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Internode structure and cell wall composition in maturing tillers of switchgrass (*Panicum virgatum*. L)

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Abstract

This work examined cell composition gradients in maturing tillers of switchgrass (*Panicum virgatum* L.) with the aim of developing baseline information on this important forage and biomass crop. Flowering tillers were collected from plants raised from seeds in a greenhouse and field, harvested at soil level and separated into internodes beginning with the node subtending the peduncle. Internodes were analyzed using microscopy, by fiber digestion, high-performance liquid chromatography and by gas chromatography–mass spectrometry to obtain anatomical and compositional data. Microscopy demonstrated the development and maturation of cortical fibers which eventually became confluent with the fiber sheath surrounding vascular bundles in the lower internodes. Detergent fiber analysis indicated increasing cellulose and lignin contents and decreases in cell solubles and hemicelluloses with increasing distance of the internodes from the top of the plant. Soluble phenolics were greatest in amounts and complexity in top internodes. The lower internodes contained greater levels of wall-bound phenolic acids, principally as 4-coumarate and ferulate.

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1. Introduction

Switchgrass, (*Panicum virgatum* L.) is a C₄ perennial forage grass native to most of North America except for the areas west of the Rocky Mountains and north of 55°N latitude (Vogel, 1996). It is currently being evaluated as a biomass/biofuel crop in the US (Vogel et al., 2002; Sharma et al., 2003; McLaughlin and Kszos, 2005; Sanderson et al., in press) and in Europe (Lewandowski et al., 2003). Realization of this potential could significantly enhance rural revenue in the coming decades.

Several studies have documented the variability that exists within available switchgrass populations with respect

to important agronomic traits, such as tillering, digestibility and biomass production (Redfearn et al., 1997b; Das et al., 2004; Smart et al., 2004). Although, these studies have documented the potential for germplasm improvement in this important forage and biofuel crop there is still limited information on the anatomical, biochemical and physiological processes affecting these attributes. A dominant theme in breeding herbaceous crops for biomass and forage are traits that control cell wall components. These changes frequently target lignin content and can affect the relative concentration of hemicelluloses, celluloses and extractable cell wall phenolics (Vogel and Jung, 2001). Lignin is an important antinutritive in forage crops and decreasing lignin content through breeding significantly enhances digestibility as determined through in vitro dry matter digestibility (IVDMD) protocols (Vogel et al., 1999; Chen et al., 2002) as well as cattle weight gain (Anderson et al., 1988). Lignin is a polymer of cross-linked phenolics derived from the

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phenylpropanoid pathway (Boerjan et al., 2003; Raes et al., 2003), and is an integral part of the secondary cell walls of plants. There are several key enzymes in this complex pathway (Dixon et al., 2001) and contribute to the overall lignin content and signature for a given species (Raes et al., 2003). For example, natural mutations in the genes (Casler et al., 2002; Cardinal et al., 2003; Pedersen et al., 2005) or antisense suppression (Anterola and Lewis, 2002; Peter and Neale, 2004) of enzymes controlling phenylpropanoid metabolism can alter plant anatomy, lignification and impact plant fitness. During normal plant development lignification occurs when the secondary cell wall is formed, although lignification of primary cell walls can occur in dicots (Oh et al., 2003) and monocots (Jung, 2003). In most grasses, cells containing extensive secondary cell walls constitute the bulk of the support tissues for the tillers and account for dominant fractions of lignin, hemicellulose and cellulose in the biomass, especially in mature plants (Chen et al., 2002; Jung and Casler, 2006a,b). Switchgrass tillers consist of solid nodes and hollow internodes, and mechanical support is provided by vascular bundles and cortical fibers. Whereas the gross characteristics of switchgrass tillers and fibers have been investigated in some detail (Grabber et al., 1991; Jung and Vogel, 1992; Law et al., 2001), many aspects of plant anatomy and biochemical changes in cell walls during tiller maturation have not been investigated.

