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Stem characteristics of two forage maize (*Zea mays* L.) cultivars varying in whole plant digestibility. IV. Changes during the growing season in anatomy and chemical composition in relation to fermentation characteristics of a lower internode

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ABSTRACT

Improving digestibility of forage maize (*Zea mays* L.) through breeding is important to optimize the efficiency of ruminant's rations. It can partly be achieved by improving the digestibility of stem tissue, a genetically complex and diverse trait changing drastically during the growing season. We tried to gain insight into this trait by analysing the changes during the growing season in the anatomy, chemical composition and fermentation characteristics of a lower internode (internode 7) of two forage maize cultivars differing in whole plant digestibility. These two cultivars, known to differ in digestibility, were grown in the Netherlands for two growing seasons. Cell wall thickness of the sclerenchyma tissue in the rind of internode 7 increased linearly with the temperature sum until reaching final cell wall thickness several days before anthesis. Volens, the less digestible cultivar, had a higher final cell wall thickness than Vitaro, the cultivar with a better digestibility. Chemical analyses included determination of NDF, ADF, ADL, crude protein, sugar content, and ash. Lignin content increased until shortly after anthesis in both cultivars, in both years. Lignin content was higher for Volens than for Vitaro and higher in 2000 than in 1999. Crude protein content decreased from 15–21% in early July to 2–5% in late September with no clear differences between cultivars or years. Fermentation characteristics showed that maximum gas production of cell wall components was highest immediately before anthesis and subsequently decreased. At all stages of development, Vitaro had a higher maximum gas production than Volens. The cultivar differences in digestibility could not be confirmed by differences in rate of cell wall disappearance. Seasonal changes showed an increase in fermentable cell wall material until anthesis; thereafter fermentability decreased. Differences in cell wall thickness and in lignin content reflected the changes in digestibility during the growing season best; the differences between the two contrasting cultivars were best reflected by the differences in cell wall thickness, lignin content and the decline of the potential digestibility in the period before anthesis.

1. Introduction

Forage maize (*Zea mays* L.) is used extensively as a ruminant feedstuff. Unlike grasses, which are often repeatedly mown, forage maize generally is harvested only once per growing season. The maize is often provided to ruminants in the form of whole-plant silage. Although much of the feeding value of the maize plant is accounted for by the ear, which is high in starch, a considerable proportion comes from cell wall material, present in stem and leaves. The proportion of stem and leaf material in the total dry matter can be as high as 30–50% at the end of the season [1,2].

Breeding forage maize for improved digestibility is important to improve the utilization by the ruminant of the roughage taken in [3–8]. An increase in digestibility of forage maize can partly be achieved by increasing the fraction of the ear in the total dry matter [5], the ear being the most digestible part of the plant at the end of the growing season [1]. Another approach could be improving the digestibility of stem tissue [4,6–8], especially through stem cell-wall digestibility [3,4,6]. Both the fraction of the ear and the digestibility of the stem tissue are genetically complex and diverse traits that change drastically during the growing season [1].

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The digestibility of the stem material is highly variable, partly because of genetic variation [1,9–11], and is associated with a large variation in cell wall content and cell wall degradability [1,9–11]. This variation is associated with differences in anatomy and in chemical composition of the various tissues, both influenced by the genetic make-up of the plant, the phenology of the crop, environmental conditions (including those related to crop management) and their interactions.

Types of tissues in Poaceae differ in rate and extent of digestion [12–15], the tissue differences partly depending on environmental conditions [16]. Differences between tissues have also been found in forage maize [17,18]. Moreover, differences between maize tissues change with crop maturity [18–20], mainly due to differences in level of lignification [21]. These differences between tissue types and the changes during crop development are strongly, but not merely related to chemical composition [16]. This means that both chemical and biological tools are required to unravel the causes of these differences.

There is also a strong genetic component, but the anatomy of stems, the cell wall composition and the cell wall digestibility of stem material are also genetically complex traits. Genetic variation in anatomy is relatively poorly investigated in large populations because such studies are very laborious. But for both cell wall composition and cell wall digestibility of stalk material there are many quantitative trait loci identified, each of the quantitative loci having a small or at most moderate effect [22–25].

The internodes of a maize stem differ in length, thickness, anatomy, chemical composition and digestibility [26]. The lower internodes start to grow first, as they are the oldest. From the base to the top of the stem, internode elongation follows a sigmoidal pattern [27]. So lower internodes have older cell wall material and will reflect changes during phenology earlier in time and also longer, provided internodes keep changing until harvest. The lowest internodes of the maize stem are not very suitable for detailed studies as they remain relatively short [26,27].

This paper is about the changes during the growing season in the quality of stem tissue in a particular internode, i.e., internode 7 counting from below, a relatively thick internode positioned between soil level and the insertion point of the main ear, but rather close to the soil. This internode, although closer to the base, is probably similar in anatomy to the fourth elongated internode above ground used by Jung and Casler [18,19] to characterize changes occurring during maize stalk development. They observed that the changes in quality of a maize internode during growth of the stalk were caused by secondary cell wall development in the sclerenchyma and parenchyma of the rind [18,19].

This paper is part of a larger study on the fermentation of the maize stem, in which fermentation of stem tissue at different positions within a selected internode at anthesis, and fermentation of internodes from the top to the base of the maize stem at anthesis were examined [2,26,28] for the contrasting forage maize cultivars Vitaro and Volens, which differ in whole plant digestibility by 9% (Advanta Seeds B.V., unpublished results, measured with Near Infrared Reflectance Spectroscopy). Vitaro was commercially grown in the Netherlands and Volens was commercially grown in France [29,30]. These previous studies clearly indicated that internode 7 would be a suitable one to investigate the changes in anatomy of cell wall characteristics during the growing season, because of its relatively large increase in diameter and its relatively large increase in diversity of tissue quality.

In the current study, this lower internode of both cultivars was sampled throughout the growing season, from late June until mid-September. Anatomical and chemical features of the selected internode were examined and analysed in relation to its fermentation characteristics. The objectives were (1) to characterize the genotype-specific changes in anatomy, chemical composition and digestibility of this lower internode during growth and development of the forage maize crop, (2) to assess which type of characteristics could explain the large difference in digestibility between these two contrasting cultivars best, and (3) to assess how consistent this explanation could be during the growing season and the two years of cultivation.

