Stem characteristics of two forage maize (*Zea mays* L.) cultivars varying in whole plant digestibility. II. Relation between *in vitro* rumen fermentation characteristics and anatomical and chemical features within a single internode

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Abstract

Internode 7 of the stem of two forage maize (*Zea mays* L.) cultivars was studied anatomically and chemically at anthesis and subjected to fermentation tests in rumen fluid, using an automated gas production system. For anatomical studies internode 7 was sectioned at the top, middle and base. For chemical analyses and fermentation studies, the internode was divided into five sections. The number of sclerenchyma layers increased from top to base of the internode, whereas cell wall thickness of the sclerenchyma decreased from top to base. The highest and lowest sections of the internode had a higher sugar content and lower levels of neutral detergent fibre, acid detergent fibre, and acid detergent lignin, and slightly higher levels of crude protein than the middle three sections. Also their hemicellulose and cellulose contents were different but the differences with the middle three sections were small. Maximum gas production from fermentation in rumen fluid was higher for cell wall material from the highest and lowest than from the middle three sections. This was mainly due to differences in chemical composition: the highest and lowest section had a higher lignin and a relatively high hemicellulose content compared with the middle three sections. Digestibility of the cultivar with the higher whole plant digestibility, Vitaro, exceeded that of the cultivar Volens for each of the sections examined within the selected internode. Secondary cell wall disappearance was only weakly correlated with lignin content or gas production. This suggests that other factors are important in the fermentation process. These

may include anatomical factors influencing the accessibility of the tissues to micro-organisms and chemical and/or physical factors such as lignin composition, lignin localization within the cell wall and nature and extent of cross-linking lignin to other cell wall components.

Additional keywords: sclerenchyma, gas production system, chemical composition, section fermentation, cell wall disappearance

Introduction

Maize (*Zea mays* L.) is important forage for winter feeding of ruminants. Its feeding value is largely determined by the digestibility of the different components of the maize plant. Its stem contributes between 42 and 44% to total plant weight early in the growing season, against 18% at the end of it (Wilman *et al.*, 1996c; Boon *et al.*, 2005), whereas its *in vitro* digestibility is relatively low and variable (Deinum & Struik, 1989) and declines during the growing season (Struik, 1983). Stem digestibility is variable because of the variation in cell wall content and cell wall digestibility (Struik, 1983). A major source of this variation in stem digestibility is genetically determined (Dolstra *et al.*, 1993).

The maize stem is made up of vascular bundles, sclerenchyma, epidermis, chlorenchyma and parenchyma. Digestibility of these tissues varies (Deinum & Struik, 1986; Cone & Engels, 1990). Degradation of chlorenchyma and parenchyma is almost complete after 24 hours of fermentation, whereas breakdown of vascular bundles, sclerenchyma and epidermis is far from complete even after 96 hours (Engels & Schuurmans, 1992). As vascular bundles and sclerenchyma make up about 80% of the weight of the stem (Engels & Schuurmans, 1992), whole plant digestibility can be considerably improved by improving the digestibility of these tissues.

Most of the large vascular bundles and sclerenchyma can be found in the rind, i.e., the outer layer of the stem. The inner part, referred to as pith, consists mainly of parenchyma with small vascular bundles.

Part of the tissues cannot be digested due to their advanced stage of lignification. Being indigestible in itself, lignin reduces the digestibility of the cell wall parts it has incrusted (Chesson, 1993; Hatfield, 1993). However, usually the unlignified, potentially digestible part of the cell wall is not fully digested either. Fermentation of the potentially degradable (secondary) cell wall is influenced by the accessibility of the cell wall to micro-organisms. Therefore, to improve fermentability, the fraction of potentially degradable secondary cell wall should be increased and its accessibility to micro-organisms enhanced. Moreover, forage particles remain in the rumen for about 25–33 hours (Minson, 1982). Only a limited portion of the potentially fermentable cell wall can actually be broken down during that period (Wilson & Mertens, 1995). Therefore, fermentation rate should be as high as possible.

Potential degradation of the stem and its internodes

Digestibility of cell walls is closely related to their chemical composition and to the distribution of its chemical components. Cell wall material is usually much less digestible than cellular contents. Cellular contents are high in soluble sugars and protein. Cell walls are made up of pectins, hemicellulose, cellulose, protein, phenolics and lignins. Cell walls can differ in lignin content, lignin composition and localization of lignin. As crop maturity progresses, digestibility drastically declines as lignification of the cell wall proceeds (Jung & Deetz, 1993). This trend is observed for the entire crop, within each plant and within each individual stem internode.

Maize internodes pass through different successive development stages: cell division, cell elongation and cell differentiation. During cell division, new cells are produced by the meristem at the base of the internode. Younger cells are located at the base of the internode, whereas older, more mature cells are located at the top. Chemical composition and physical characteristics of the cell walls change during development. All parts of the internode ultimately reach a final stage of differentiation and maturity. Lignification is thought to begin when secondary cell wall material starts being deposited; it serves to stiffen the cell wall, thereby fixing cell size once the cell itself is fully elongated. Deposition of lignin starts in the primary cell wall, and is then slowly extended in the direction of the cell lumen, following secondary wall build-up. Lignin content, composition and localization are genetically determined, but can be influenced by environmental factors such as temperature. Lignin is an essential component of the plant, protecting cells from pathogens, and the plant as a whole against climatic influences. Lignin provides structural strength to the plant.