Future improvement of switchgrass for forage and/or biofuel crop will require a detailed understanding of the molecular processes that impact desired phenotypes. Cellular pathways that improve forage quality may be different than those needed for biomass (Casler and Vogel, 1999; Mitchell et al., 2001; Lemus et al., 2002; Stroup et al., 2003). Thus, it is critical to obtain cellular and molecular information on switchgrass to more fully guide existing and future breeding programs. This paper examines the anatomy, digestibility and other cell wall characteristics in flowering tillers of switchgrass as a function of internode position along the length of the tiller.

2. Methods

2.1. Plant material

Switchgrass plants (*Panicum virgatum* L. cv. *Kanlow*) were grown from seed in a greenhouse in Lincoln, NE under a 16 h ~26–30 °C day/8 h ~22–26 °C night growth regimen, using supplemental lighting from halide lamps (200 mol photons m⁻² s⁻¹) in a soil mixture consisting of 40% Canadian peat, 40% coarse vermiculite, 15% masonry sand and 5% screened topsoil, amended with 4.45 kg Waukesha fine lime m⁻³. Plants were watered biweekly with a nutrient solution containing 200 ppm N and with tap water as needed otherwise. Plants were grown to post-anthesis and harvested when the peduncle was fully elongated on most of the older tillers. Tillers (3–4) from twelve plants were selected to contain at least 6 internodes below the fully extended peduncle and cut 4 cm above the soil surface.

Internodes were excised in order of position (1 = top, 6 = bottom) and the nodes and sheaths were removed and discarded. Internodes of the same position were pooled and handled as described below for microscopy, fiber extraction and high performance liquid chromatography (HPLC).

For gas chromatography–mass spectrometry (GCMS) analyses, five tillers were collected from approximately 30 field grown plants and separated into internodes. Most tillers had only 5 internodes, and when a sixth internode was present it was pooled with the fifth internodes and analyzed as together as one sample, labeled as 5+ in the text.

All analyses were repeated at least thrice. Statistical analyses were performed using the statistical routines available in Microsoft Excel. Critical values of the *t*-distribution were obtained from published tables (Steel and Torrie, 1982).

2.2. Microscopy

Approximately 0.5 cm sections were cut from the center of each internode and fixed in a solution containing 2.5% glutaraldehyde and 4% paraformaldehyde in 0.1 M potassium phosphate buffer, pH 7.0 (Ruzin, 1992), processed through a graded alcohol series embedded in JB-4 plastic sectioned and stained with 0.1% aqueous toluidine blue to determine extent of lignification and observed using an Olympus BX 51 (Olympus America, Melville, NY, USA) light microscope.

2.3. Fiber extraction

Dried, ground plant material was analyzed for cell wall components using a detergent digestion protocol as described by Vogel et al. (1999). Triplicate samples were placed in pre-weighed bags and processed by the fiber extraction protocol (Vogel et al., 1999), and the ANKOM ADL procedure (ANKOM Technology-9/99, Method for Determining Acid Detergent Lignin (ADL); ANKOM Technology Corp., Fairport, NY, USA). These procedures estimate neutral detergent fiber (NDF), acid detergent fiber (ADF) acid detergent lignin (ADL). A final combustion step determined ash concentration. The relative percentage of cell wall components, namely cell-solubles, hemicellulose, cellulose and lignin, were calculated using component concentrations expressed on a dry weight basis. On a oven-dry weight basis (g kg⁻¹), cell solubles = sample weight – NDF; hemicellulose = NDF – ADF; cellulose = ADF – ADL; and lignin = ADL – ash (Jung and Vogel, 1992).

