Stem characteristics of two forage maize (*Zea mays* L.) cultivars varying in whole plant digestibility. III. Intra-stem variability in anatomy, chemical composition and *in vitro* rumen fermentation

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Abstract

The internodes of forage maize (*Zea mays* L.) stems were studied at anthesis for variation in anatomy and chemical composition in relation to digestibility. The study was carried out with a short (Vitaro) and a tall (Volens) cultivar differing in whole-plant digestibility, both of which were grown in the field in the growing seasons of 1999 and 2000. Internode diameter increased from the top to the base of the stem and Vitaro had shorter and thicker internodes than Volens. The cell walls of the sclerenchyma tissue in the rind were thicker and the numbers of sclerenchyma layers around vascular bundles in the rind higher in lower than in upper internodes. The neutral detergent fibre content (NDF%) of the internodes increased from the top to the base of the stems of both cultivars, but was very high for the peduncle. NDF% was lower for Vitaro than for Volens in all internodes. The sugar content of the dry matter was highest for Internode 12, i.e., the internode near the position of the ear, and was very low for the peduncle. Vitaro always had a higher sugar content than Volens. When subjected to fermentation tests with rumen fluid in an automated gas production system, gas production values after 3, 20 or 72 h of incubation were higher for internodes from the top than for internodes from the base of the stem, and were lower for the peduncle than for Internode 14. The values were consistently higher for Vitaro than for Volens internodes; in general, this difference was most apparent for Internode 10. The differences in gas production amongst internodes and between cultivars were in line with differences expressed by *in vitro* digestibility measurements. Fermentation results of cross sections suggest that the cell walls in lower internodes disappeared faster and to a greater extent than the cell walls in upper internodes, except for Volens in 1999, and with the exception of the peduncle. The rate of cell wall disappearance was higher for Volens

than for Vitaro, but ultimately similar amounts of cell wall material disappeared.

Additional keywords: fermentability, gas production system, sclerenchyma, section fermentation

Introduction

In Europe, but also elsewhere in the world, maize (*Zea mays* L.) silage is frequently and widely used as roughage for ruminants, especially during wintertime. Maize silage has a high energy content, moderate protein content and an adequate structural value. Ruminant nutritionists are therefore strongly interested in optimizing fermentation of silage maize. Improving the nutritive value of maize would imply that ruminants would need less maize to obtain the same amount of energy, thus reducing the area of land needed to grow maize to provide the same energy to the ruminant.

Improving the digestibility of forage maize is another important though not easily achievable breeding goal (Struik, 1983; Deinum & Struik, 1985; 1989; Geiger *et al.*, 1992; Dolstra *et al.*, 1993; Barrière *et al.*, 1997; Méchin *et al.*, 2001). The most valuable plant trait when improved nutritive value is the main breeding objective, is the digestibility of the stover cell walls (Deinum & Struik, 1989; Struik & Deinum, 1991; Struik & Dolstra, 1991; Dolstra *et al.*, 1993). Cell-wall digestibility estimates are also the quality traits with the highest number of detectable quantitative trait loci (Méchin *et al.*, 2001).

In the past, selection for improved feed value of forage maize mainly led to short cultivars with a heavy ear, and, in countries where the season is short, early flowering and ripening of the grain. This type of selection enhanced the energy content but could reduce cell wall digestibility (Struik, 1983). Digestibility is negatively correlated with important agronomic traits such as lodging resistance and yield (e.g., Geiger *et al.*, 1992; Barrière & Argillier, 1998; Méchin *et al.*, 2001). However, maize plants do not only consist of an ear, but also of stems and leaves and especially the digestibility of the stem parts is highly variable (Struik, 1984). Stem digestibility is mainly determined by the digestibility of its cell walls (Struik, 1983; Deinum & Struik, 1989), which declines when plants become older (Struik, 1983; Deinum & Struik, 1989). More insight into the mechanisms underlying the plant-related genetic and environmental variation in cell wall degradation of the stem is therefore essential. However, as discussed by Boon *et al.* (2005a, b), insight into the plant-related aspects of cell wall degradation is not sufficient, especially as access of rumen micro-organisms to the cell wall after ingestion is also playing a significant role (Wilson & Hatfield, 1997).

We studied the degradation of the cell wall by comparing tissues from different stem internodes at anthesis. Development of the stem internodes of maize starts in the lowest internodes and elongation of internodes takes place in a sigmoidal pattern from the base to the top (Morrison *et al.*, 1998). So the internode located at the base of the stem completes its development first (the 'oldest' internode), followed by younger internodes higher up. As a consequence there is a gradient of physiological cell wall age from the top to the base of the stem. A comparison of these internodes gives insight into differences in cell wall degradability caused by differences in physiological maturity (Morrison *et al.*, 1998). In this study we used an array of morphological, anatomical,

chemical and fermentation techniques to obtain a complete view of the different factors involved in the differences in stem digestibility between two contrasting forage maize cultivars, Vitaro and Volens. The first one is known for its relatively high, the second one for its relatively low digestibility.

Material and methods

Plant growth and development

The maize cultivars Vitaro and Volens were sown on a clay soil near Wageningen in 1999 and 2000. Their stems were harvested in early August when plants were at developmental stage 5 (anthesis) (Groot *et al.*, 1986). More details on how the crops were grown and how they developed can be found in Boon *et al.* (2005a, b).

The stems were cut just below Internode 7, which is the first or second internode above ground level. Internodes were numbered from the base of the stem: the internode accompanying Leaf 1 was designated as Internode 1. Internode number was verified by measuring the length of the leaf accompanying the internode (Bos, 1999; Boon *et al.*, 2005a). Stem samples were stored at –20 °C until further analysis. Nodes were discarded.

Morphological and anatomical studies

Internode length and diameter were assessed using a measuring tape. Volume of internodes was calculated using the formula $\pi r^2 l$, where r is the radius and l the length of the internode, ignoring the deviation from the assumed shape in lower internodes and especially in the internodes near the ears.

Cross sections with a thickness of 100 µm were made of each of the Internodes 7–16, using a sledge microtome (Ernst Leitz, Wetzlar, Germany). Sections were taken at the exact middle of the internode.

In each cross section the number of vascular bundles was recorded. Also the number of layers of sclerenchyma cells beneath the epidermis and on the adaxial side of the vascular bundles in the rind was recorded. Cell wall thickness was measured at 1000 × magnification on recorded video images, using the analySIS 3.0 software package (Soft Imaging Systems GmbH, Münster, Germany).

Chemical studies

NDF (neutral detergent fibre), ADF (acid detergent fibre) and ADL (acid detergent lignin) contents of Internodes 8, 10, 12, 14, and 16 were determined in g per g dry matter (DM) according to the methods described by Goering & Van Soest (1970). Dry matter content was determined after drying at 103 °C for 24 h. Ash was determined after incineration for 4 h at 500 °C. Reducing-sugar content was determined colorimetrically with the neocuproin method (Dygert *et al.*, 1965).

Hemicellulose was calculated as (NDF – ADF), cellulose as (ADF – ADL), and lignin was determined as the ADL fraction.

Fermentation tests on ground material

The isolated whole internodes were oven-dried at 70 °C for at least 48 h and ground to pass a 1-mm screen before fermentation.