Accessibility of the degradable cell wall in the stem

Accessibility of the cell wall to micro-organisms is a complex issue, and can be thought to consist of three components: (1) accessibility of a tissue particle, (2) accessibility of the cell wall, and (3) accessibility of cell wall components.

If the size of the particle is large, only outer cell walls are available for fermentation by the micro-organisms in the rumen. Size reduction of a large particle by fermentation is slow, as primary cell walls are often highly lignified and undegradable (Engels & Schuurmans, 1992). Particle size reduction mainly takes place by mastication (Wilson *et al.*, 1989).

Structural factors related to the accessibility of tissues to rumen microbes play a role in the fermentation process (Engels & Schuurmans, 1992; Wilson, 1993). Sclerenchyma cells can increase their cell wall thickness in such a way, that the lumen of the cell becomes too narrow for more than one microbe at a time. The ratio of cell volume to available cell wall surface area will then be a limiting factor for the colonization of the cell wall (Wilson, 1993). An increased accessibility of cells could drastically improve feeding value (Wilson & Kennedy, 1996).

Digestible cell wall components can be incrusted by lignin, making it difficult for the enzymes from micro-organisms to reach such components. Reduced accessibility of cell wall components will reduce digestibility of the cell wall as a whole.

Rate of fermentation of the stem

Because of the availability of fermentable cell walls, the rate of fermentation in unlignified cell walls will be high throughout fermentation until available degradable cell wall material runs out. In contrast, rate of fermentation in the lignified cell wall will initially be substantial, but will slow down once micro-organisms reach the lignified part of the secondary cell wall. When this stage is reached the rate of fermentation will decrease considerably, depending on lignin content, on lignin composition within the lignified part of the cell wall, and on the way lignin is bound to other parts of the cell wall.

So the fermentation process is influenced by a complex system of physical, chemical and anatomical factors: plant maturity, particle size, tissue structure, rumen retention time, mastication, physical and chemical composition of the secondary cell wall, lignin composition, lignin content and lignin localization. Each element plays a role (often at a different organizational level) and interacts with other factors. The plant factors find their origin in plant development, and the large genetic component makes it interesting to compare different cultivars. Furthermore, the relationships between these plant factors have never been studied in detail in the same study. We present a detailed study of two forage maize cultivars that differ in whole plant digestibility, showing anatomical data, chemical analyses, and fermentation tests *in vitro* with 1 mm particle size and with 100 µm-thick sections. The results allow us to clarify the relationships of anatomy, chemistry and fermentation that eventually determine the feeding value of the maize stem.

In this paper we focus on differences at anthesis within a selected internode of the maize stem, in order to relate possible differences in (rate and extent of) fermentation to differences in anatomy and/or chemical composition. A single internode developed within a limited time frame shows different stages of development simultaneously and has a well-arranged pattern of very contrasting tissues.

Materials and methods

Plant growth conditions and sampling

The maize cultivars Vitaro and Volens were grown in the field on a heavy river clay soil, using standard cultural practices. The crops were sown in May 1999 and 2000. Samples of internode 7 of both cultivars were collected at anthesis (Developmental Stage 5; Groot *et al.*, 1986; T-sum 483 °C.d in 1999, T-sum 503 °C.d in 2000).

Internodes were counted from the base of the stem, the internode accompanying leaf 1 being designated as internode 1. As leaf length increases with leaf insertion number according to a characteristic pattern, the number of an internode was verified by measuring the length of the leaf accompanying the internode (Bos *et al.*, 2000; Boon *et al.*, 2005). Leaf length of Vitaro and Volens was measured for all leaves in 1999, and for leaves 6 to 8 in 2000 to check for similarity to the previous season. Plant height and internode lengths were measured.

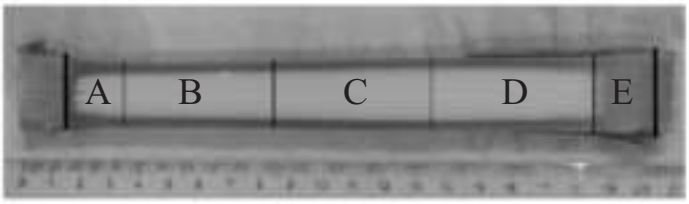

Figure 1. Division of internode 7. The upper (A) and lower (E) segments are 2 cm long. The remainder was divided into 3 segments (B–D) of equal length. The thick lines indicate the position of the nodes.

Sampled internodes and stems were kept at –20°C until further use.

Before chemical and *in vitro* analyses, samples were dried and ground to pass a 1 mm sieve.

Anatomical studies

Sections with a thickness of 100 µm were made of the top, middle and base of internode 7 using a sledge microtome (Ernst Leitz Wetzlar, Germany). Top and base sections were taken at 2 cm from node 8 and 7, respectively. Middle sections were taken from the exact middle.

The number of layers of sclerenchyma (Scl) cells was counted underneath the epidermis, and near the vascular bundle in the rind of the sections, using a light microscope. Cell wall thickness of Scl was measured at 1000 × magnification on recorded video images with the use of the analySIS 3.0 software package (Soft Imaging Systems GmbH, Münster, Germany).

Chemical analyses

The internodes were divided into five sections: A–E (Figure 1). Sections A and E consisted of the upper and lower 2 cm of the internode and included the actual node plus adjacent material. The remaining part was divided into three equal sections: B, C, and D.

Neutral detergent fibre (NDF), acid detergent fibre (ADF), acid detergent lignin (ADL), crude protein (CP) and ash contents were determined for each of the five sections at the ID-TNO Animal Nutrition laboratories according to the techniques described by Goering & Van Soest (1970). Hemicellulose was calculated as NDF–ADF and cellulose as ADF–ADL.

