds per row at a spacing of 23-cm within and 76-cm between rows, respectively. The plots were fertilized prior to planting according to soil test results and University of Minnesota recommendations for maize production. A pre-emergence herbicide was applied to the plots prior to planting and mechanical weed control was done during the growing season.

Beginning in mid-June of both years, random maize plants were examined for stage of development of the fourth elongated, above-ground internode. Sample harvests were initiated when the fourth internode was 10–15 mm in length. Ten harvests of the fourth internode were made over the course of each growing season. The initial harvests were made on 24 and 28 June in 1998 and 1999, respectively. Harvest intervals after the first harvest averaged 2, 2, 4, 4, 7, 7, 14, 28, and 28 days over the two years for the subsequent nine harvests. To provide sufficient sample material for subsequent analyses, number of plants from which internodes were collected ranged from 44 plants at the earlier stages of development to eight plants at the later harvests.

Maize plants were harvested at ground level and the position of the fourth elongated, above-ground internode was determined by visual inspection of the basal stem internodes. The fourth internode was excised from the stem by cutting through the nodes. The length of four randomly chosen internodes was measured with a ruler. The diameter of these four internodes was measured with calipers in the middle of the internode, across both the long and short axis. Internode cross-sectional area was calculated using the area formula for an ellipse. The remaining internodes from each plot were frozen and lyophilized. The dried internodes were weighed prior to grinding through a 1-mm screen in a cyclone-type mill.

4.2. Chemical analysis

Dried and ground internode samples were analyzed for cell wall polysaccharide sugar residues and Klason lignin by the Uppsala Dietary Fibre two-stage sulfuric acid hydrolysis method (Theander et al., 1995). Neutral sugar residues (glucose, xylose, arabinose, mannose, galactose, rhamnose, and fucose) were quantified by GC as alditol acetate derivatives. Total uronic acids were measured by colorimetry prior to the second stage of the acid hydrolysis using glucuronic acid as the standard (Ahmed and Labavitch, 1977). Monosaccharide data were converted to an anhydro-sugar basis.

Total (ester- and ether-linked) ferulate in the cell wall was liberated with 4 M NaOH at 160 °C for 3 h from starch-free, alcohol insoluble residues (Iiyama et al., 1990). Ester-linked ferulate and *p*-coumarate were extracted from similar starch-free, alcohol insoluble residues with 2 M NaOH at 39 °C for 24 h (Jung and Shalita-Jones, 1990). Ferulic and *p*-coumaric acids released by the alkaline treatments were quantified by HPLC (Jung and Shalita-Jones, 1990). Ether-linked ferulate was calculated as the difference between total and ester-linked ferulate concentrations of each sample (Iiyama et al., 1990).

All data were corrected to an organic matter basis by drying ground internodes samples overnight at 100 °C and subsequent ashing at 450 °C for 6 h. All chemical assays were done in duplicate. Maize internode cell wall concentration was calculated as the sum of glucose, xylose, arabinose, mannose, galactose, rhamnose, fucose, uronic acids, Klason lignin, total ferulic acid, and ester-linked *p*-coumaric acid for each sample. The amount of each cell wall component in the internode was calculated from the compositional and internode weight data for each sample.

4.3. Statistical analysis

Data for internode cell wall components were analyzed as a randomized complete block design with two replicates and a split-split arrangement of treatments. Year was the main effect, maize hybrid and the interaction with year represented the split plot, and harvest and interactions with hybrid and year were in the split-split plot. To compare depositional patterns of the cell wall components, the fractional rate of deposition for each wall component and the lag time after the first harvest date before accumulation of the cell wall component could be observed were calculated using the first-order kinetic model of Mertens (1973). The fractional rate values represent the proportional increase of cell wall components that occurs on a daily basis during the exponential portion of cell wall deposition. These kinetic data were statistically analyzed using a randomized complete block design with a split-split plot arrangement where cell wall components replaced harvests as a model parameter. For both models, each statistical parameter was tested with the appropriate error term. The least significant difference method was used

to compare treatment means for parameters that had a significant F-test. Model parameter differences were considered significantly different if $P < 0.05$. All statistical analyses were done using the procedures in SAS (1985).

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