Fermentation characteristics of Internodes 7 to 16 were determined using the gas production technique described by Cone *et al.* (1996). Gas production profiles were fitted with a three-phasic model (Groot *et al.*, 1996), describing the fermentation of the soluble constituents (subcurve 1), the non-solubles (subcurve 2) and the microbial turnover (subcurve 3) (Cone *et al.*, 1997). Each subcurve is described by the parameters A (asymptotic maximum gas production), B (time in h to reach 50% of A) and C (determining the shape of the curve) (Groot *et al.*, 1996). In this paper, the numbers behind A, B and C refer to the numbers of the subcurves. The gas production after 3 h (GP3) is referred to as A1 and the gas production between 3 and 20 h (GP20 – GP3) as A2. Both A1 and A2 are expressed in ml per g organic matter.

Samples of individual internodes of Vitaro and Volens were also tested for percentage digestibility using the technique of Tilley & Terry (1963) (T&T) as a widely accepted reference value for quality.

Fermentation of sections

Cross sections of 100 μm thickness were mounted on double-sided tape attached to microscope slides. The sections were fermented in a 1.5-litre container with buffered rumen fluid (as used for the gas production technique), and 12.5 g of maize stem meal as additional substrate. All sections were fermented in the same container, and slides were removed from the fermentation container after 12, 24, or 48 h of fermentation. Unfermented adjacent sections (so-called mirror sections) were used as a reference to assess the decrease in cell wall thickness (Engels & Schuurmans, 1992).

Statistical methods

Statistical significance was assessed with Student's t-test or with a general analysis of variance, using Statistix for Windows version 2.0 (Analytical Software, Tallahassee, USA).

Results

Morphology

The length of the internodes showed a consistent pattern in both cultivars and both years (Table 1). Internode 16, the peduncle, was always 5–10 cm longer than Internode 15. In Volens, internode length increased from Internode 14 downwards to Internode 8 (Internode 9 in Vitaro in 2000). However, Internode 7 was shorter than Internode 8. Internode 11, the internode near the ear, was an exception to this general pattern, as it was always shorter than the adjacent internodes. Usually, Volens had significantly

Table 1. Length and diameter (± SEM) of Internodes Number 16 (top) to 7 (base) of the forage maize cultivars Vitaro and Volens in 1999 (n = 10) and 2000 (n = 5).

Internode Number	Length				Diameter			
	1999		2000		1999		2000	
	Vitaro	Volens	Vitaro	Volens	Vitaro	Volens	Vitaro	Volens
	(cm)							
16	24.4 ± 1.9	25.0 ± 1.5	21.3 ± 2.0	28.1 ± 0.6	0.68 ± 0.05	0.51 ± 0.02	0.65 ± 0.03	0.56 ± 0.02
15	19.2 ± 2.1[1]	14.8 ± 0.6	16.4 ± 2.0	21.6 ± 1.0	0.88 ± 0.07	0.75 ± 0.02	0.95 ± 0.03	0.84 ± 0.03
14	15.5 ± 0.6	15.0 ± 0.6	21.1 ± 1.8	20.7 ± 1.5	1.17 ± 0.05	0.94 ± 0.02	1.10 ± 0.11	1.04 ± 0.04
13	16.8 ± 0.5	17.6 ± 0.6	21.1 ± 1.2	21.7 ± 1.3	1.43 ± 0.04	1.14 ± 0.02	1.24 ± 0.17	1.06 ± 0.05
12	17.9 ± 0.2	19.5 ± 0.5	21.6 ± 1.0	23.0 ± 0.4	1.58 ± 0.04	1.33 ± 0.03	1.70 ± 0.06	1.32 ± 0.07
11	17.3 ± 0.3	19.0 ± 0.3	21.0 ± 1.2	21.3 ± 0.1	1.71 ± 0.08	1.44 ± 0.02	1.96 ± 0.07	1.46 ± 0.04
10	18.9 ± 0.5	21.7 ± 0.4	21.4 ± 0.7	22.4 ± 0.4	2.10 ± 0.05	1.88 ± 0.05	2.20 ± 0.09	1.94 ± 0.07
9	19.8 ± 0.3	22.4 ± 0.3	22.0 ± 0.4	23.7 ± 0.6	2.43 ± 0.04	2.13 ± 0.04	2.58 ± 0.12	2.13 ± 0.07
8	20.3 ± 0.4	24.0 ± 0.5	19.6 ± 0.7	25.2 ± 0.4	2.57 ± 0.04	2.28 ± 0.04	2.76 ± 0.09	2.26 ± 0.07
7	16.4 ± 0.6	21.7 ±0.3	16.9 ± 0.3	22.2 ± 0.5	2.72 ± 0.05	2.38 ± 0.04	2.84 ± 0.12	2.43 ± 0.08
Mean	18.5 ± 0.4	20.1 ± 0.4	20.3 ± 0.4	23.2 ± 0.4	1.75 ± 0.07	1.47 ± 0.06	1.84 ± 0.11	1.48 ± 0.08

[1] Length of Internode 15 excluding the peduncle: 15.3 ± 2.4 cm.

longer internodes than Vitaro ($P < 0.001$), although occasionally – like for Internode 14 in 1999 – an internode of Volens was shorter than the corresponding Vitaro internode. In 2000 internodes were significantly longer than in 1999 ($P < 0.001$).

Internode diameter increased very consistently from the top to the base of the stem for both cultivars and in both years (Table 1): each internode usually differed significantly from the previous and the following one. The relation between internode number and diameter was linear with R^2 values between 0.9791 and 0.9908 (n = 10). Despite this linear relationship, the difference in diameter between Internode 11 – the one carrying the ear – and Internode 12 was large. Vitaro had significantly thicker internodes than Volens ($P < 0.001$). Internodes of Vitaro were significantly thicker in 2000 than in 1999 ($P = 0.008$). No year effect was found for Volens ($P = 0.08$). Internode volume calculated from the data in Table 1 was always larger for Vitaro than for Volens. Internode volume increased from the top to the base of the stem. When year had an effect on internode volume, the volume was higher in 2000 than in 1999.

Anatomical differences

The number of vascular bundles per cross section increased from the top to the base of the stem (data not shown). The peduncle had between 100 and 200 vascular bundles