2. Materials and methods

2.1. Plant growth

The maize cultivars Vitaro and Volens were grown on a heavy river clay soil near Wageningen (51°6′N; 5°40′E), in plots of 18 m × 20 m at a density of 10 plants per m^2. Sowing took place on 19 May in 1999 and 3 May in 2000, and both cultivars flowered in the first week of August in both years. Uniform plants were sampled to monitor leaf growth and development. Internode 7 samples were taken twice a week until anthesis, once a week during the first 3–4 weeks after anthesis, and once every fortnight during the last month of growth. Internode 7 was the first or second fully elongated internode above soil level. It was selected because of its large (changes in) diameter and large (changes in) diversity of tissue quality. Internodes were numbered from the base of the stem upward. The internode subtended by leaf 1 was designated internode 1. Internode numbering was verified by measuring the length of the leaf subtending the internode [31]. The method was supported by measuring the length of leaves 6, 7 and 8 of Vitaro and Volens in 2000 to identify internode 7. Leaf lengths in 2000 were similar to those in 1999.

In total, each cultivar was sampled 15 times in each year. Internode and stem samples were kept at −20 °C until further analysis.

2.2. Temperature sum and sunshine hours

The progression of maize development is closely related to the temperature during the growing season [32]. In order to be able to compare the growing seasons of 1999 (relatively dry, warm season) and 2000 (relatively wet, cold season), results were related to the temperature sum, which was calculated for each day from the time of sowing and expressed in °Cd [33], using the following equation:

$$\text{T-sum} = \Sigma\left(\left(\frac{T_{\max} + T_{\min}}{2}\right) - T_{base}\right)$$

where T_{max} = daily maximum temperature; T_{min} = daily minimum temperature; T_{base} = 10 °C.

Temperature sums for the sampling dates are listed in Table 1. In 1999 and 2000, the daily average temperatures after sowing were always above the base temperature, although occasionally values were close to 10 °C.

During and after flowering, incoming radiation can significantly influence the chemical composition of stem parts [1]. We therefore assessed the number of sunshine hours for August and September of the years 1999 and 2000. In 1999, August had 179 h of sunshine and September had 164 h. In 2000, August had 215 h of sunshine and September had 110 h. This means that in 1999, August and September were almost equally sunny, whereas in 2000, September had only about half the number of sunshine hours of August.

2.3. Anatomy

The sampled internodes were divided into two equal parts. Sections (100 μm) were cut from the top of the lower half of the internode, using a sledge microtome (Ernst Leitz, Wetzlar, Germany). The upper half was discarded. The sections were used to count the number of layers of sclerenchyma (SCL) in the subepi-

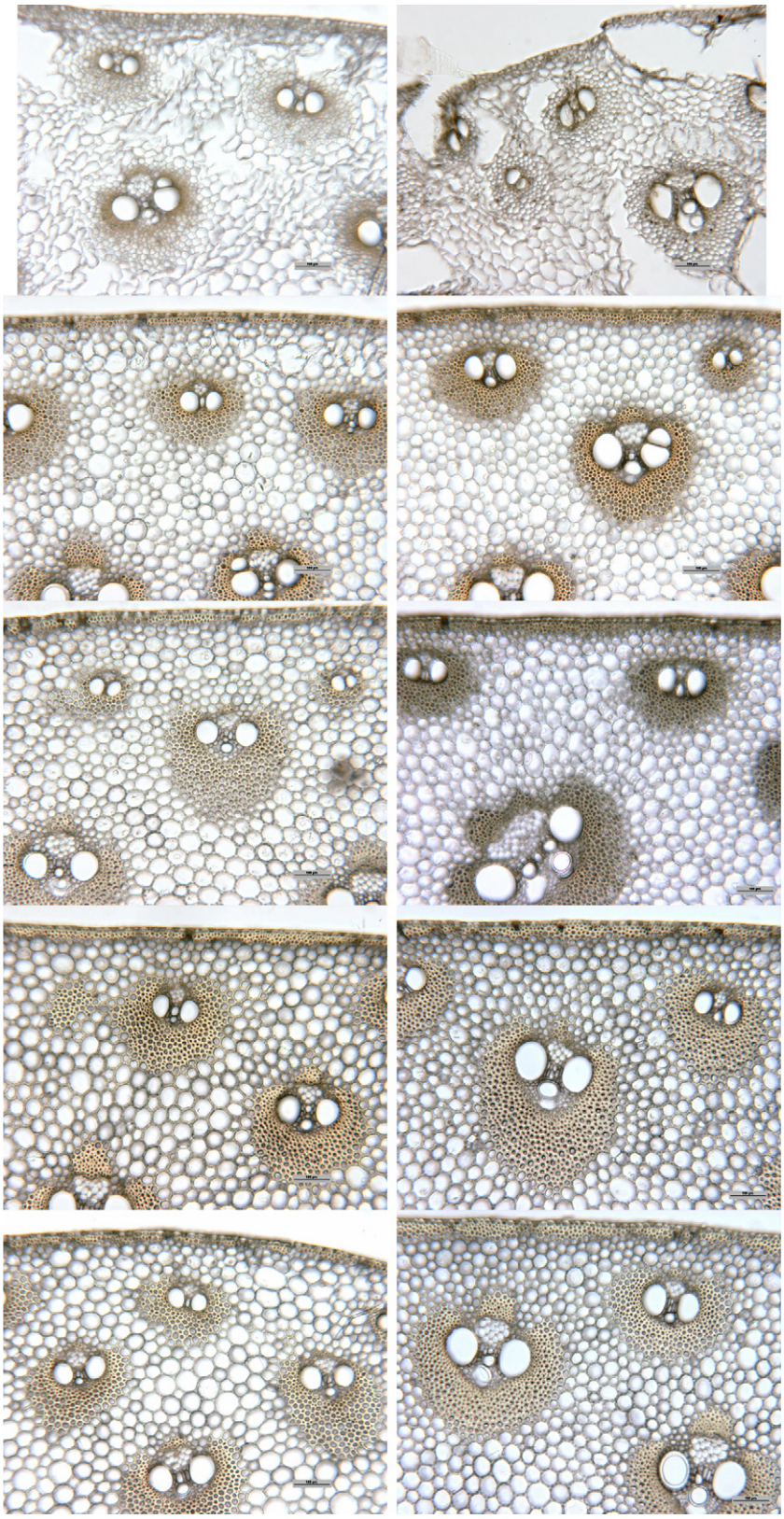

Fig. 1. Cross-sections of internode 7 of forage maize cultivars Vitaro (left) and Volens (right) sampled in 1999 on 12 July (top), 19 July, 22 July, 9 August and 13 September (bottom), corresponding to temperature sums of approximately 300, 360, 385, 550 and 800 °Cd.

dermal layer and the number of layers of SCL adaxial to rind vascular bundles, and were mounted on slides for fermentation tests as described in detail by Engels and Schuurmans [34]. Cell wall thickness of SCL was measured at 1250× magnification using the UTHSCSA ImageTool programme (developed at the University of Texas Health Science Center at San Antonio, Texas and available from Internet by anonymous FTP from maxrad6.uthscsa.edu). The number of observations per section was 30.

Table 1
Sampling dates and corresponding temperature sums (°Cd). Dates of sowing, anthesis and usual harvests for silage use are indicated.