2.4. Analyses of phenolics by high performance liquid chromatography (HPLC)

Cell wall phenolics were analyzed as described by Franke et al. (2002) using lyophilized and oven-dried tissues. Initial experiments did not reveal a significant difference in extractable phenolics between lyophilized or oven-dried materials. Analyses were subsequently performed on oven-dried (50 °C) material. Dried plant material was ground and milled

to pass 1 mm sieves. Aliquots of 100 mg (± 1 mg) ground plant materials were extracted twice with 1.3 ml of 1.5% acetic acid in 50% methanol with continuous agitation for 1 h at room temperature ($22 \pm 3^\circ\text{C}$). Pooled supernatants were vacuum-dried and dissolved in 1 ml of 50% methanol and used directly for HPLC analysis. The residual plant material was resuspended in 300 μl of 2.0 M NaOH and allowed to stand at room temperature for 2 h (low temperature hydrolysis). After acidification with 6 M HCl, the aqueous phase was extracted thrice with 500 μl of ethyl acetate. The pooled ethyl acetate extracts were vacuum dried, and the pellet was dissolved in 1 ml 50% methanol and analyzed by gas chromatography–mass spectrometry (see below) to obtain chromatographic profiles of wall-bound phenolics.

All samples and standards were filtered through 0.22 μm filters and analyses were performed on a Beckman programmable gradient HPLC equipped with a Beckman 508 automated injector and a Beckman 168 diode-array detector (Beckman Coulter, Inc., Fullerton, CA, USA). Extracts were separated on a 300 \times 4.6 mm C-18 column (Microsorb cv) using a linear gradient of acetonitrile in 1.5% phosphoric acid, at a flow rate of 1 ml min⁻¹ as described by Franke et al. (2002). Column effluent was monitored at 320 nm and by spectral scans using the diode array detector.

Gas chromatography–mass spectrometry (GCMS) analyses were performed using an Agilent G2570A integrated GCMS system equipped with a G2913A autoinjector module. Phenolic acids were extracted from samples generated for HPLC and from purified cell walls as described by Iiyama et al., 1990 (hydrolysis at 90 $^\circ\text{C}$ for 3 h). In all cases, extracts were first treated with alkali to release free phenolic acids, acidified with HCl and extracted thrice with ethyl acetate as described above. Ethyl acetate was removed by vacuum drying and the resulting pellet containing phenolic acids were analyzed for their silyl esters using N,O-bis-(trimethylsilyl)trifluoroacetamide and trimethylchlorosilane reagent using manufacturer recommended protocols (Pierce Chemical Company; Rockford, IL). GCMS parameters were essentially as described by Zuo et al. (2002). Standard curves for different phenolics were generated using authentic compounds. Triplicate samples were analyzed for each internode.

3. Results

3.1. Lignification of cell walls in internodes

In the internodes closest to the peduncle, the developing fiber cells (DF) and fiber sheaths (F) surrounding the vascular bundles can be discerned (Fig. 1A). There was limited lignification of these cells as observed by the low intensity of blue¹ staining. Parenchyma cells (PC) exhibited thin cell walls with limited secondary cell wall deposition. In the third internode from the peduncle, fiber cells exhibited lig-