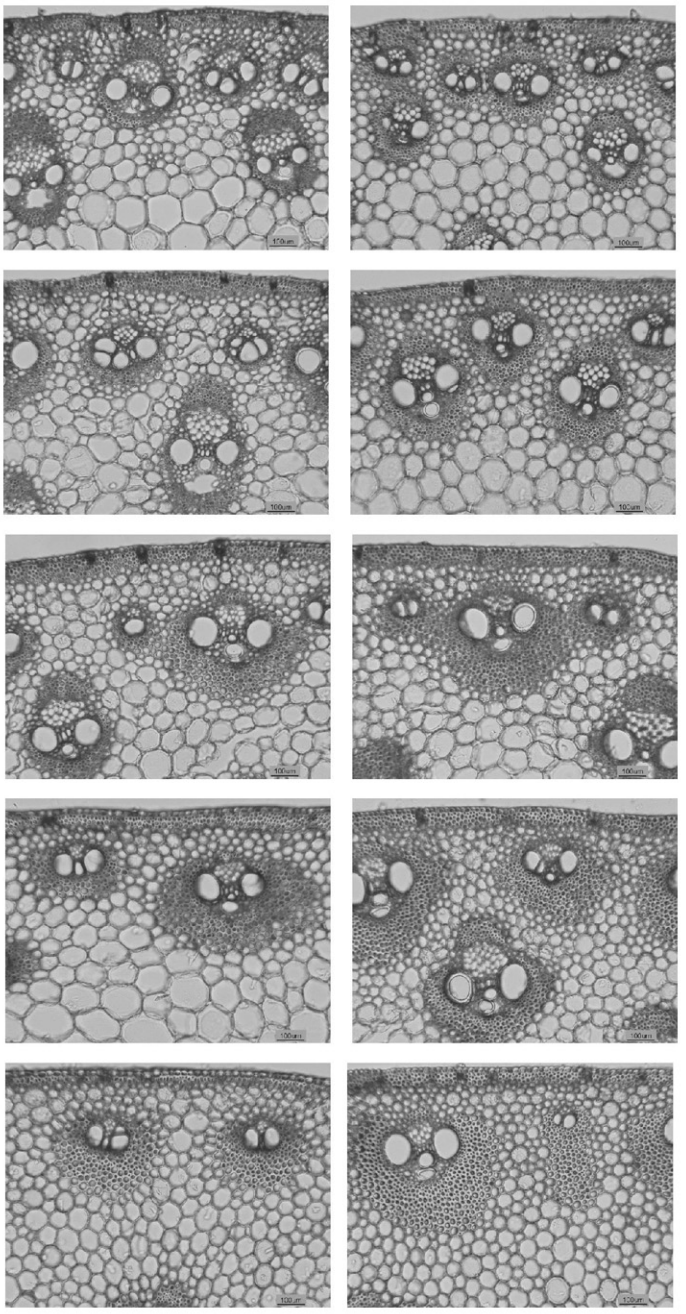

Figure 1. Anatomy of cross sections of Internodes 16 (top), 14, 12, 10, and 8 (bottom) before fer of the forage maize cultivars Vitaro (left) and Volens (right).

per cross section, whereas as many as 400 were recorded at the base of the stem. The increase was linear and similar for both cultivars; in 1999 the crops had significantly more vascular bundles than in 2000. This pattern and the trend in diameter along the stem, however, make it possible to calculate that there were more vascular bundles per unit area in the top than in the bottom of the stem.

In the stem sections a shift in the position of the sclerenchyma surrounding the vascular bundles was observed from the top to the base of the stem (Figure 1). At the top of the stem the sclerenchyma was roughly equally positioned on the abaxial and adaxial sides of the vascular bundles (Figure 1, top left and right). More towards the base of the stem, sclerenchyma was predominant on the adaxial side of the vascular bundles

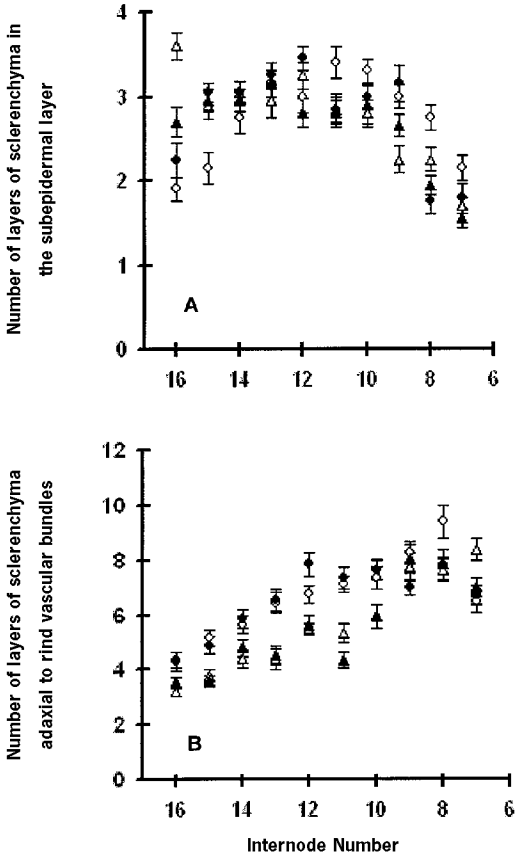

Figure 2. Number of layers of sclerenchyma tissue in the subepidermal layer (A) and number of layers of sclerenchyma tissue adaxial to rind vascular bundles (B) in the middle of Internodes 16 to 7 of the forage maize cultivars Vitaro and Volens at anthesis in 1999 and 2000 (n = 20; values ± SEM as indicated by bars). Δ Vitaro 1999; ▲ Vitaro 2000; ○ Volens 1999; • Volens 2000.

(Figure 1, bottom left and right). The area occupied by phloem tissue decreased from the top to the base of the stem, which is consistent with the reduction in number of vascular bundles per unit area mentioned above. At the base of the stem, vascular bundles appeared to lie further apart, separated by an apparently larger volume of pith parenchyma.

The subepidermal sclerenchyma consisted of 1–4 layers, with the number of layers apparently increasing slightly from Internode 16 to Internode 12, and subsequently decreasing slightly towards the base of the stem (Figure 2A). The number of layers of sclerenchyma adaxial to rind vascular bundles increased from the top to the base of the stem, levelling off at about Internode 8 (Figure 2B). There were no consistent effects of year or cultivar on the relationships between internode number and number of sclerenchyma layers.

Cell wall thickness of the sclerenchyma increased from Internode 15 towards the base of the stem, both in the subepidermal layers and adaxial to the rind vascular bundles (Figures 3A, B). The increase in cell wall thickness of sclerenchyma was more apparent in 2000, particularly in Internodes 10 to 7 (Figure 3). The cell wall of the sclerenchyma was significantly thicker in Internode 16, i.e., the peduncle, than in Internode 15. In general, Vitaro had thinner cell walls than Volens, especially in the lower internodes.

From the analysis of these results on the morphology and the anatomy it can be concluded that the effects of the position of the internode were large and consistent, with internodes in the lower part of the stem having more bundles per unit area, more layers of sclerenchyma and thicker cell walls than internodes in the higher part. The obvious exception was the peduncle. Cultivar differences in these morphological and anatomical trends were small. However, averaged over the internodes, Vitaro had shorter and thicker internodes than Volens. Vitaro also had thinner cell walls than Volens.

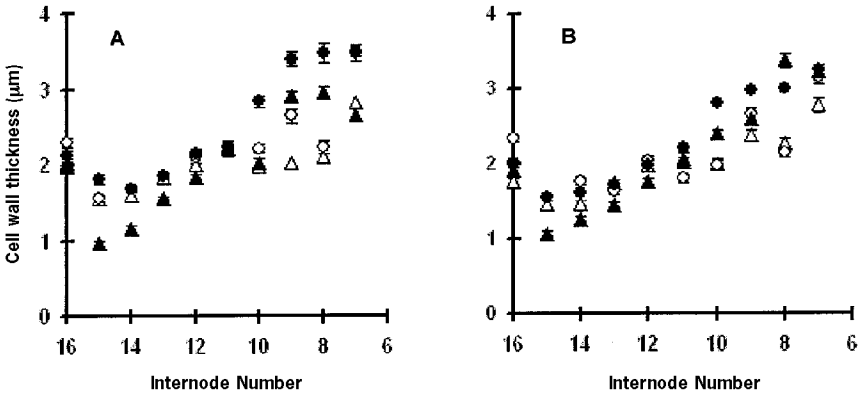

Figure 3. Cell wall thickness (µm) of subepidermal sclerenchyma cells (A), and of sclerenchyma cells adaxial to rind vascular bundles (B), of Internodes 16 to 7 of the forage maize cultivars Vitaro (triangles) and Volens (circles) at anthesis in 1999 (open symbols) and 2000 (closed symbols) (n = 30). Bars indicate SEM.