1999		2000	
Sampling date	T-sum (°Cd)	Sampling date	T-sum (°Cd)
19 May (sowing)	0	3 May (sowing)	0
9 July	266	26 June	295
12 July	301	3 July	324
14 July	318	7 July	349
16 July	330	10 July	359
19 July	359	13 July	369
22 July	383	17 July	385
26 July	414	20 July	400
29 July	437	24 July	426
2 August (anthesis)	483	27 July	448
9 August	548	3 August (anthesis)	503
16 August	587	10 August	551
23 August	618	14 August	590
30 August	673	21 August	643
13 September (harvest)	796	4 September	717
27 September	895	18 September (harvest)	799

2.4. Chemical analyses

Neutral detergent fibre (NDF), acid detergent fibre (ADF), acid detergent lignin (ADL), crude protein (CP), ash content (Ash), and sugar content (SU) were determined in duplicate at the Animal Sciences Group (Lelystad, the Netherlands) using the techniques described by Goering and van Soest [35]. Cellulose was calculated as ADF–ADL, hemicellulose as NDF–ADF, and lignin was assumed to be equal to ADL.

2.5. Fermentation of ground material

Separate samples of internode 7 were oven dried at 70 °C for at least 48 h and ground to pass a 1-mm screen. Fermentation characteristics of these samples were determined using the gas production technique as described by Cone et al. [36]. The gas production profiles obtained were fitted with a three-phase model, describing the fermentation of the soluble components (subcurve 1), the non-soluble components (subcurve 2) and the microbial turnover (subcurve 3) [37]. 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 sharpness of the curve) [38].

In this paper, the gas production after 3 h (gp3) was defined as *a1* and the gas production between 3 and 20 h (gp20–gp3) as *a2*. Additional to the fit, *b2* (time to reach 50% of *a2*, i.e., (gp20–gp3)) was also determined directly from measured data. Total duration of fermentation was 72 h (gp72).

2.6. Fermentation of sections

The 100-μm sections were mounted on double-sided tape that in turn was mounted on microscope slides [34]. The sections were fermented in a single container with 1.5 l of buffered rumen fluid (as used for the gas production technique [36]), with 12.5 g of maize internode material (dried and ground to pass a 1-mm screen) as additional substrate. The sections were removed after 12, 24, or 48 h of fermentation. Mirror sections were used as a reference to evaluate the decrease in cell wall thickness.

The set-up of the fermentation of the sections allowed for only a limited number of samples. So a selection was made of samples taken at T-sums of about 300, 360, 385 (before anthesis), 550 (shortly after anthesis), and 800 °Cd (end of season).

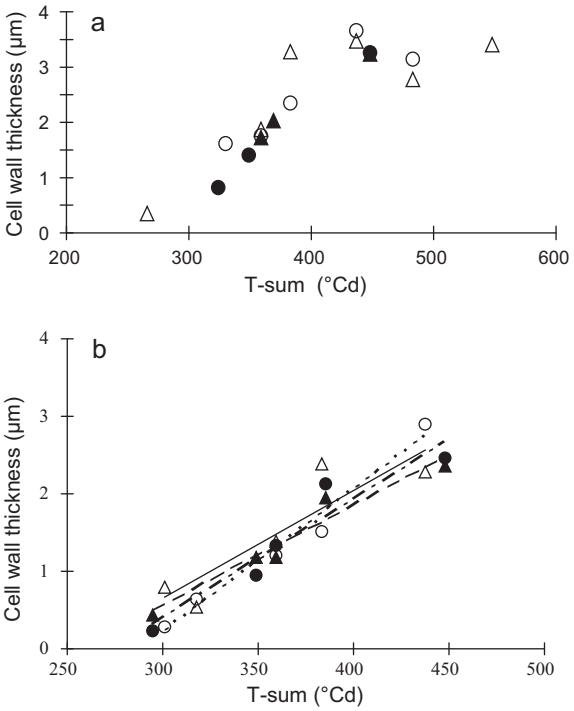

Fig. 2. (a) Cell wall thickness of sclerenchyma tissue in the rind of internode 7 of forage maize cultivars Vitaro (triangles) and Volens (circles) sampled in 1999 (open symbols) and 2000 (closed symbols), in relation to temperature sum. (b) Linear fits of cell wall thickness increase between T-sum 300 °Cd and 450 °Cd (solid line: Vitaro 1999, dotted line: Volens 1999, dashed line: Vitaro 2000, dashed and dotted line: Volens 2000), in relation to temperature sum.

Cell wall thickness of unfermented and fermented sections was measured as described for unfermented sclerenchyma cell walls in Section 2.3.

2.7. Statistical methods

All chemical analyses were done in duplicate, averaged and the standard errors of the means calculated. Values (means and standard errors of the mean) for cell wall thickness of sections (either before or after fermentation) were based on 30 observations per section. Regression analyses were based on average values for both the dependent and the independent variable. We used Statistix for Windows version 2.0 for statistical data processing.

3. Results

3.1. Anatomy

The number of cell layers in the rind and in the sclerenchyma close to the vascular bundles of internode 7 increased during the development of the crop; this trend was stronger in Volens than in Vitaro (Fig. 1). Moreover, cell wall thickness of the sclerenchyma tissue adaxial to the rind vascular bundles in internode 7 tended to increase with T-sum (Figs. 1 and 2). Although this increase in cell wall thickness levelled off after flowering (Fig. 2a), the increase was linear over the range of T-sum 300 °Cd to T-sum 450 °Cd (Fig. 2b), i.e., during rapid internode growth. Individual linear fits for this range showed statistically significant r^2 values of 0.80, 0.97, 0.94 and 0.91 for Vitaro 1999, Volens 1999, Vitaro 2000 and Volens 2000, respectively (Fig. 2b). The increase in cell wall thickness per T-sum unit was different for the four combinations of variety × year. The rate of increase was higher for Volens than for Vitaro in both years, but the difference was somewhat larger in 1999. Maximum cell