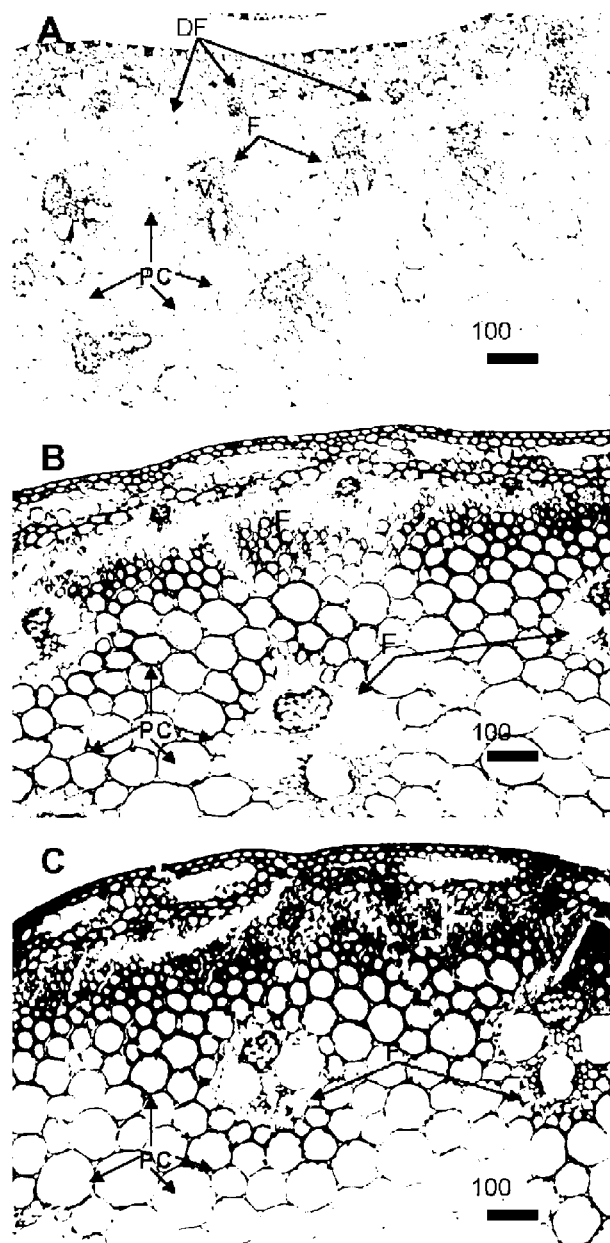


Fig. 1. Light micrographs of internode thin sections select stained with toluidine blue. A, internode 1; B, internode 3; C, internode 6. df = developing fiber cells; F = mature fiber cells; PC = parenchyma cells; Bars = 100 μm .

nification and the fiber sheath surrounding the vascular bundles were well defined and appeared to be extensively lignified (Fig. 1B). The lowest internode (sixth from the peduncle) exhibited extensive lignification of the cortical fibers and a clear confluence of the fiber sheaths and the lignified areas of the cortex. Significant cell wall thickening of the parenchyma cells (PC) was also evident at this stage of internode development (Fig. 1C).

3.2. Composition analyses of internodes as a function of distance from the peduncle

Data from compositional analyses were obtained from three replicate samples and analyzed by single factor

¹ For interpretation of the references in colour in Fig. 1, the reader is referred to the web version of this article.

Anova using the calculated percentages of the different fiber constituents. Cell solubles comprised about 21% (w/w) in top internodes and decreased significantly with increasing distance of the internodes from the peduncle, with a 16% decrease in cell solubles between internodes 1 and 2 (21.0% versus 17.7%) respectively ($df=5$; $F=24.4$, $P=0.001$) (Fig. 2). Cell solubles continued to decrease slightly in internodes 3 and 4 (17.3% and 17.1%, respectively). There was a significant loss of cell solubles in internode 5 (15.5%) and an increase in internode 6 (17.5%). Hemicellulose levels were highest in the internode 1, then decreased through internode 6 (~33.7% versus 28.3%, respectively; $df=5$, $F=11.8$, $P=0.007$). Cellulose accounted for 37% of the dry weight in internode 1, increased to approximately 41% (w/w) in internode 2 and then appeared to stabilize around 43% (w/w) in the lower internodes ($df=5$, $F=12.2$, $P=0.006$). Lignin levels were lowest in the highest internode (1; 6.5%) and increased in concert with internode distance from the top of the plant. In the 6th internode, lignin content was significantly greater than in internode 1 (6.5% versus 12.9%, respectively; $df=5$, $F=24.4$, $P=0.001$). These data were consistent with our light microscopy observations indicating significant secondary cell wall thickening and lignification of the different cell types in internodes furthest from the peduncle.

3.3. Free and wall-bound phenolics during internode development

Levels and complexity of soluble phenolics analyzed by HPLC decreased with increasing distance of the internodes from the top of the plant (Fig. 3). In the top internodes, the phenolic profiles of methanol extracts were quite complex,