The differences in both morphology (through its effect on rind–pith ratio) and cell wall thickness could contribute to the cultivar difference in digestibility.

Chemical differences

NDF content increased from Internode 14 to Internode 8, but was higher for the peduncle (i.e., Internode 16) than for Internode 8 (Table 2). Volens had a higher NDF content than Vitaro and NDF content was higher in 2000 than in 1999. Ash content was relatively constant (Table 2). Sugar content (Su) did not show a clear trend along the stem, but was always lowest in Internode 16 (Table 2). Volens had a lower sugar content than Vitaro. This difference between the two cultivars was especially striking in 2000. In general, sugar content was lower in 2000 than in 1999.

The calculated content of hemicellulose in the NDF decreased from the top to the base of the stem, whereas cellulose content in the NDF increased (Figure 4). Hemicellulose content was generally lower and cellulose content generally higher in 2000 than in 1999. Although the samples in 2000 showed a general pattern of a higher hemicellulose content and lower cellulose content for Vitaro than for Volens, this apparent cultivar difference was not observed in the 1999 samples. Lignin content increased from around 30 g per

Table 2. NDF, ash and sugar contents (± SEM) of Internodes Number 16 to 8 of the forage maize cultivars Vitaro and Volens (n = 2) in 1999 and 2000.

	Internode Number				
	16	14	12	10	8
			(g per kg DM)		
1999 Vitaro					
NDF [1]	596 ± 4.0	466 ± 2.8	517 ± 3.2	541 ± 4.2	558 ± 0.9
Ash	45 ± 3.3	53 ± 4.1	41 ± 4.0	34 ± 6.1	37 ± 4.0
Su [1]	190 ± 4.4	280 ± 14.7	305 ± 1.6	288 ± 5.5	297 ± 1.1
1999 Volens					
NDF	628 ± 2.8	481 ± 4.5	545 ± 1.8	596 ± 0.6	604 ± 3.2
Ash	38 ± 4.2	44 ± 0.7	46 ± 2.0	32 ± 0.0	26 ± 2.3
Su	149 ± 5.5	262 ± 1.1	250 ± 6.0	246 ± 3.3	257 ± 1.9
2000 Vitaro					
NDF	645 ± 1.3	532 ± 0.9	549 ± 3.0	594 ± 4.1	612 ± 3.2
Ash	38 ± 3.7	46 ± 5.4	47 ± 1.7	38 ± 1.9	43 ± 0.7
Su	139 ± 0.3	233 ± 1.9	253 ± 1.9	240 ± 1.5	222 ± 5.1
2000 Volens					
NDF	679 ± 2.5	577 ± 0.2	569 ± 3.3	643 ± 1.9	679 ± 1.7
Ash	32 ± 6.4	46 ± 0.7	48 ± 3.4	42 ± 0.1	42 ± 5.0
Su	116 ± 2.4	174 ± 5.0	186 ± 8.1	156 ± 8.0	152 ± 3.6

[1] NDF = neutral detergent fibre; Su = sugar content.

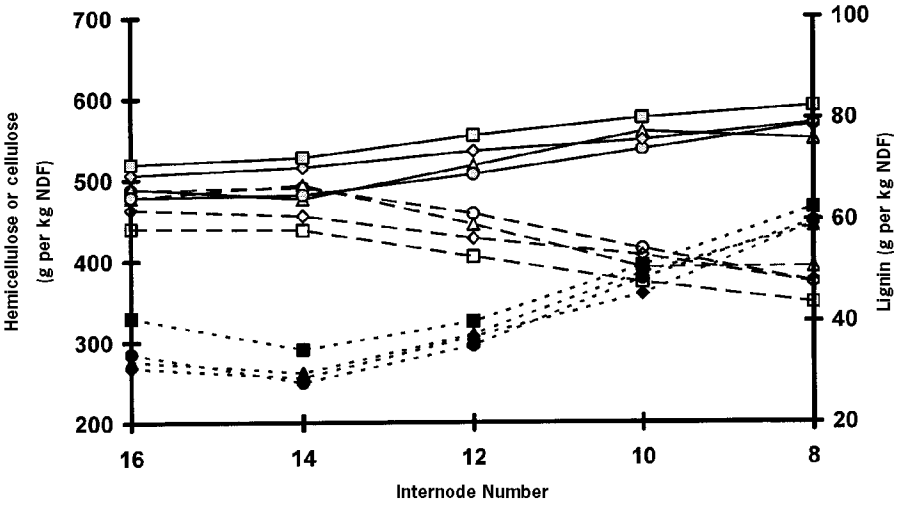

Figure 4. Hemicellulose (open symbols), cellulose (grey symbols), and lignin (black symbols) content (in g per kg NDF) in Internodes 16 to 8 of the forage maize cultivars Vitaro and Volens at anthesis in 1999 and 2000 (Δ Vitaro 1999; ◊ Vitaro 2000; ○ Volens 1999; □ Volens 2000).

kg NDF in Internode 14 to around 60 g per kg NDF in Internode 8. The lignin content of Internode 16 was higher than that of Internode 14. Vitaro, which had the higher whole-plant digestibility, had slightly lower lignin content than Volens. However, lignin content was lower in 1999 than in 2000 for Vitaro, but for Volens the opposite was found. As a result, the cultivar difference in lignin content was higher in 2000 than in 1999 (Figure 4).

The chemical analyses showed a strong influence of internode position on NDF, Su and the chemical composition of the cell wall constituents. Cultivar differences were large: the better digestible cultivar Vitaro had lower NDF and higher Su content in the dry matter and lower lignin content in the NDF, in line with its better digestibility. Cultivar differences in chemical composition were larger in 2000 than in 1999.

Fermentation

Total gas production (gas production after 72 h; GP72), gas production caused by fermentation of the soluble fraction (GP3) and gas production caused by fermentation of the cell walls (gas production between 3 and 20 h, (GP20 – GP3), decreased consistently from Internode 14 to Internode 8 (Table 3). Internode 16 always had a lower GP3, GP20 – GP3 and GP72 than Internode 14. GP3 and GP72 were higher in 1999 than in 2000, whereas GP20 – GP3 was lower in 1999 than in 2000. GP3, GP20 – GP3 and GP72 were consistently higher for Vitaro than for Volens. The T&T data (Tilley & Terry, 1963) closely matched the patterns found in total gas production (GP72) (Table 3; $R^2 = 0.83$,

n = 20), but less than those of GP3 (R^2 = 0.66, n = 20) or GP20 – GP3 (R^2 = 0.66, n = 20).