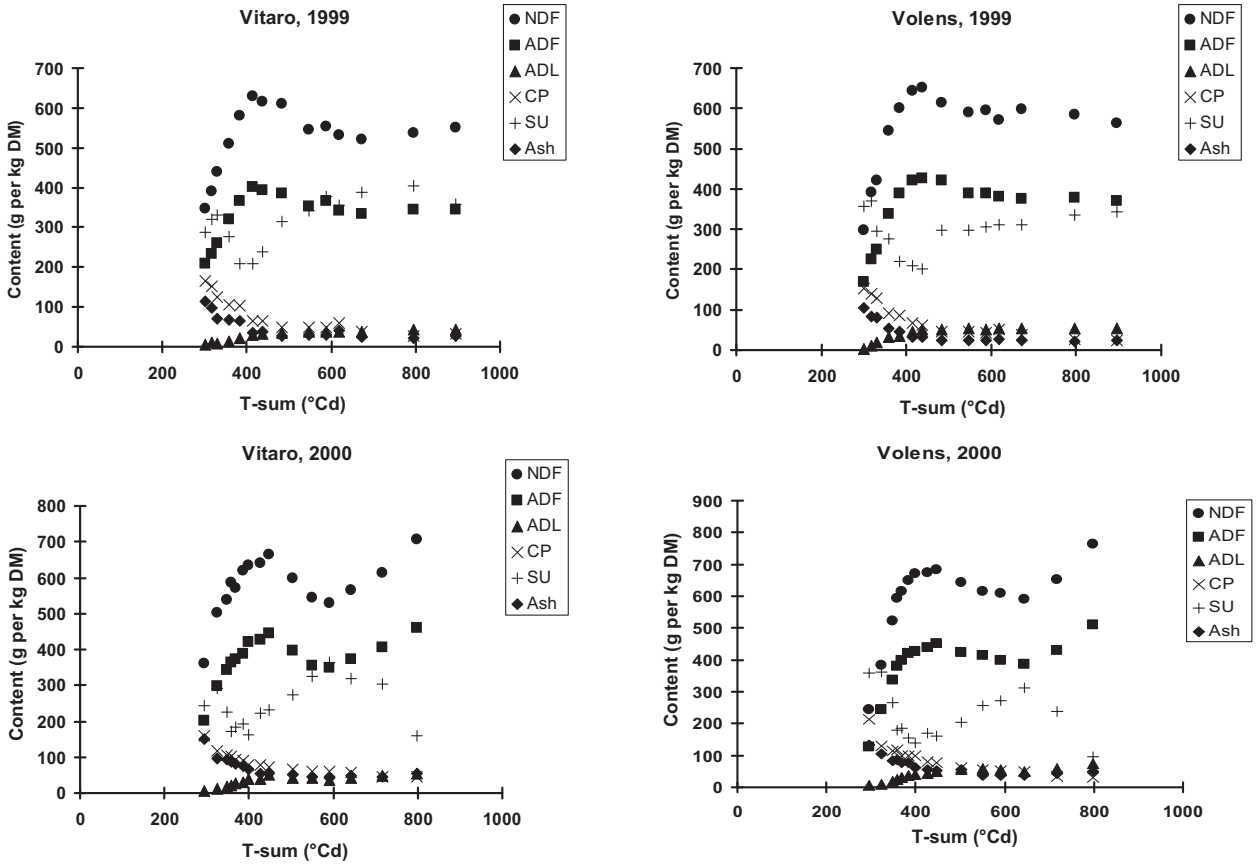

Fig. 3. Chemical composition (in g per kg dry matter) of internode 7 of forage maize cultivars Vitaro and Volens stems sampled in 1999 and 2000 against temperature sum (T-sum in °Cd). Values are averages of duplicate analyses; standard errors of the mean averaged across sampling date, year and cultivar were (in g per kg DM): NDF: 2.3; ADF: 2.1; ADL: 1.0; CP: 0.7; SU: 5.1; Ash 0.4. NDF = neutral detergent fibre; ADF = acid detergent fibre; ADL = acid detergent lignin; CP = crude protein; SU = sugar content; Ash = ash content. DM = dry matter.

wall thickness was reached at about 450 °Cd and was 3.0–3.3 μm in all cases, values similar to data observed previously [28].

3.2. Chemical analyses

The chemical composition of internode 7 on a dry matter (DM) basis is given in Fig. 3. Contents of cell wall components on the basis of NDF are shown in Fig. 4.

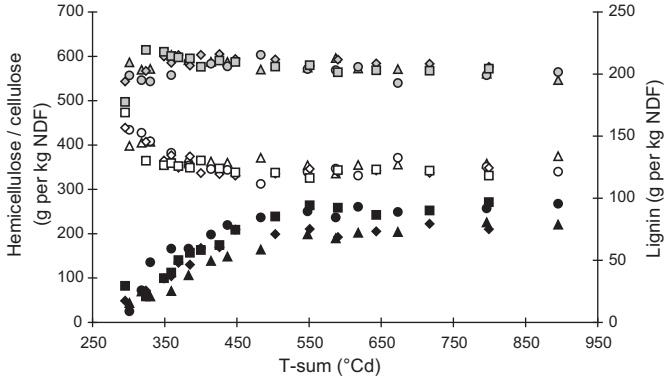

Fig. 4. Hemicellulose (open symbols), cellulose (grey symbols), and lignin (closed symbols) content of internode 7 of forage maize cultivars Vitaro and Volens in relation to temperature sum (Vitaro 1999 Δ, Volens 1999 ○, Vitaro 2000 ◊,Volens 2000 □). Values are averages of duplicate analyses. Standard errors of the mean usually smaller than size of the marker and therefore not shown.

NDF of internode 7 increased from about 300 g per kg dry matter (DM) to about 650 g per kg DM just before anthesis (Fig. 3). In 1999, NDF then slightly decreased, late-season measurements being around 550 g per kg DM. An initial decrease in NDF after anthesis was also observed in 2000, but late-season measurements showed an increase to about 700–750 g per kg DM. NDF was always higher in Vitaro than in Volens, with the exception of the earliest sampling dates.

ADF (g per kg DM) followed a trend similar to that for NDF, i.e., an increase from about 200 g per kg DM to about 450 g per kg DM just before anthesis. Late-season measurements were about 350 g per kg DM in 1999 and about 480 g per kg DM (after a decrease to about 380 g per kg DM) in 2000. ADF was also higher in Vitaro than in Volens with the exception of the earliest sampling dates.

ADL (g per kg DM) increased until anthesis and then more or less remained constant at about 50 g per kg DM.

Crude protein decreased steadily throughout the season from about 150 g per kg DM to about 30 g per kg DM (Fig. 3). No clear year or cultivar effects were observed.

Sugar content (SU) varied throughout the season: the sugar content initially increased to a maximum value shortly after anthesis. In 1999, SU remained constant thereafter, but in 2000 there was a strong decline at the end of the growing season. Sugar content was consistently higher in 1999 than in 2000. While Vitaro had a lower SU than Volens early in the season, from a T-sum of about 400 °Cd onwards Vitaro always had a higher SU than Volens.

Ash content decreased throughout the season. Vitaro tended to have a higher ash content than Volens. In 2000, ash content was consistently higher than in 1999.