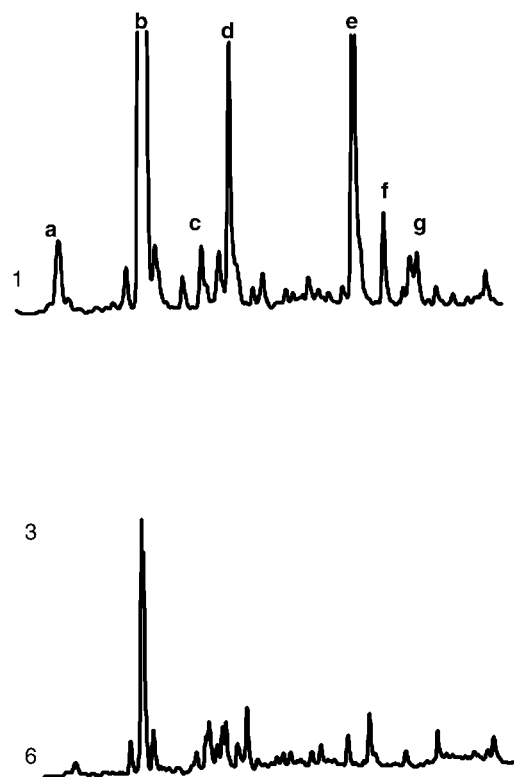


Fig. 3. Analysis of phenolics present in the soluble fractions from Kanlow internodes 1, 3 and 6 by HPLC ($n=3$). For comparisons, select peaks are labeled with smaller case letters.

and were dominated by three major peaks (labeled b, d, e on chromatogram) and several minor peaks, (i.e., a, c, f and g). In progressively further internodes, the levels of the three major phenolic peaks decreased dramatically.

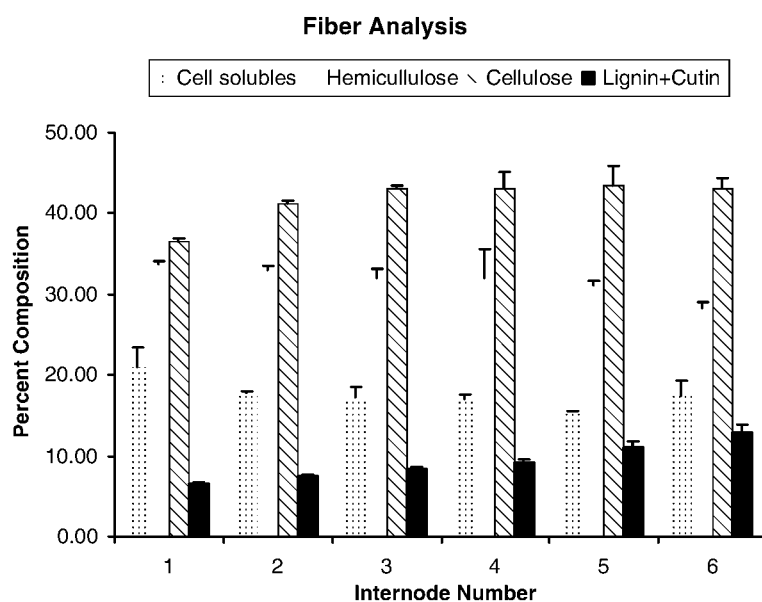


Fig. 2. Detergent fiber analysis of Kanlow internodes; percent composition of the four fractions, cell solubles, hemicelluloses, cellulose and lignin are shown for internodes 1 through 6 from the peduncle. Bars represent S.D. ($n=3$).

Although peak b was present in extracts of internodes 3 and 6, peak areas were substantially less. Peaks d and e were essentially absent in the internodes 3 and 6 (Fig. 3). A similar change in chromatographic profiles was observed for the compounds present in smaller amounts as well. For example, peaks a, c, f and g were strongly diminished in extracts from internode 3 as compared to extracts of internode 1, and were barely detected in extracts of internode 6 (Fig. 3).