Fermentation tests using the gas production technique

Fermentation characteristics for the 5 sections of internode 7 of both cultivars were determined with the gas production technique described by Cone *et al.* (1996). 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) (Cone *et al.*, 1997). Each

subcurve is described by the parameters a (asymptotic maximum gas production), b (time in hours to reach 50% of a) and c (determining the steepness of the curve) (Groot *et al.*, 1996). The subnumbers of the parameters a and b in this paper refer to the number of the subcurve. The data of subcurve c are not shown. Data presented in this paper refer to a1 as gas production after 3 hours (GP3) and to a2 as gas production between 3 and 20 hours (GP20–GP3).

An *in vitro* essay was carried out on isolated sections of internode 7 of the two cultivars, using the technique of Tilley & Terry (1963).

Fermentation of sections

Sections 100 µm thick were mounted on double-sided tape, which in turn was mounted on microscope slides. Fermentation of the sections was done in 1.5 l of buffered rumen fluid (as described in Fermentation tests using the gas production technique), with 12.5 g of maize internode material (ground to pass a 1 mm sieve) as additional substrate. All sections were fermented in the same container, and slides were taken out after 12, 24 or 48 hours of fermentation. A mirror sectioning procedure was used (i.e., sections were taken from exactly the same part of the internode). By this method more or less the same cells could be examined before and after fermentation but from opposite ('mirror') sides of the cut. Mirror sections were used as a reference to assess the condition of the material before fermentation, thus allowing for the evaluation of the decrease of cell wall thickness.

Statistical methods

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

Results

Anatomy

Examination of the sections taken from internode 7 revealed structural differences in the sclerenchyma (Scl) tissue from top to base of the internode for both cultivars (Figure 2). In general, an increase in the number of Scl layers, accompanied by a decrease of Scl cell wall thickness was observed from top to base.

The number of Scl layers in the subepidermal layer was low, but increased from between 1.1 and 1.6 at the top to between 2.4 and 3.1 at the base of the internode (Table 1). The number of subepidermal Scl layers was significantly influenced by the factors year, position and cultivar, and by the interactions year × position, year × cultivar and position × cultivar ($P < 0.01$ for year; $P < 0.001$ for the other factors and interactions).

The number of Scl layers on the central side of vascular bundles in the rind approximately doubled from top to base of the internode in both cultivars in both

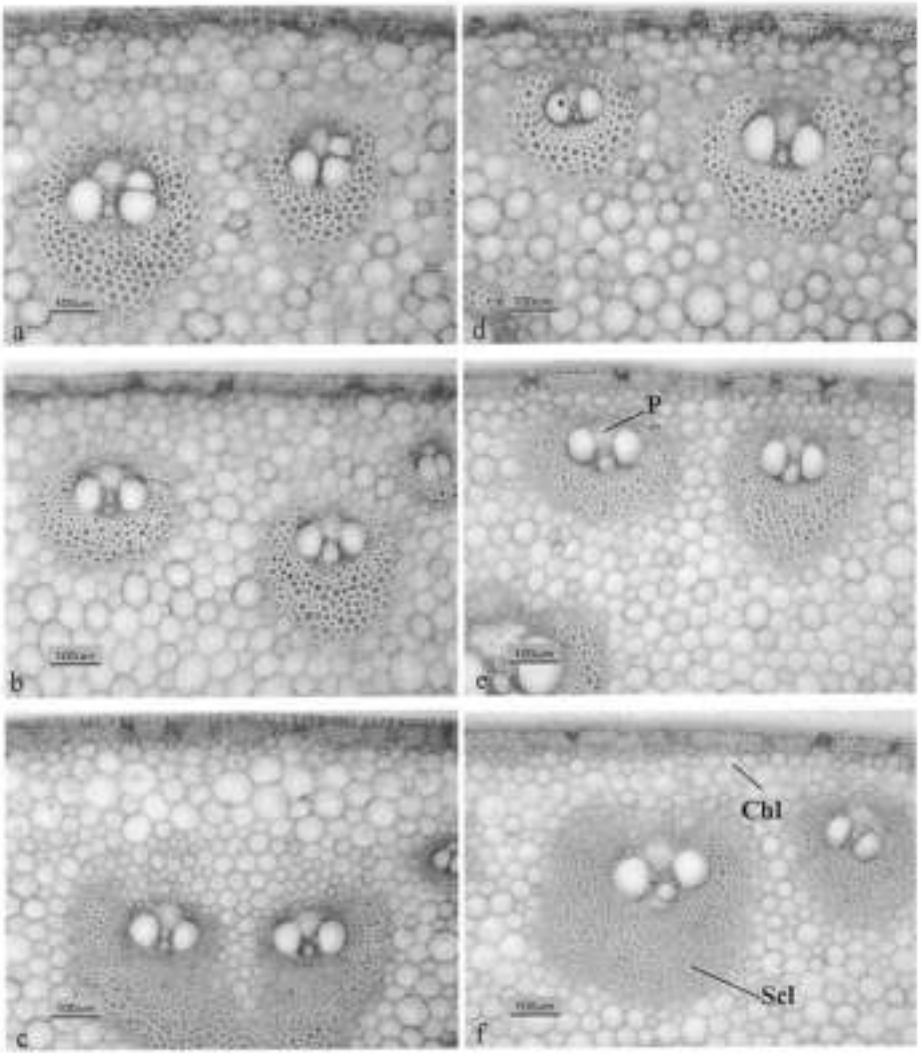

Figure 2. Cross-sections of the rind tissue from segments B (a, d), C (b, e) and D (c, f) of internode 7 of the maize cultivars Vitaro (a, b, c) and Volens (d, e, f) at anthesis. P = phloem; Chl = chlorenchyma; Scl = sclerenchyma.

years. The difference between top and middle was relatively small. A much larger change took place from the middle to the base of the internode. The effects of year, position and cultivar on number of sclerenchyma layers on the central side of vascular bundles in the rind were statistically significant ($P < 0.001$ for year and position; $P = 0.04$ for cultivar).