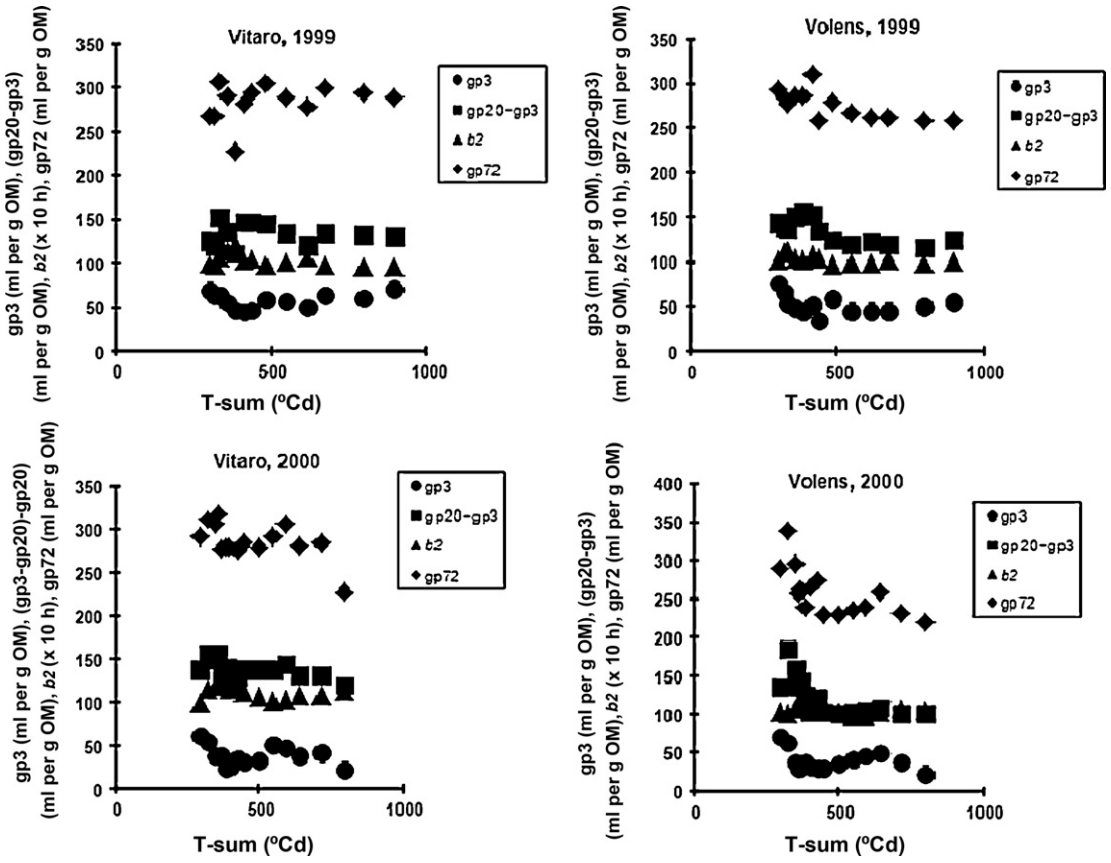

Fig. 5. Fermentation characteristics of internode 7 of forage maize cultivars Vitaro and Volens stems sampled in the growing seasons of 1999 and 2000, as measured during gas production tests, and plotted against temperature sum (T-sum in °Cd). Values are averages of duplicate analyses; standard errors of the mean averaged across sampling date, year and cultivar were 6.1 ml per g organic matter for gp3, 8.4 ml per g organic matter for gp20–gp3, 0.4 h for *b2*, and 28.3 ml per g organic matter for gp72. gp3 is gas production after 3 h of fermentation (in ml per g organic matter), gp20–gp3 is gas production between 3 and 20 h of fermentation (in ml per g organic matter), *b2* is the time necessary to reach half of the gas production between 3 and 20 h (in h); gp72 is gas production after 72 h of fermentation (in ml per g organic matter); OM = organic matter.

Cell wall components were also expressed as g per kg NDF (Fig. 4). Cellulose content varied little and averaged about 600 g per kg NDF throughout the season (Fig. 4), although there was a very slight downward trend. Hemicellulose decreased until anthesis to a constant level thereafter of about 350 g per kg NDF. For both cellulose and hemicellulose, no differences between cultivars or years were observed. Lignin also increased from the earliest sampling date until (shortly after) anthesis. The lignin levels of Volens were higher (ca. 10 g per kg NDF) than those of Vitaro. The cultivars clearly differed in lignin content from an early stage onwards in both years. In 2000, the difference between cultivars was relatively small until anthesis but increased thereafter. Lignin was generally lower in 1999 than in 2000.

3.3. Fermentation of ground material

Fermentation characteristics including gas production of soluble and insoluble components of internode 7 material are shown in Fig. 5. Fermentation of soluble components (gp3) declined in the first part of the growing season in both cultivars and in both years. In 1999, this decrease was followed by a small increase after which the values stabilized in both cultivars. In 2000, the decrease was also followed by an initial increase but at the end of the growing season this was followed by another (relatively strong) decline in both cultivars. Although there was a trend towards higher gp3 in Vitaro than in Volens in 1999, no cultivar difference was observed in 2000. These trends very much reflect the development over time of SU (see also below).

Fermentation of insoluble components (gp20–gp3) was variable, but tended to decrease slightly after anthesis. A year effect was only observed for Volens (lower gp20–gp3 in 2000). Volens had a relatively high gp20–gp3 early in the season, followed by a strong decline; from a T-sum of about 400 °Cd onwards, Vitaro had the higher gp20–gp3. This cultivar effect was more pronounced in 2000.

The time to reach half of gp20–gp3 (*b2*) – as determined from measured data – varied only little throughout the season, ranging from 9.8 to 12.4 h. There were no clear trends or differences observed.

Trends in fermentation after 72 h (gp72) were different. For Vitaro 1999, the values remained more or less stable, with one outlier at about 360 °Cd, but for Volens 1999, values gradually declined until 587 °Cd (well beyond anthesis). For Vitaro 2000, values remained relatively stable until the end of the growing season when the bad weather caused a decline. For Volens 2000 (like for Volens 1999), values initially declined until 503 °Cd (anthesis) followed by another decline at the end of the growing season.