Although we observed well resolved chromatograms by HPLC, we could not readily identify the large numbers of peaks seen in the soluble fractions. To obtain a better insight into the levels and complexity of phenolics, we utilized a robust GCMS protocol. These analyses showed that the soluble fractions obtained from top internodes had substantial levels of caffeic, 4-coumaric and ferulic acids (approximately 82% of total acids; $4.9 \text{ mg acids g}^{-1}$), and relatively lower amounts of protocatechuic, sinapic, syringic and vanillic acids (approximate total of 0.96 mg g^{-1} or 17% of total; Table 1). In successive internodes, the levels of caffeic acid declined from a value of $2.3 \pm 0.1 \text{ mg g}^{-1}$ for the top internode to $1.0 \pm 0.1 \text{ mg g}^{-1}$ for the bottom internodes. In contrast, the levels of 4-coumaric acid remained relatively constant for the top three internodes, and increased from $1.5 \pm 0.07 \text{ mg g}^{-1}$ in internode 1– $1.7 \pm 0.1 \text{ mg g}^{-1}$ in internode 4, levels decreased slightly in the lowest internodes analyzed (Table 1). Ferulic acid levels were similar in internodes 1 and 2, decreased in internodes 3, 4 and 5. Vanillic acid composed approximately $0.3 \pm 0.04 \text{ mg g}^{-1}$ in internode 1 and increased to 0.5 mg g^{-1} in the lowest internodes analyzed. The total amount of free phenolics decreased with distance of the internodes from the top of the plant as was observed by HPLC.

Low temperature hydrolysis of internode samples in alkali predominantly released caffeic, 4-coumaric and ferulic acid (Table 1). Trace levels of other phenolics were also

detected, but these were present in levels lower than could be accurately quantitated by our GCMS protocols. Caffeic acid composed approximately 1% of the phenolics in internodes (range from 0.3 mg g^{-1} in internode 1– 0.2 mg g^{-1} in internode 4). Levels of 4-coumaric acid increased with increasing distance of the internodes from the top of the plant and contributed to about 76% of the total phenolics released by low temperature alkali extraction (ranged from approximately 17 mg g^{-1} in the top internodes to around 20 mg g^{-1} for the lowest internode; Table 1). In contrast, ferulic acid levels were higher in the top internodes and steadily decreased in the lower internodes ($10.5 \pm 1.0 \text{ mg g}^{-1}$ in internode 1 and approximately $6\text{--}7 \text{ mg g}^{-1}$ in internodes 4 and below; Table 1).

In high-temperature hydrolysis of pretreated tissues, 4-coumaric acid content increased significantly with increasing distance of the internodes from the top of the plant (Fig. 4). There was approximately a 50% increase in wall-bound 4-coumarate levels between internodes 1 and 2 (6.4 mg g^{-1} versus 9.6 mg g^{-1} , respectively; $df = 4$, $F = 27.7$, $P = < 0.00001$). Levels of bound 4-coumarate increased in internode 3 as compared to internode 2, but was present in highest levels in internodes 4 and 5+ (Fig. 4). The overall increase in 4-coumarate levels of 107% was observed from internode 1 (6.4 mg g^{-1}) through internode 5+ (13.3 mg g^{-1}).

In contrast, wall-bound ferulate levels did not exhibit a substantial change with increasing distance of the internodes from the top of the plant. There was a trend for decreased ferulate content in internode 3 as compared to the other internodes, however the result was not statistically significant ($P < 0.097$). Overall, the ratio of 4-coumarate to ferulate increased with increasing distance of internodes from the top of the plant. This value was 1.8 for internode 1, increasing to 3.3 in internode 3 and 4.0 in internodes 5+ ($df = 4$, $F = 92.5$, $P = < 0.00001$) (Fig. 4).

Table 1
Phenolic acid composition of switchgrass internodes harvested from field grown plants