Fermentation of the cell wall fraction of the internodes at the base of the stem was slower, which is indicated by an increase of B2 from Internode 14 to Internode 8 (Table 3). Variable B2 is a measure for the time at which half of the maximal gas production caused by fermentation of the cell walls was reached. Internode 16 had a much higher B2 than Internode 14. Only small differences were observed in this parameter between the two years and between the two cultivars.

Table 3. Gas production parameters [1] (± SEM) for Internodes Number 16 to 8 of the forage maize cultivars Vitaro and Volens harvested in 1999 and 2000 (n = 2).

	Internode Number				
	16	14	12	10	8
1999 Vitaro					
GP3	53 ± 0.2	78 ± 0.4	75 ± 0.1	66 ± 1.3	61 ± 0.6
GP20 – GP3	154 ± 2.0	173 ± 0.1	153 ± 1.6	143 ± 0.0	126 ± 1.3
GP72	312 ± 2.6	348 ± 2.4	327 ± 0.7	320 ± 1.0	299 ± 5.3
B2	10.3 ± 0.1	9.0 ± 0.0	9.2 ± 0.0	9.7 ± 0.1	10.7 ± 0.0
T&T	66 ± 1.2	79 ± 0.6	76 ± 0.6	71 ± 0.6	67 ± 0.4
1999 Volens					
GP3	46 ± 1.3	70 ± 0.2	62 ± 0.3	62 ± 0.3	56 ± 0.1
GP20 – GP3	142 ± 1.1	172 ± 0.5	146 ± 0.3	126 ± 0.7	120 ± 1.9
GP72	281 ± 2.2	342 ± 1.8	309 ± 0.4	288 ± 0.4	291 ± 3.8
B2	10.6 ± 0.1	9.6 ± 0.0	9.8 ± 0.1	9.7 ± 0.0	10.5 ± 0.0
T&T	61 ± 1.0	74 ± 1.3	67 ± 1.3	61 ± 0.3	59 ± 0.4
2000 Vitaro					
GP3	47 ± 0.6	64 ± 0.7	62 ± 0.5	58 ± 0.3	48 ± 0.8
GP20 – GP3	159 ± 0.4	166 ± 1.8	160 ± 0.4	153 ± 4.2	131 ± 2.3
GP72	307 ± 0.6	321 ± 1.0	315 ± 1.3	320 ± 4.8	290 ± 0.8
B2	10.1 ± 0.0	9.1 ± 0.0	9.2 ± 0.1	9.8 ± 0.0	11.3 ± 0.1
T&T	65 ± 0.3	76 ± 0.6	75 ± 0.2	72 ± 0.2	67 ± 0.3
2000 Volens					
GP3	40 ± 0.9	56 ± 0.8	53 ± 0.2	43 ± 1.8	36 ± 1.1
GP20 – GP3	127 ± 0.0	161 ± 1.0	160 ± 1.9	136 ± 3.2	122 ± 0.8
GP72	255 ± 4.2	312 ± 1.3	302 ± 0.9	272 ± 4.9	267 ± 2.8
B2	10.3 ± 0.0	9.6 ± 0.0	9.2 ± 0.1	9.7 ± 0.1	11.1 ± 0.0
T&T	57 ± 0.2	70 ± 0.3	69 ± 0.4	61 ± 0.2	58 ± 0.6

[1] GP3 = cumulative gas production after 3 h of incubation, GP20 = cumulative gas production after 20 h of incubation, GP72 = cumulative gas production after 72 h of incubation (in ml per g organic matter). B2 = time (h) in which half of GP20 – GP3 was reached. T&T = % digestibility according to Tilley & Terry (1963).

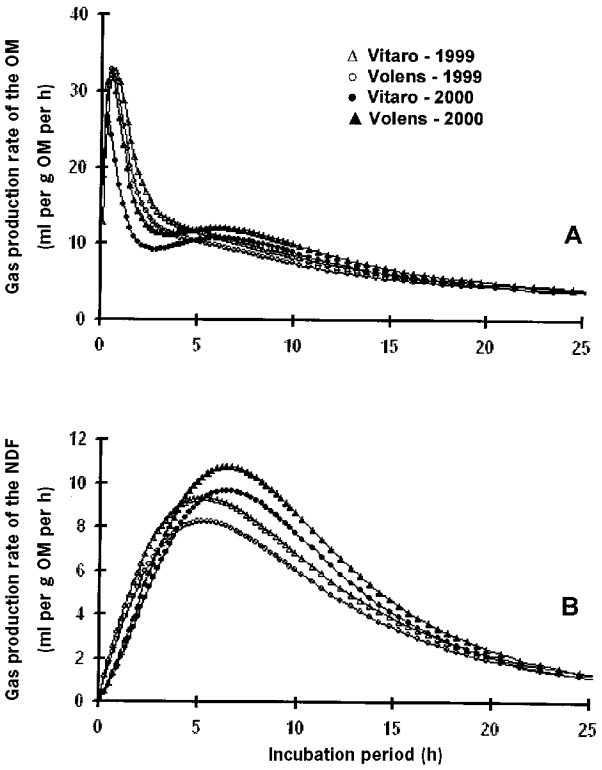

Figure 5. Relationship between gas production rate (ml per g OM per h) and incubation period (h) of samples of Internode 10 of the forage maize cultivars Vitaro and Volens. A: the entire sample; B: cell wall constituents only. OM = organic matter.

Gas production data were used to visualize the gas production rate for the whole sample (Figure 5A), or for the cell wall constituents only (Figure 5B). The gas production data were in line with the three-phasic model of Groot *et al.* (1996). Rapid fermentation of the cell contents is reflected by the initial peak in Figure 5A. Cell wall fermentation is reflected by the second peak, isolated in Figure 5B. Differences in maximal gas production between samples originated from an early stage of fermentation. The maximal gas production rate of the cell wall constituents was higher for Vitaro than for Volens, and higher for the 2000 samples than for the 1999 ones. Although the B2 parameter was the same for the samples in Figure 5, compared with the 1999 samples, the peak rate for the 2000 samples clearly occurred at a later stage of the incubation. This was related to a steeper gas production curve and a higher maximal gas production for the 2000 samples.

Fermentation data clearly show the large differences in digestibility between internodes. Whereas the trends along the stem were the same for both cultivars, all fermentation