3.4. Fermentation of sections

Sections fermented in buffered rumen fluid for 48 h showed a rapid decrease in cell wall thickness during the first 12 h (Figs. 6 and 7). The phloem and most of the parenchyma had disappeared from the samples taken at T-sum 300 °Cd, and few sclerenchyma cell walls remained. So a reliable assessment of cell wall thickness could no longer be made. Therefore, the data from T-sum 300 °Cd are not shown. For samples from T-sum 360–800 °Cd,

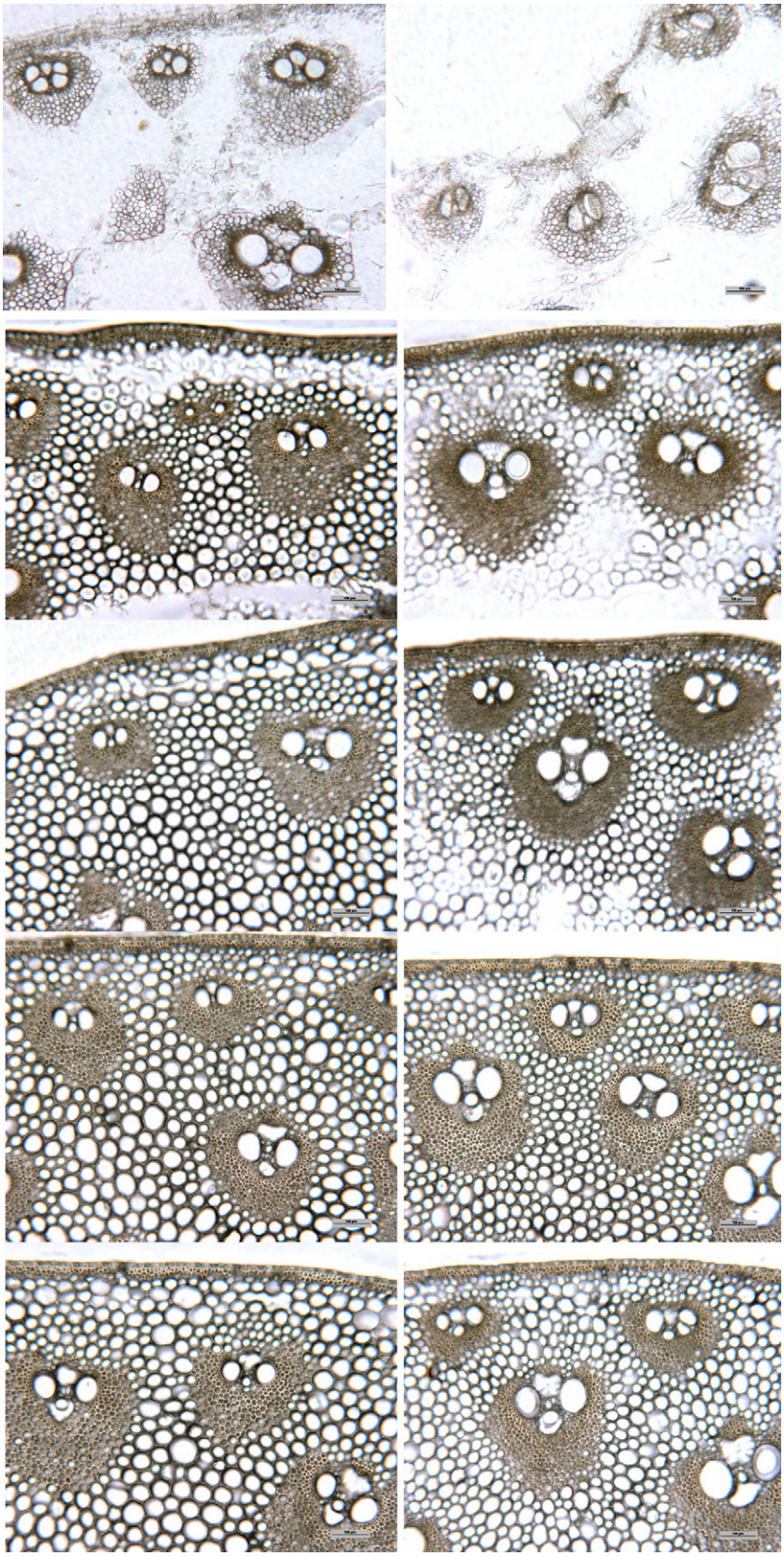

Fig. 6. Cross-sections of internode 7 of forage maize cultivars Vitaro (left) and Volens (right) 7 sampled in 1999 on 12 July (top), 19 July, 22 July, 9 August and 13 September (bottom), corresponding to temperature sums of approximately 300, 360, 385, 550, and 800 °Cd, after 24 h of fermentation in buffered rumen fluid.

Table 2
Cell wall thickness (μm ± sem[a]) and rate of decrease of cell wall thickness (nm h^{-1}) of sclerenchyma tissue adaxial to rind vascular bundles after 12, 24 or 48 h of fermentation in rumen fluid, measured in 100-μm sections of internode 7 of forage maize cultivars Vitaro and Volens sampled in 1999 and 2000 on dates with similar T-sum values (see Table 1 (30 observations per section)). See Figs. 1 and 4 for cross sections before and after 24 h of digestion. In contrast to these figures data from T-sum of about 300 °Cd are not shown.

	Vitaro				Volens			
T-sum (°Cd) year 1999/year 2000	359/359	383/385	548/551	796/799	359/359	383/385	548/551	796/799
1999								
0 h	1.8 ± 0.1	2.0 ± 0.1	2.9 ± 0.1	2.8 ± 0.1	1.6 ± 0.0	2.1 ± 0.1	3.2 ± 0.1	3.6 ± 0.1
12 h	1.3 ± 0.1	0.9 ± 0.1	1.4 ± 0.0	1.6 ± 0.1	0.6 ± 0.0	1.0 ± 0.1	2.5 ± 0.1	2.0 ± 0.1
Difference with 0 h	0.5	1.1	1.5	1.2	1.0	1.1	0.7	1.6
Decrease (nm h^{-1})	44	90	123	98	85	93	58	129
Decrease (%)	29	53	51	42	65	53	22	43
24 h	0.6 ± 0.0	0.6 ± 0.0	0.8 ± 0.0	1.1 ± 0.1	0.5 ± 0.0	0.4 ± 0.0	1.4 ± 0.1	1.0 ± 0.1
Difference with 0 h	1.2	1.4	2.1	1.7	1.1	1.7	1.8	2.6
Decrease (nm h^{-1})	51	59	88	70	47	69	75	108
Decrease (%)	68	70	73	60	71	80	57	72
48 h	0.5 ± 0.0	0.7 ± 0.1	0.7 ± 0.0	0.9 ± 0.1	0.4 ± 0.0	0.4 ± 0.0	0.5 ± 0.0	0.7 ± 0.0
Difference with 0 h	1.3	1.3	2.2	1.9	1.2	1.7	2.7	2.9
Decrease (nm h^{-1})	27	27	46	40	24	34	56	60
Decrease (%)	72	64	76	69	72	79	85	81
2000								
0 h	1.6 ± 0.0	2.0 ± 0.1	3.3 ± 0.1	3.9 ± 0.2	1.3 ± 0.1	2.1 ± 0.0	2.1 ± 0.1	3.6 ± 0.2
12 h	0.6 ± 0.0	0.9 ± 0.0	2.1 ± 0.2	2.4 ± 0.1	0.6 ± 0.0	1.8 ± 0.1	1.1 ± 0.1	1.9 ± 0.1
Difference with 0 h	1.0	1.0	1.2	1.6	0.7	0.4	0.9	1.6
Decrease (nm h^{-1})	87	85	102	130	59	32	76	137
Decrease (%)	65	52	37	40	53	18	44	46
24 h	0.4 ± 0.0	0.4 ± 0.0	1.1 ± 0.1	1.5 ± 0.1	0.6 ± 0.0	1.8 ± 0.1	0.8 ± 0.0	1.2 ± 0.1
Difference with 0 h	1.2	1.6	2.2	2.4	0.7	0.3	1.3	2.4
Decrease (nm h^{-1})	48	67	92	101	29	14	54	98
Decrease (%)	72	82	67	62	52	16	62	66
48 h	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	1.0 ± 0.1	0.5 ± 0.0	0.5 ± 0.0	0.4 ± 0.0	0.7 ± 0.1
Difference with 0 h	1.2	1.6	2.9	2.9	0.8	1.6	1.7	2.8
Decrease (nm h^{-1})	26	33	61	60	17	33	35	59
Decrease (%)	78	82	89	73	62	75	82	79