I. Soluble fraction				Phenolic acid (mg g^{-1} dry weight)			
Internode	Caffeic	4-Coumaric	Ferulic	Protocatechuic	Sinapic	Syringic	Vanillic
1	2.3 ± 0.10	1.5 ± 0.07	1.1 ± 0.02	0.3 ± 0.02	0.06 ± 0.01	0.3 ± 0.03	0.3 ± 0.04
2	1.7 ± 0.03	1.5 ± 0.10	1.1 ± 0.04	0.2 ± 0.04	0.08 ± 0.01	0.3 ± 0.09	0.3 ± 0.01
3	1.7 ± 0.08	1.5 ± 0.10	0.9 ± 0.06	0.2 ± 0.06	0.08 ± 0.01	0.3 ± 0.02	0.3 ± 0.02
4	1.4 ± 0.08	1.7 ± 0.10	0.9 ± 0.06	0.1 ± 0.01	0.06 ± 0.01	0.3 ± 0.1	0.4 ± 0.02
5+	1.0 ± 0.10	1.4 ± 0.07	0.8 ± 0.07	0.2 ± 0.04	0.07 ± 0.01	0.3 ± 0.02	0.5 ± 0.01
II. Low temperature hydrolysis							
Internode	Caffeic		Coumaric		Ferulic		
1	0.30 ± 0.02		17.7 ± 1.9		10.5 ± 1.0		
2	0.29 ± 0.03		17.3 ± 0.4		8.9 ± 0.2		
3	0.25 ± 0.02		17.7 ± 0.7		6.9 ± 0.4		
4	0.20 ± 0.03		18.3 ± 2.1		5.8 ± 0.9		
5+	0.23 ± 0.05		20.2 ± 0.6		6.3 ± 0.2		

Internodes were extracted as described in the methods section and analyzed for phenolics by GCMS. Data are the means of single injections of triplicate samples. Values \pm s.e.m. are shown for each sample.

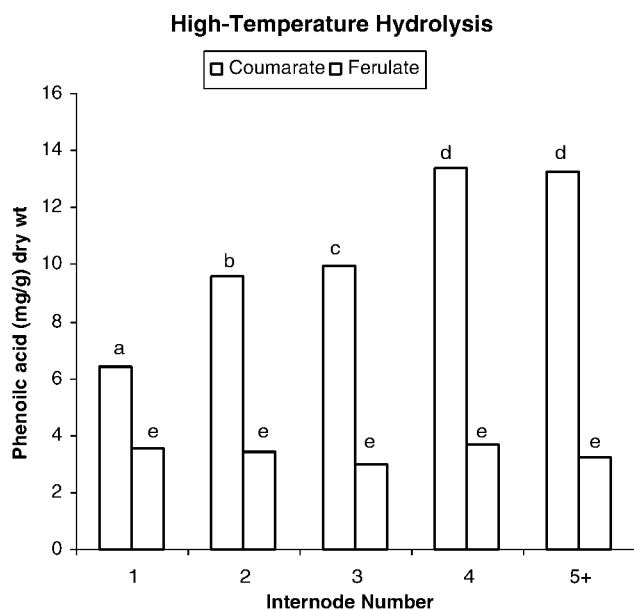


Fig. 4. Relative abundance of cell wall-bound 4-coumaric and ferulic acids in switchgrass internodes as determined by GCMS ($n=9$). Bars with different letters were significantly different ($P \leq 0.05$).

4. Discussion

Previously published analyses of switchgrass have concentrated on bulk-isolated cell types or pooled plant material consisting of a mixture of plant parts or separated plant parts (Grabber et al., 1992; Casler et al., 2002; Redfearn et al., 1997a; Mitchell et al., 2001). In these studies stage-specific structural and composition changes were masked and the results would be more indicative of whole-plant changes occurring at the level of the standing crop. In the present study we have attempted to correlate anatomical changes observed in internodes obtained from flowering switchgrass tillers to corresponding changes in cell wall composition. These data are important for basic understanding of cell wall development in switchgrass, and for management practices in terms of harvesting for optimal forage or biomass quality. Although we have not directly addressed changes in the development for a specific internode as has been published for corn (*Zea mays* L.) (Jung, 2003; Jung and Casler, 2006a), we have done so indirectly by the analyses of internodes along the length of a tiller (the basic unit of harvested switchgrass biomass). The expectation was that anatomical and compositional differences observed in internodes successively along the tiller would be reflective of developmental changes occurring in an individual internode over time. Our study did not directly assess whether any of the upper internodes would actually develop a structural and chemical identity similar to the more basal ones over time. However, anatomical data shown in Fig. 1 would suggest that these internodes follow a programmed developmental pattern and could be expected to display similar changes in cell wall composition as well. In the longer-term, data on developmentally related

changes in cell wall composition along a tiller and for specific internodes can aid in selecting cellular targets for breeding improved cultivars.