The number of Scl layers on the central side of vascular bundles varied, especially

Table 1. Average number of layers of sclerenchyma cells present in the sub-epidermal layer and in the layer adaxial to the rind vascular bundles in the top, middle and base of internode 7 of the forage maize cultivars Vitaro and Volens at anthesis in 1999 and 2000.

Year/ Cultivar	Sub-epidermal layer			Layer adaxial to rind vascular bundles		
	Top	Middle	Base	Top	Middle	Base
1999 (n = 25)						
Vitaro	1.2 ± 0.1[1]	1.7 ± 0.1*	2.4 ± 0.1*	7.3 ± 0.4	8.4 ± 0.4	13.3 ± 0.9
Volens	1.2 ± 0.1	2.5 ± 0.1	3.1 ± 0.1	7.0 ± 0.3	9.4 ± 0.5	14.3 ± 0.8
2000 (n = 40)						
Vitaro	1.6 ± 0.1*	1.5 ± 0.1	2.6 ± 0.1	6.4 ± 0.3	8.0 ± 0.3	12.3 ± 0.6
Volens	1.1 ± 0.1	1.6 ± 0.1	2.8 ± 0.1	6.6 ± 0.2	7.7 ± 0.2	12.9 ± 0.5

[1] Average ± standard error of the mean.

* Statistically different ($P < 0.05$) from Volens

Table 2. Cell wall thickness of the sub-epidermal sclerenchyma cells and of the sclerenchyma cells adaxial to the rind vascular bundles in the top, middle and base of internode 7 of the forage maize cultivars Vitaro and Volens in 1999 and 2000.

Year/ Cultivar	Sub-epidermal layer			Layer adaxial to rind vascular bundles		
	Top	Middle	Base	Top	Middle	Base
	(µm)					
1999 (n = 50)						
Vitaro	3.0 ± 0.1*[1]	2.5 ± 0.1*	2.3 ± 0.0	4.7 ± 0.1*	2.7 ± 0.1*	2.3 ± 0.1
Volens	4.0 ± 0.1	3.6 ± 0.1	2.2 ± 0.1	4.2 ± 0.1	3.6 ± 0.1	2.3 ± 0.1
2000 (n = 30)						
Vitaro	3.4 ± 0.1	3.4 ± 0.1	2.1 ± 0.1*	4.5 ± 0.2	3.9 ± 0.1*	2.2 ± 0.1
Volens	3.4 ± 0.1	3.6 ± 0.1	2.7 ± 0.1	4.3 ± 0.2	3.2 ± 0.1	2.3 ± 0.1

[1] Average ± standard error of the mean.

* Statistically different ($P < 0.05$) from Volens.

at the base of the internode (Figure 2f). The number of Scl layers of some bundles was as high as 21 whereas other bundles only had 3 to 5 layers. Bundles with many Scl layers seemed to alternate with those with only few Scl layers, each type of vascular bundle apparently making up about half of the total number of bundles in the rind.

Cell wall thickness of the Scl cells was related to the position of the Scl within the

Table 3. Chemical composition of sections A–E of internode 7 of the forage maize cultivars Vitaro and Volens at anthesis in 1999 and 2000.

Year	Cultivar	Component[1]	Internode section				
			A	B	C	D	E
			(g per kg dry matter)				
1999	Vitaro	NDF	554 ± 3[2]	570 ± 3	574 ± 5	565 ± 4	536 ± 1
		CP	67 ± 0	37 ± 2	38 ± 2	39 ± 0	67 ± 0
		Ash	36 ± 1	23± 1	25 ± 1	32 ± 0	42 ± 1
		SU	266 ± 2	305 ± 2	316 ± 3	319 ± 2	285 ± 1
	Volens	NDF	588 ± 1	626 ± 3	633 ± 1	597 ± 2	543 ± 1
		CP	60 ± 0	37 ± 0	39 ± 0	47 ± 1	79 ± 0
		Ash	27 ± 1	12 ± 1	16 ± 1	24 ± 1	49 ± 2
		SU	236 ± 1	263 ± 1	255 ± 1	263 ± 2	263 ± 12
2000	Vitaro	NDF	600 ± 2	603 ± 2	602 ± 1	588 ± 6	582 ± 0
		CP	72 ± 0	64 ± 0	69 ± 0	72 ± 0	76 ± 0
		Ash	46 ± 2	46 ± 0	49 ± 1	57 ± 0	62 ± 1
		SU	279 ± 6	284 ± 2	299 ± 1	282 ± 4	274 ± 2
	Volens	NDF	632 ± 3	669 ± 4	652 ± 2	642 ± 3	616 ± 3
		CP	63 ± 0	51 ± 0	54 ± 1	65 ± 1	77 ± 0
		ASH	37 ± 2	30 ± 0	33 ± 2	45 ± 1	54 ± 0
		SU	242 ± 6	245 ± 2	235 ± 8	228 ± 4	245 ± 1

[1] NDF = neutral detergent fibre; CP = crude protein; SU = sugars.