[a] sem = standard error of the mean.

cell wall thickness after 12, 24 or 48 h of fermentation is shown in Table 2. After anthesis (T-sum 550 and 800 °Cd) the decrease in cell wall thickness upon fermentation was more rapid than early in the season for both years and both cultivars.

Averaged over all samples per time-point, the rate of fermentation decreased rapidly from 89 nm h^{-1} between 0 and 12 h, to 45 nm h^{-1} between 12 and 24 h and becoming as low as 13 nm h^{-1} between 24 and 48 h.

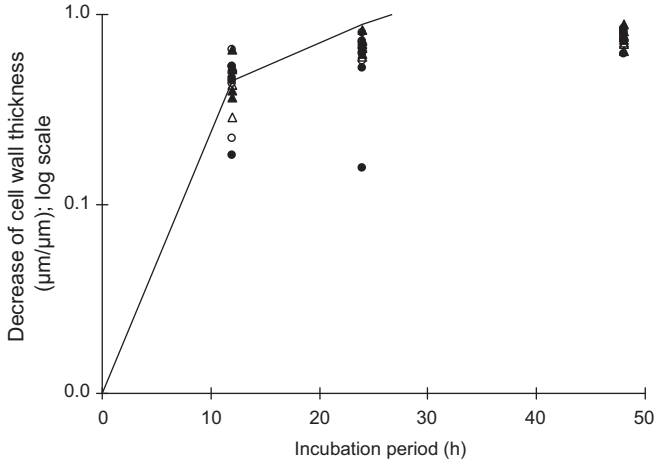

Fig. 7. Decrease in cell wall thickness (μm per μm original cell wall thickness; log scale) of sclerenchyma in cross-sections of internode 7 of forage maize cultivars Vitaro and Volens after 12, 24 or 48 h of fermentation in buffered rumen fluid (Vitaro (Δ) and Volens (○) in 1999 (open symbols) and 2000 (closed symbols)). Dotted line indicates decrease extrapolated from the mean rate in the first 12 h.

3.5. Correlation between chemical composition and gas production characteristics

The sugar content of maize stem samples was correlated with gas production after 3 h of incubation in buffered rumen fluid (r^2 = 0.63; Fig. 8). The relationships between hemicellulose, cellulose and lignin contents and gp20–gp3 were expressed on the basis of NDF (Fig. 9a–c). Hemicellulose content correlated positively with gp20–gp3, with an overall r^2 of 0.61 (r^2 values for Vitaro 1999, Volens 1999, Vitaro 2000 and Volens 2000 were 0.55; 0.76; 0.62 and 0.64, respectively). Cellulose content did not correlate with gp20–gp3. Lignin was negatively correlated with gp20–gp3, with

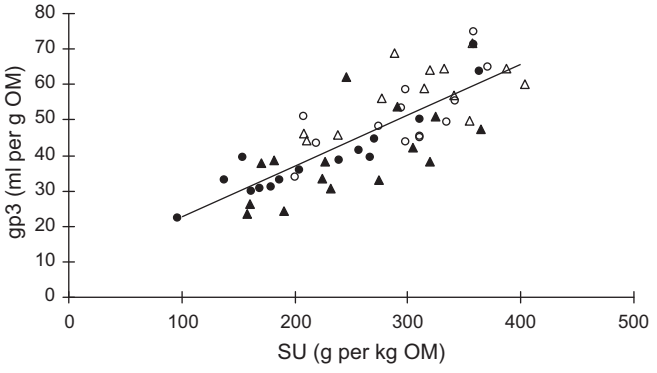

Fig. 8. Gas production after 3 h of incubation in buffered rumen fluid (gp3) as affected by sugar content (SU) of internode 7 of forage maize cultivars Vitaro (Δ) and Volens (○) sampled in 1999 (open symbols) and 2000 (closed symbols). The overall linear regression is: gp3 = 8.52 + 0.16 × SU; r^2 = 0.63, n = 56; OM = organic matter.

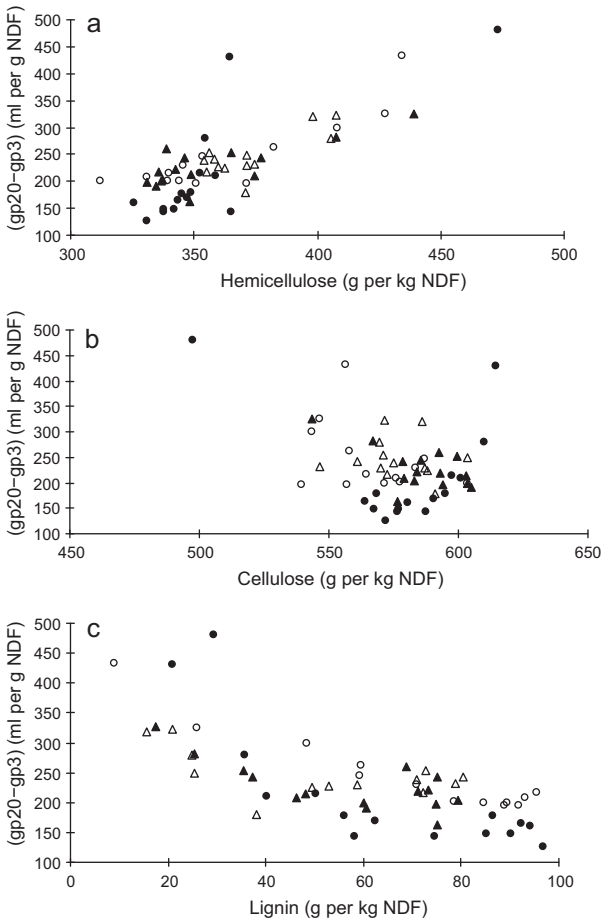

Fig. 9. Gas production between 3 and 20 h of incubation in buffered rumen fluid (gp20–gp3) as affected by hemicellulose (a), cellulose (b) and lignin content (c) of internode 7 of forage maize cultivars Vitaro (△) and Volens (○) sampled in 1999 (open symbols) and 2000 (closed symbols).

an overall r^2 of 0.51 (r^2 values for Vitaro 1999, Volens 1999, Vitaro 2000 and Volens 2000 were 0.30; 0.91; 0.51 and 0.62, respectively).