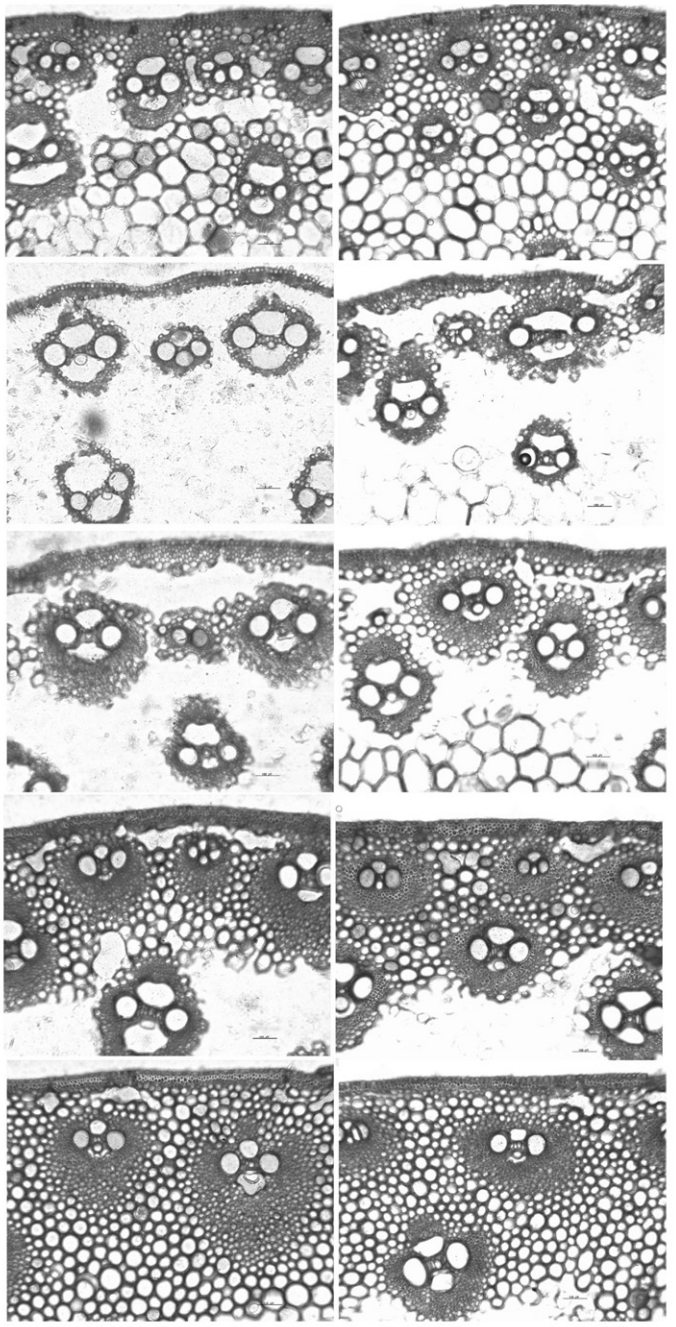

Figure 6. Cross sections of Internodes 16 (top), 14, 12, 10, and 8 (bottom) of the forage maize cultivars Vitaro (left) and Volens (right) after 24 h of fermentation in buffered rumen fluid.

Table 4. Cell wall thickness (μm; ± SEM) and rate of decrease in cell wall thickness (nm h^{-1}) of sclerenchyma cells adaxial to rind vascular bundles in Internode Numbers 16 to 08 of the forage maize cultivars Vitaro and Volens after 0, 12, 24, or 48 h of incubation in buffered rumen fluid, in 1999 and 2000 (n = 30).

	Vitaro					Volens				
Intermode:	16	14	12	10	08	16	14	12	10	08
1999										
0 h	1.4 ± 0.1	1.3 ± 0.1	1.9 ± 0.1	2.1 ± 0.1	2.8 ± 0.1	2.3 ± 0.1	1.7 ± 0.1	2.1 ± 0.1	2.2 ± 0.1	2.7 ± 0.1
12 h	0.6 ± 0.1	0.5 ± 0.1	0.9 ± 0.1	1.3 ± 0.1	1.7 ± 0.2	1.1 ± 0.1	0.6 ± 0.1	1.1 ± 0.1	1.6 ± 0.2	2.2 ± 0.2
Difference	0.9	0.8	1.0	0.8	1.1	1.2	1.0	1.0	0.6	0.5
Decrease h^{-1}	72	69	83	65	90	103	87	82	48	45
% decrease	61	64	53	37	39	54	62	47	26	20
0 h	1.4 ± 0.1	1.3 ± 0.1	1.8 ± 0.1	2.0 ± 0.1	2.6 ± 0.2	2.3 ± 0.1	1.5 ± 0.1	2.0 ± 0.1	1.9 ± 0.1	2.8 ± 0.1
24 h	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.5 ± 0.0	0.8 ± 0.1	0.5 ± 0.0	0.3 ± 0.0	0.4 ± 0.0	0.7 ± 0.1	1.0 ± 0.1
Difference	1.2	1.0	1.5	1.5	1.8	1.8	1.3	1.7	1.2	1.8
Decrease h^{-1}	49	41	61	63	74	75	52	69	52	76
% decrease	82	79	84	76	70	78	83	82	66	64
0 h	1.6 ± 0.1	1.3 ± 0.1	1.8 ± 0.1	2.2 ± 0.1	2.5 ± 0.2	2.1 ± 0.1	1.6 ± 0.1	2.1 ± 0.1	1.9 ± 0.1	2.6 ± 0.1
48 h	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.4 ± 0.0	0.6 ± 0.1	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.6 ± 0.1
Difference	1.3	1.0	1.5	1.8	1.9	1.9	1.3	1.8	1.7	2.0
Decrease h^{-1}	27	21	31	39	39	39	27	38	35	43
% decrease	82	79	84	84	75	88	84	87	86	77
2000										
0 h	1.6 ± 0.1	1.3 ± 0.1	1.7 ± 0.1	2.4 ± 0.1	2.7 ± 0.2	2.1 ± 0.1	1.6 ± 0.1	2.3 ± 0.1	2.4 ± 0.1	3.2 ± 0.2
12 h	0.9 ± 0.1	0.7 ± 0.1	0.9 ± 0.1	1.3 ± 0.1	1.6 ± 0.2	1.5 ± 0.1	1.0 ± 0.1	1.2 ± 0.1	1.4 ± 0.1	2.4 ± 0.2
Difference	0.8	0.6	0.8	1.2	1.1	0.6	0.6	1.1	1.0	0.8
Decrease h^{-1}	65	53	70	96	95	53	49	93	80	67
% decrease	48	49	49	47	41	29	36	49	40	25
0 h	1.7 ± 0.1	1.4 ± 0.1	1.6 ± 0.1	2.4 ± 0.1	3.0 ± 0.2	2.0 ± 0.1	1.6 ± 0.1	2.0 ± 0.1	2.7 ± 0.2	3.4 ± 0.2
24 h	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.5 ± 0.0	0.9 ± 0.1	0.4 ± 0.1	0.3 ± 0.0	0.6 ± 0.1	1.0 ± 1.1	1.3 ± 1.0
Difference	1.4	1.1	1.4	1.9	2.1	1.6	1.2	1.4	1.7	2.1
Decrease h^{-1}	59	46	57	77	88	68	52	57	72	87
% decrease	83	80	83	77	69	81	80	69	65	62
0 h	1.8 ± 0.1	1.3 ± 0.1	1.9 ± 0.1	2.2 ± 0.2	3.0 ± 0.2	1.7 ± 0.1	1.6 ± 0.1	2.2 ± 0.1	2.7 ± 0.1	3.5 ± 0.2
48 h	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.4 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.6 ± 0.1	0.5 ± 0.0	0.8 ± 0.1
Difference	1.5	1.0	1.6	1.9	2.6	1.4	1.4	1.6	2.2	2.7
Decrease h^{-1}	32	22	33	40	54	28	28	33	46	56
% decrease	85	79	85	85	87	81	85	72	81	78

parameters, except B2, were higher for Vitaro than for Volens, thus confirming the better digestibility of Vitaro.