[2] Average ± standard error of the mean (n = 2).

internode, and decreased from between 3.0 and 4.7 µm at the top to between 2.1 and 2.7 µm at the base of the internode (Table 2). Scl at the top of the internode had a higher cell wall thickness on the central side of vascular bundles in the rind (4.2–4.7 µm) than in the subepidermal layer (3.0–4.0 µm). Scl at the base of the internode had the same cell wall thickness on the central side of vascular bundles in the rind (2.2–2.3 µm) as in the subepidermal layer (2.1–2.7 µm). Although there were statistically significant cultivar differences in Scl cell wall thickness, these differences were not consistent. In the subepidermal Scl layers, Volens mostly had the thicker cell wall, whereas in the Scl adaxial to rind vascular bundles Vitaro had the thicker cell wall in many cases.

Chemical analyses

Sections A and E generally differed in chemical composition from sections B, C and D (Table 3; Figure 3). Note that standard errors in Table 3 only reflect sampling error and analytical error in the laboratory phase. Therefore, no further statistical analyses were performed on these data. Ash and crude protein contents were higher in sections A

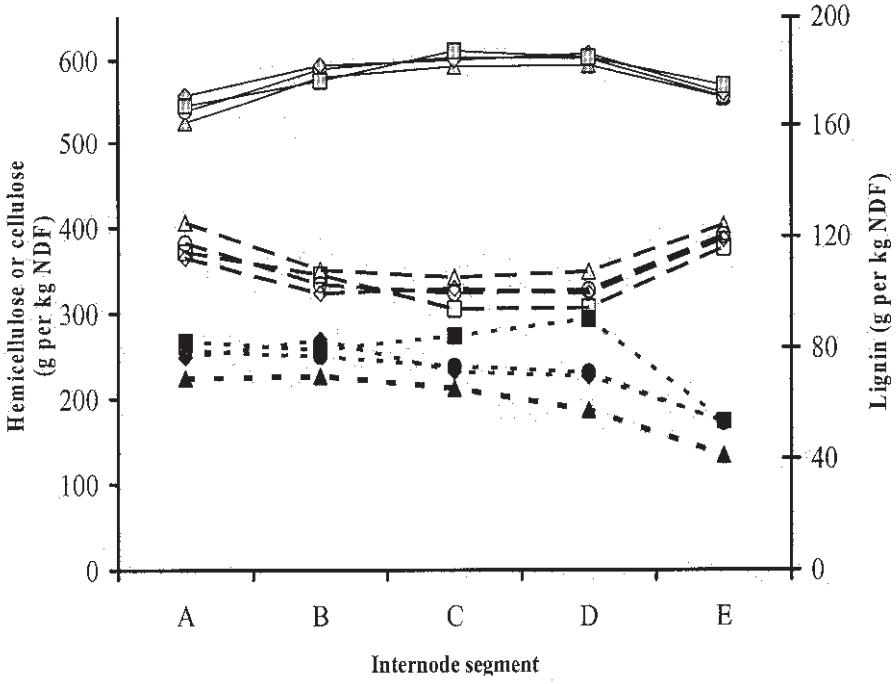

Figure 3. Contents of cellulose (shaded symbols), hemicellulose (open symbols) and lignin (black symbols) for the segments A–E of internode 7 of the maize cultivars Vitaro and Volens at anthesis, in 1999 and 2000. △ Vitaro 1999; ○ Volens 1999; ◇ Vitaro 2000; □ Volens 2000.

and E than in sections B, C and D, whereas NDF content was lower. In 1999, sugar content was lower in sections A and E than in the other sections, but in 2000 it was similar (Table 3).

Hemicellulose content expressed per unit NDF was higher for sections A and E than for the middle sections, and was accompanied by a lower cellulose content (Figure 3). Lignin content was lower for section E than for any other section (Figure 3). Although sections B through D varied little in chemical composition, differences in lignin content between cultivars increased from section B to D. NDF was slightly lower and CP slightly higher in section D compared with sections B and C (Table 3).

Cultivar Vitaro had a lower NDF, a higher sugar and a lower lignin content than Volens (Table 3; Figure 3). Chemical composition in the first year of sampling differed from that in the second. In 1999, NDF and lignin contents were lower than in 2000; especially sections B, C and D showed much lower values for ash and crude protein contents in 1999 than in 2000. Note that also the sum of NDF, crude protein, ash and sugar contents was much lower in 1999 (on average 935 g per kg dry matter) than in 2000 (on average 992 g per kg dry matter); this sum was also slightly lower for Volens (957) than for Vitaro (970).

Fermentation

The parameters for the different phases of fermentation were estimated from gas production tests with samples from 1999 and 2000 (Table 4). Significant effects of cultivar, year and position were found for GP3, GP20–GP3, and GP72. However, also

Table 4. Gas production parameters of the soluble (GP3) and insoluble (GP20–GP3 and GP72; cell wall) fraction, and organic matter digestibility according to Tilley & Terry (T&T) of sections A–E of internode 7 of the forage maize cultivars Vitaro and Volens at anthesis in 1999 and 2000.