4. Discussion

4.1. Anatomy

Cell wall thickness of sclerenchyma in the rind increased linearly until several days before anthesis (Fig. 2a). From the fitted equation it was calculated that an increase in cell wall thickness from 0.5 μm to 3.5 μm would take (446–283 =) 163 °Cd. Combined with temperature data, this indicates that cell wall deposition in internode 7 took approximately 20 days in 1999 and 35 days in 2000. The weather, especially the temperature, was notably different in the two years, with an average T-sum of 8.9 °Cd in the relevant period in 1999 compared with 4.7 °Cd in 2000. These data are consistent with the data found by Morrison et al. [27], which showed a period of 15 days for elongation, differentiation and secondary cell wall formation at an average daily contribution of 13 °Cd to the T-sum.

4.2. Chemical analyses

NDF content decreased after anthesis, both in 1999 and 2000. Sugar content increased after anthesis but later declined again, at least in 2000. Accumulation of structural carbohydrates in the upper internodes can continue for some time after anthesis, but accumulation of non-structural carbohydrates shortly after anthesis is much stronger because during this period the main ear has not yet become a strong sink whereas whole-plant photosynthesis is at its peak. The result is an increase in SU and a decrease in NDF content. The absolute amount of NDF in lower internodes such as internode 7 remains the same after anthesis. As a measure to monitor cell wall development throughout the growing season, cell wall components (hemicellulose, cellulose and lignin) expressed as g per kg NDF are considered to be more appropriate than contents based on dry matter.

Sugar content was consistently higher in 1999 than in 2000. The strong decline in SU at the end of the growing season of 2000 was associated with the lack of sunshine in that month compared with the situation in 1999. In general, the weather in 2000 allowed a high ear growth rate at a relatively low crop growth rate, causing a significant redistribution of water soluble carbohydrates from the stem to the ear (cf. [1]).

Ash was consistently higher in 2000 than in 1999, which could be related to the wetter sampling conditions during the early and very late parts of the growing season in 2000. Under wet sampling conditions, it is likely that some residual soil adheres to the samples. At the end of the growing season the drop in SU associated with the relatively poor light conditions in September 2000 could also have played a role.

Lignin levels of Vitaro were consistently lower than those of Volens, which is in accordance with a higher whole plant digestibility.

4.3. Fermentation of ground internode material

Fermentation data were fitted to a three-phasic model (as also used by Groot et al. [38]). This model fits each subcurve to a sigmoid curve. Maximal gas production values of the soluble and non-soluble components of the sample, i.e., cell content (+ pectin) and cell wall, *a1* and *a2* respectively, were defined as gp3 and (gp20–gp3). The definition of *a2* as (gp20–gp3) assumes that maximal gas production for non-soluble components is reached after 20 h of fermentation. Fitted values for *b2*, the parameter representing half of the time to reach maximum gas production of the non-soluble components, were close to 10 h for many of the samples. However, a substantial number of the samples had higher fitted values of *b2*, some even as high as 17.8 h (data not shown). Given the definition of *a2*, this would imply that the second half of gas production is attained in only 2.2 h. However, this would be incompatible with a sigmoid curve. The data presented in Fig. 5 for *b2* were measured, not fitted values. Although a time interval of 20 h to reach maximal gas production of non-soluble components can be a good estimate for some feedstuffs [37], after 20 h the samples used here probably had not finished fermentation of non-soluble components. The isolated internodes had a high fibre content and in the later stages of the growing season had high lignin contents. A comparison of gas production after 20 h is relevant to illustrate certain differences between samples. However, it is suspected that maximum gas production of non-soluble components for the more mature samples could be reached only after about 36 h (twice 17.8 h). In an *in vivo* situation, the amount of time needed to ferment cell wall material could exceed the retention time in the rumen. Thus, the feed value of potentially fermentable cell wall material would be limited by its retention time in the rumen.

Vitaro had a higher NDF, but a lower ADL content than Volens. The relative amount of fermentable cell wall is expected to be higher in Vitaro than in Volens.

As expected, the seasonal development showed an increase in fermentable material (increasing cell wall thickness) until anthesis, offset by a decrease in fermentability (influenced amongst other by increasing ADL content). Whereas lignin content was maximal after

anthesis, optimal cell wall fermentation (indicated by gp20–gp3 as shown in Fig. 5) appeared to be between T-sum 320 and 380 °Cd. As discussed, it is possible that not all cell wall material was fermented after 20 h. The patterns over time for gp72 showed a very distinct difference between the two cultivars during the first part of the growing season, with a strong decrease before anthesis for cultivar Volens, suggesting that differences in potential digestibility of internode 7 are mainly realized before anthesis.

4.4. Fermentation of sections

As expected, cell wall fermentation rate decreased after 12 h and decreased further after 24 h. Differences between younger and older stages of internode 7 in rate of decrease of cell wall thickness were small or inconsistent (Table 2). As lignin content was maximal after anthesis, samples from 550 to 800 °Cd were expected to ferment more slowly and to have a less rapid decrease in cell wall thickness than samples harvested before anthesis (360 and 385 °Cd). Data from internode 8 at anthesis [26] were comparable with data found in this study at 550 °Cd for internode 7.

The set-up used for the section fermentation experiments allowed for a relatively quick qualitative assessment of the samples. For an accurate quantitative analysis, the impact of between plant variation (given the small number of samples per assessment) must be taken into consideration.

Measurements on fermented sections were further hampered by the thickness of the sections, as discussed previously in Boon et al. [28]. Interpretation of the results would have benefited greatly by using thinner sections but this would have required embedding the material prior to sectioning. However, in that case it should be warranted that the embedding material does not interfere with the fermentation, for example through washing out the embedding material before incubation in the buffered rumen fluid. This requires further study.

5. Conclusions

Identified internode features that have an impact on the development of fermentation characteristics during the season include the absolute and relative amounts of cell wall material, influenced by cell wall composition and cell wall thickness, which in turn influence the accessibility of the tissue to rumen micro-organisms, composition of cell wall material and the relationship between these features over time.

Differences in cell wall thickness and in lignin content reflected the changes in digestibility during the growing season best; the differences between the two contrasting cultivars were best reflected by the differences in cell wall thickness, lignin content and the decline of the potential digestibility in the period before anthesis.

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