Cross section fermentation

In the cross sections major changes in cell wall thickness were observed 24 hours after incubation in rumen fluid (Figure 6). As expected, cell wall thickness decreased rapidly during the initial stages of fermentation. In general, the first cell walls to disappear were those of phloem tissue and parenchyma tissue surrounding the vascular bundles. Phloem was fully broken down after 12 h of incubation, whereas for the parenchyma and chlorenchyma tissues it took longer to disappear. Cell wall disappearance was relatively low in Internode 8 and became faster and larger towards the top of the stem, with only xylem rings, epidermis and sclerenchyma cell walls left in Internode 14. Cell wall degradation in Internode 16, the peduncle, was low compared with Internode 14. Secondary sclerenchyma cell walls with an initial cell wall thickness of 2.0 µm or less appeared to be fermented completely down to the primary wall and middle lamella (Figure 6).

Initial cell wall thickness was always higher in Volens than in Vitaro (Table 4). This consistent difference is in line with the data presented in Figure 3 for the lower part of

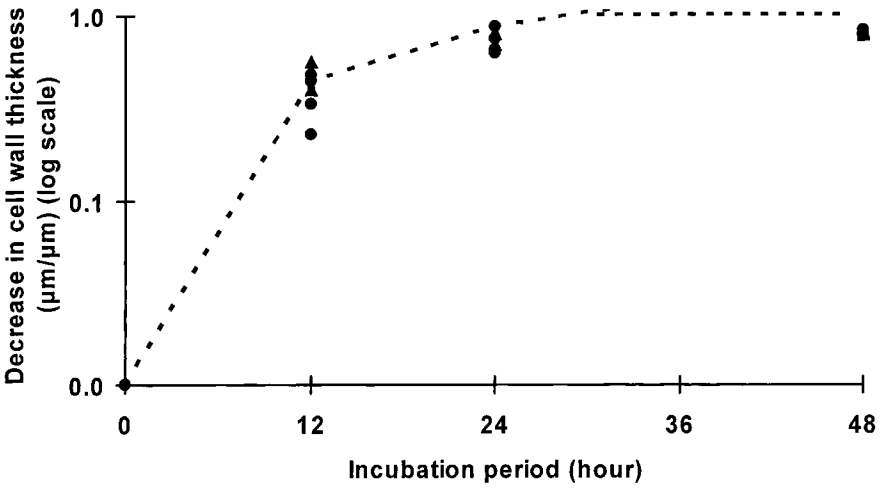

Figure 7. Decrease in cell wall thickness (µm per µm original cell wall thickness) of internode samples of the forage maize cultivars Vitaro (triangles) and Volens (circles) after 12, 24 or 48 h of fermentation in buffered rumen fluid. Dashed line indicates the decrease extrapolated from the mean rate during the first 12 h. Data points are from Internodes 8, 10, 12, 14 and 16.

the stem. The relative rate at which cell wall material disappeared did not differ much between cultivars (Figure 7), although the absolute decrease in cell wall thickness was slightly higher in Volens than in Vitaro with longer duration of fermentation (Table 4). The highest total cell wall thickness disappearance was 2.7 μm in samples of Internode 8 in Volens.

Discussion

The stem characteristics investigated will be evaluated with a focus on the changes from the top to the base of the stem, their correlation, and their impact on fermentation of the cell wall. The year and cultivar effects will be discussed in separate sections.

Differences between internodes from top to base of the stem

Morphology

The main apparent difference in morphology from top to base of the stem was the large increase in internode volume due to an increase in both internode length and diameter. The increase in diameter was linear over the entire length of the stem, except for the internode carrying the ear, which was markedly longer.

Anatomy

The contribution of sclerenchyma tissue to the weight of an internode is likely to increase towards the base of the stem. To be able to support the weight of the stem above them the lower parts of the stem (especially below the main ear) need more strength, which makes it plausible why lower internodes have more sclerenchyma than upper internodes. The larger amount of sclerenchyma in the lower internodes compared with the upper internodes is reflected by a greater number of layers of sclerenchyma and a thicker cell wall of this tissue.

Chemical analyses

The chemical analyses provide strong evidence for the large differences in digestibility of the different internodes. NDF, which represents the cell walls, consists of three constituents: hemicellulose, cellulose, and lignin. Despite the fact that hemicellulose can be removed from the complex with acid detergent, lignin is assumed to be primarily linked to hemicellulose (Hatfield, 1993). Figure 4 shows a decrease in hemicellulose and an increase in lignin towards the base of the stem, suggesting an increased frequency of linkages between the two constituents in the lower internodes. The presence of such linkages may not only prevent the degradation of hemicellulose, due to shielding, it may also hamper the degradation of cellulose (Chesson, 1993). Gas production results as well as T&T results are in line with the observed changes in cell wall composition.

Fermentation

A larger amount of available secondary cell wall could result in more gas production

from sclerenchyma tissue. Gas production data and chemical data together indicated that the secondary cell wall was not equally digestible in all internodes, and that digestibility was lower towards the base of the stem. A reduced fermentation of the cell wall fraction from the top to the base of the stem could be due to a higher physiological age of the tissues lower in the stem, which often is associated with more advanced incrustation of lignin into the cell walls. Data from fermented sections show that the available sclerenchyma tissue at the base of the stem was not fully digested after 48 h of fermentation, even though the cells in these sections were fully accessible to micro-organisms (Table 4).

Microbial colonization of small particles increases as particle size decreases (Gerson *et al.*, 1988). Therefore, digestion of sclerenchyma cell walls might have been less in the particles used in gas production tests than in the sections fermented with rumen fluid.

In wheat straw, pore size of the cell walls was insufficient to allow optimal fermentation rates (Chesson *et al.*, 1997). This could mean that there is a potential for improvement of fermentation rate at cell wall level, which would imply altering the physical structure of the cell wall. This could for example be done by changing the particle size of the forage through finer chopping or milling, or by altering the chewing behaviour of the ruminant during eating or rumination (see also Wilman *et al.*, 1999).

Section fermentation

The mean rate of cell wall disappearance (averaged over all samples) during the initial 12 h of fermentation was 73 nm h^{-1}. Assuming this to be the optimal rate of cell wall disappearance, as much as 3.5 μm could be broken down in 48 h. However, in our 48-h-fermented samples a minimal cell wall thickness of 0.3 μm was found, which approximates reported values of the primary wall / middle lamella complex. In lignified tissues such as the sclerenchyma, the primary wall / middle lamella complex is very resistant to degradation, and therefore a cell wall with a thickness of 0.3 μm can be considered not degradable.

Year effects

The longer internodes in 2000 compared with 1999 reflected differences in growing conditions between the two years, at least during stem elongation. Differences in internode length will affect chemical composition. Internode length will affect the rind–pith ratio and long internodes will need relatively more sclerenchyma tissue to provide adequate stem support. Compared with the rather cold and wet growing season of 2000, the warm and sunny growing season of 1999 resulted in shorter internodes and consequently in a higher sugar content.

The crops of 1999 and 2000 were grown on different fields. Struik (1983) already suggested large differences in cell wall digestibility of forage maize between locations. In general, in our experiments the cell walls contained more hemicellulose and less cellulose in 1999 than in 2000.

Differences between years had little effect on the anatomical characteristics of the stems.