Year	Cultivar	Parameter[1]	Internode section				
			A	B	C	D	E
1999	Vitaro	GP3	75 ± 1[2]	75 ± 1	75 ± 2	76 ± 0	83 ± 0
		GP20–GP3	116 ± 0	110 ± 0	109 ± 3	122 ± 1	137 ± 6
		GP72	284 ± 1	289 ± 1	283 ± 6	298 ± 2	318 ± 9
		T&T (%)	66 ± 0	62 ± 0	63 ± 0	66 ± 0	73 ± 0
		GP20–GP3 (ml per g NDF)[3]	201 ± 0	188 ± 0	186 ± 5	210 ± 2	244 ± 11
	Volens	GP3	74 ± 2	71 ± 6	64 ± 1	65 ± 1	78 ± 0
		GP20–GP3	106 ± 2	87 ± 9	94 ± 2	107 ± 1	120 ± 1
		GP72	276 ± 6	256 ± 4	262 ± 3	278 ± 2	291 ± 1
		T&T (%)	60 ± 0	52 ± 0	52 ± 1	57 ± 1	68 ± 1
		GP20–GP3 (ml per g NDF)	176 ± 3	138 ± 14	146 ± 3	175 ± 3	210 ± 1
2000	Vitaro	GP3	73 ± 1	70 ± 1	66 ± 2	73 ± 2	69 ± 1
		GP20–GP3	110 ± 1	111 ± 0	110 ± 1	117 ± 1	122 ± 0
		GP72	278 ± 1	287 ± 0	275 ± 2	291 ± 3	304 ± 1
		T&T (%)	61 ± 1	64 ± 1	62 ± 0	60 ± 1	69 ± 0
		GP20–GP3 (ml per g NDF)	239 ± 3	170 ± 0	172 ± 2	171 ± 2	249 ± 1
	Volens	GP3	64 ± 0	63 ± 10	51 ± 1	52 ±1	71 ± 4
		GP20–GP3	105 ± 3	87 ± 4	95 ± 1	98 ± 2	93 ± 2
		GP72	266 ± 3	252 ± 7	253 ± 3	269 ± 5	273 ± 2
		T&T ((%)	65 ± 0	54 ± 0	51 ± 0	51 ± 0	58 ± 0
		GP20–GP3 (ml per g NDF)	221 ± 6	136 ± 7	151 ± 1	151 ± 3	199 ± 5

[1] GP3, GP20 and GP72 = cumulative gas production (ml per g organic matter) after 3, 20 and 72 h of incubation, respectively.

[2] Average ± standard error of the mean.

[3] GP20–GP3 also expressed in ml per g neutral detergent fibre (NDF).

statistically significant cultivar × position effects (and sometimes other interactions) on gas production parameters were found. So positive conclusions as to the actual influence of cultivar, year or position on the amount of gas produced are difficult to draw. Nevertheless, cultivar Vitaro had a higher gas production than Volens in all individual comparisons. Also Tilley & Terry digestibility was higher for Vitaro than for Volens samples, with the exception of sections B, which had a slightly lower digestibility in Vitaro. Maximum gas production of the soluble and insoluble fractions (GP3 and GP20–GP3), respectively) was slightly lower for sections B, C and D than that for sections A and E (Table 4). GP72 differed little between sections A–D, but was slightly higher for section E than for the other four sections. Gas production was higher for the samples taken in 1999 than for the samples taken in 2000, but the difference was only marginal.

No interactions were found for the parameters b3, c2, and c3 (half-time of gas production of microbial turnover; steepness of the curve for cell wall gas production, and steepness of the curve for microbial turnover, in that order) (data not shown). There was a statistically significant effect of year on these three parameters (*P*-values 0.0457, 0.0008 and 0.0021, respectively), as well as a statistically significant effect of cultivar ($P = 0.0001$) and position ($P = 0.0105$) on b3.

As NDF content may have had a considerable effect on gas production of the insoluble component, GP20–GP3 was also calculated per g NDF in organic matter (Table 4). Statistically significant effects of cultivar and position (but not year), and of the interactions cultivar × position and year × position on GP20–GP3 were found. If calculated per g NDF, gas production of the cell wall fraction was higher for cultivar Vitaro than for Volens, the difference even being larger than if calculated per g organic matter. Both sections A and E had a much higher gas production per g NDF than the adjacent sections (B and D, respectively).

Gas production took place mainly in the first 24 hours of fermentation (Figure 4a, b). Overall gas production from cell contents fermentation showed large peaks during the first hours of incubation (Figure 4a). In Figure 4b only the rate of fermentation of the cell wall component is plotted, which shows that the difference between cultivars in gas production from the cell wall component resulted from a difference in maximum gas production rate during the first 10 hours of incubation, which was lower for Volens than for Vitaro.

Relationship between lignin content and gas production of the insoluble fraction

The correlation between lignin content (ADL) and gas production from cell wall fermentation (GP20–GP3) is shown in Figure 5. A higher ADL content in the NDF resulted in a lower value for cell wall fermentation. Trend lines were fitted for the relationship between ADL and GP20–GP3 with values of R^2 that were much higher in 1999 than in 2000, and also higher for Vitaro than for Volens. (Values of R^2 were 0.89 for Vitaro in 1999; 0.61 for Volens in 1999; 0.27 for Vitaro in 2000; 0.18 for Volens in 2000).