A dominant feature of switchgrass internodes was the thick ring of cortical fiber cells which in mature internodes occupied a region approximately 100–125 μm thick just beneath the epidermis. Another feature was the confluence of the fiber-sheath surrounding the vascular bundles with the cortical fiber sheath. These developmental patterns probably enhance the mechanical support of the tillers as well as contributing to the increases in lignin in internodes as they mature. Similar temporal sequences in lignification have been observed in other grasses including fescue (*Festuca arundinacea* Schreb.) (Chen et al., 2002), and maize (*Zea mays* L.) (Jung, 2003; Jung and Casler, 2006a). For switchgrass, our results showed that there was the expected increase in apparent lignification as a function of internode distance from the top of the plant with strong secondary cell wall deposition in the cortical fibers, the fiber sheaths surrounding the vascular bundles, as well in portions of the cortical parenchyma. Although, we observed increases in secondary cell wall deposition in the cortical parenchyma of bottom internodes, we did not evaluate whether these depositions resulted in enhanced lignin. These data appear to be consistent with the findings of Grabber et al. (1991) who documented enhanced lignin in older stem parenchyma in switchgrass. Isolating cortical fiber and parenchyma from selected internodes could help demarcate this issue.

Changes in lignin and cellulose as a function of internode development were not unexpected, because mature tissues will contain a greater proportion of secondary cell walls as well as higher levels of lignification (Grabber et al., 1991; Jung and Casler, 2006a,b). Our data indicated that cellulose deposition ceased in the third or fourth internode, whereas lignification proceeded for a longer time. There was a corresponding steady decrease in hemicellulose that accompanied internode as a function of position along the tiller, suggesting that cell growth and cell wall extension were essentially complete for internodes three and below. Our results also suggest that whole tillers including the basal internodes (approximately 4 cm above soil surface) need to be analyzed to obtain a good estimate of crop quality for forage or biofuel feedstocks. Alternatively, a higher cutting would yield lower total biomass, but potentially one of better quality. In terms of forage utilization, it has been shown that rumen bacteria do not attach effectively to grass-stem sclerenchyma and xylem (Akin, 1989; Jung and Casler, 2006b) resulting in incomplete digestion. Akin (1989) and Chen et al. (2002) have pointed out that reducing stem sclerenchyma might be a good target for forage breeding in grasses. However, high lignin might be a good attribute in biomass-conversion technologies involving the production of syn-gases (Dr. A. Boateng, USDA-ARS, ERRC, PA, personal communication; Boateng et al., 2006). For this method of conversion to biofuel, switchgrass germplasm with earlier lignin deposition resulting in increased lignin in all internodes would be a desirable target for breeding.

The levels and complexity of wall-associated and wall-bound extractable phenolics decreased with increasing distance of the internodes from the peduncle, which support the findings from the fiber analyses. These data suggest that in the more lignified internodes, there was both a lowered total level of freely extractable phenolics and greater cross-linking of phenolics, predominantly derived from 4-coumarate and ferulate, in switchgrass cell walls. Our data are also compatible from a physiological standpoint (Jung, 2003), where relatively unligified cell walls would contain greater amounts of free phenolics, and in more lignified cell walls, phenolics compounds would be incorporated with ether and ester-linkages in the cell-wall matrix. Our results indicated that most of the 4-coumarate and ferulate were incorporated into cell-walls in the lower internodes (Fig. 4) and the levels of free phenolics decreased with increasing lignification of the internodes (Fig. 3). These findings are consistent with enhanced lignification in basal internodes and indicate that switchgrass cell walls will contain significant amounts of these two phenolic derivatives as expected for grasses (Dixon et al., 2001; Jung, 2003).

In conclusion, we have documented for the first time the changes that take place at the anatomical and physiological level in internodes of flowering tillers of switchgrass, an important forage and biofuel crop. These data will be useful for future breeding and harvest management programs, and provide a baseline against which different germplasm can be evaluated for traits influencing anatomical and compositional changes.

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