Cultivar effects

The phenological and morphological development of the cultivars have been described in more detail in earlier papers (Boon *et al.*, 2005a, b). There were large differences in many respects between the two cultivars and these were usually consistent over the different internodes. Cultivar effects included shorter and thicker internodes and thinner sclerenchyma cell walls in Vitaro than in Volens. Moreover, Vitaro had higher sugar and lower NDF contents in the dry matter than Volens. The NDF fraction contained slightly less lignin in Vitaro than in Volens. All these differences point in the same direction: Vitaro is more digestible than Volens. This was in line with a higher gas production and higher T&T values for Vitaro than for Volens.

Correlation between chemical composition and gas production characteristics

Sugar content correlated very well ($P < 0.001$) with gas production after 3 h of incubation in buffered rumen fluid (Figure 8). The intercept of 20.6 ml per g organic matter represents fermentation of non-sugar cell contents, such as protein, and of soluble constituents of the cell wall, such as pectin.

The relationships between hemicellulose, cellulose or lignin content in NDF and GP20 – GP3 are shown in Figures 9A, B, and C. A relationship between hemicellulose

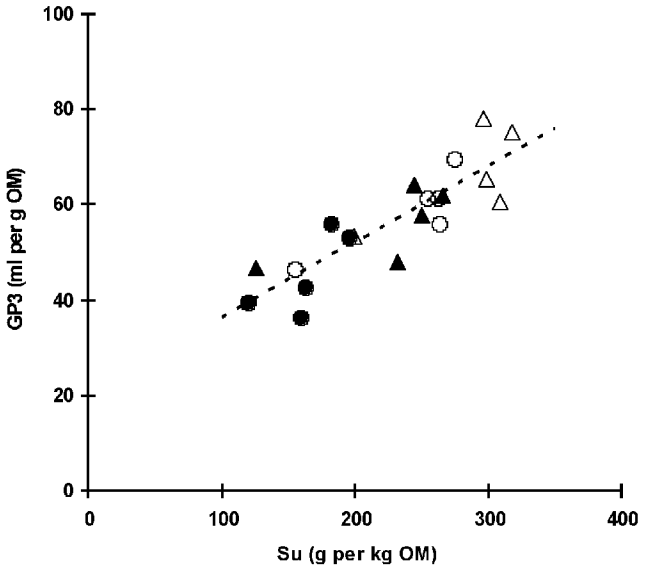

Figure 8. Relationship between sugar content (Su; g per kg OM) and gas production after 3 h of incubation in buffered rumen fluid (GP3; ml per g OM) of the forage maize cultivars Vitaro (triangles) and Volens (circles) in 1999 (open symbols) and 2000 (closed symbols). The overall linear regression represented by the dashed line is: $y = 20.64x + 0.16$; $R^2 = 0.995$, n = 20. OM = organic matter.

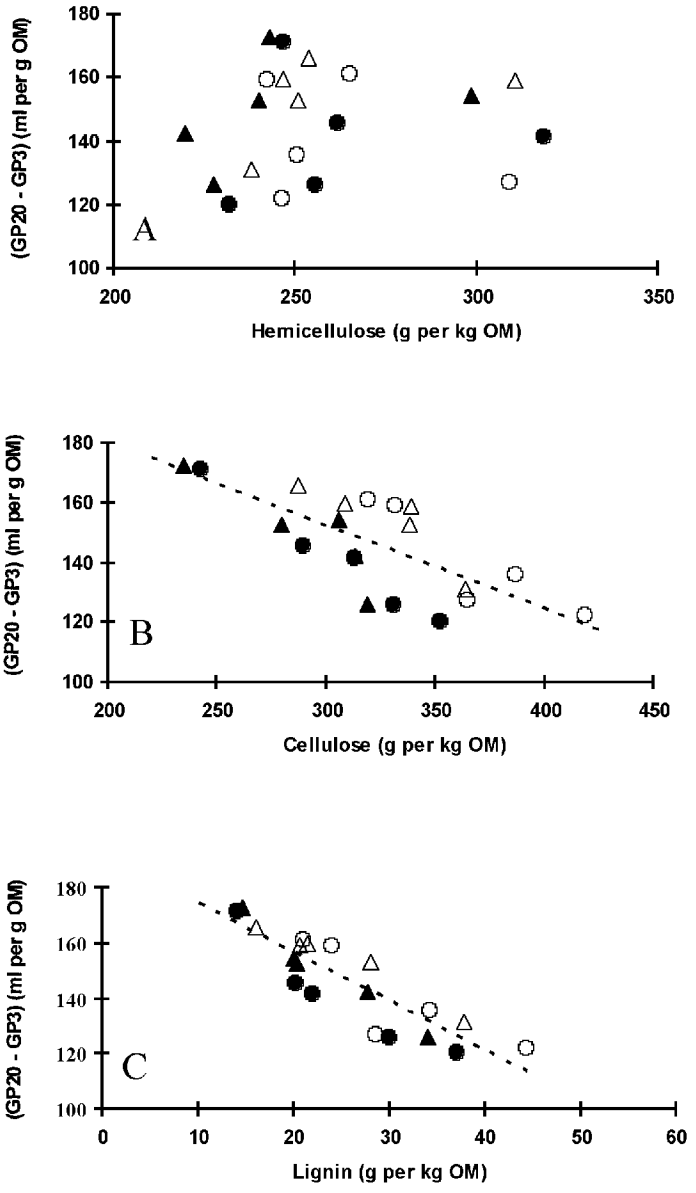

Figure 9. Relationship between hemicellulose (A), cellulose (B) and lignin content (C) (g per kg OM), and gas production between 3 and 20 h of incubation in buffered rumen fluid (GP20 – GP3) (ml per g OM) of the forage maize cultivars Vitaro (triangles) and Volens (circles) in 1999 (open symbols) and 2000 (closed symbols). Dashed lines are fitted trend lines for cellulose and lignin. Fitted lines (n = 10) for cellulose in 1999: $y = -0.468x + 284.6$ ($R^2 = 0.903$); for cellulose in 2000: $y = -0.386x + 281.0$ ($R^2 = 0.818$); for lignin in 1999: $y = -2.218x + 198.6$ ($R^2 = 0.902$); for lignin in 2000: $y = -1.709x + 194.6$ ($R^2 = 0.832$). OM = organic matter.

content and GP20 – GP3 did not exist (R^2 = 0.01). Both cellulose and lignin content were strongly and negatively correlated with GP20 – GP3, with R^2 values for the linear relationship for all 20 observations of 0.54 and 0.78 for cellulose and lignin content, respectively. However, the relationships differed between the two years, with a less negative slope in 2000 than in 1999 for both cellulose and lignin and with much higher R^2 values for the individual years than for the pooled data.

A lower gas production of insoluble constituents towards the base of the stem was correlated with a relatively higher cellulose and lignin content. Again, the higher cellulose and lignin content at the base could indicate a higher structural strength of the lower internodes, needed to prevent lodging and support the weight of the plant. The peduncle, which has the function of supporting the tassel, also had higher lignin content than Internode 14.

Our analyses show that around anthesis the differences in digestibility between internodes and the differences between cultivars are associated with differences in relative abundance of vascular bundles and sclerenchyma, in cell wall thickness, cell wall content and cell wall composition. Further research should clarify to what extent this is relevant for differences in cell-wall digestibility and whole-plant digestibility among cultivars at silage maturity.

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