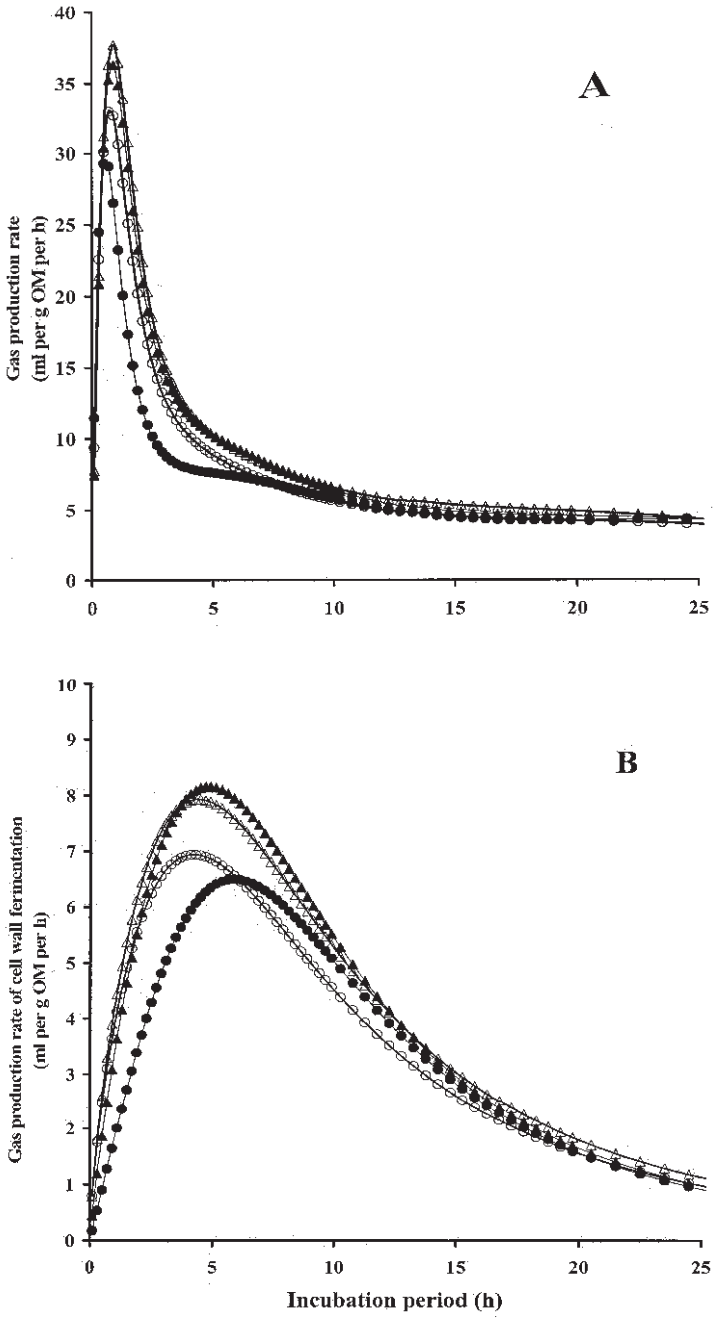

Figure 4. Gas production rate of complete samples (A) and cell wall material only (B) during incubation of segment D of internode 7 of the maize cultivars Vitaro (△, ▲) and Volens (○, ●) at anthesis, in 1999 (open symbols) and 2000 (closed symbols).

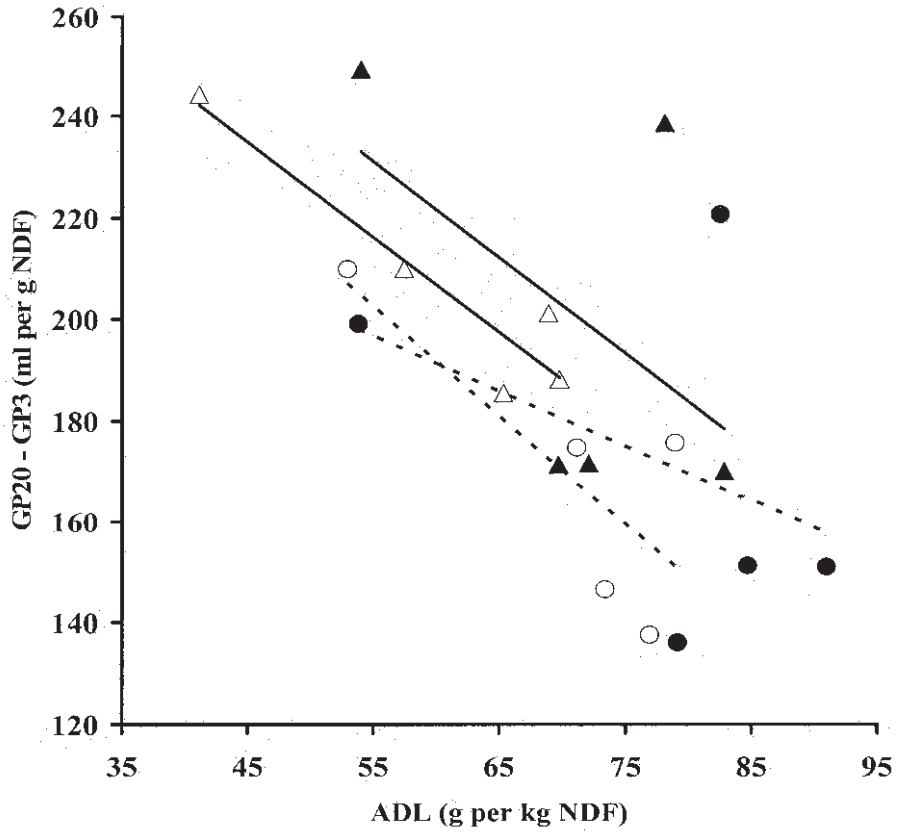

Figure 5. Gas production (GP20–GP3) during incubation of the cell wall material of internode 7 of the maize cultivars Vitaro (△, ▲) and Volens (○, ●) at anthesis, in 1999 (open symbols) and 2000 (closed symbols), in relation to lignin (ADL) content. Lines fitted: ——— Vitaro 1999; ..... Volens 1999; ——— Vitaro 2000; - - - - - - Volens 2000.

Fermentation of sections

Following 24 hours of fermentation of maize stem sections from the top, middle and base of internode 7 in buffered rumen fluid, the phloem tissue had been fully digested together with part of the chlorenchyma tissue (Figure 6; cf. Figure 2). Cell wall thickness of Scl tissue adaxial to rind vascular bundles was measured after 12, 24 and 48 hours of incubation and showed a considerable decrease (Table 5). Initially, rate of cell wall thickness decrease (as calculated from the difference between mirror sections and fermented sections, divided by the number of hours) was high, but gradually became lower (Table 5; Figure 7). Cultivars initially had a similar rate of decrease, but later on in the incubation period Vitaro showed a higher rate of cell wall decrease than Volens. This difference was especially apparent after 48 hours of incubation when cell walls of

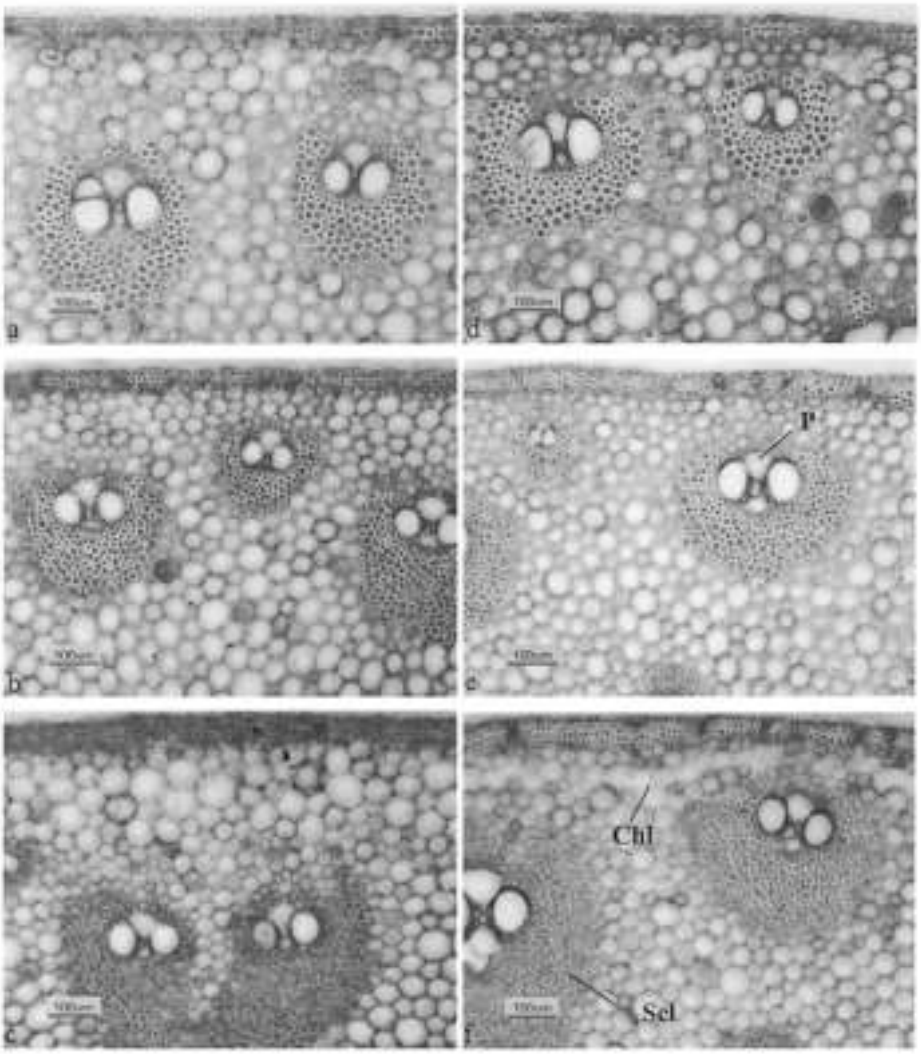

Figure 6. Cross-sections of the rind tissue from segments B (a, d), C (b, e) and D (c, f) of internode 7 of the maize cultivars Vitaro (a, b, c) and Volens (d, e, f) at anthesis, after 24 hours of incubation in buffered rumen fluid. P = phloem; Chl = chlorenchyma; Scl = sclerenchyma.

Vitaro, the cultivar with the higher whole plant digestibility, were broken down for 52% on average, against 36% on average for Volens.

The rate of cell wall decrease was highest in the top, less in the middle, and lowest in the base sections, although the proportion of cell wall that had disappeared after 48 hours was very similar for all sections.

Table 5. Cell wall thickness and decrease in cell wall thickness of sclerenchyma cells adaxial to rind vascular bundles of the top, middle and base of internode 7 of the forage maize cultivars Vitaro and Volens at anthesis after 12, 24 and 48 h of fermentation in buffered rumen fluid, in 1999 and 2000 (n = 30).

Year	Fermentation duration/changes in cell wall thickness	Vitaro			Volens		
		Top	Middle	Base	Top	Middle	Base
1999	*0–12 h*						
	Mirror (µm)[1]	3.8	2.7	2.0	4.3	3.2	2.2
	After 12 h (µm)	3.0	2.0	1.5	3.8	2.4	1.5
	Difference (µm)	0.8	0.7	0.5	0.5	0.8	0.6
	% decrease	22	27	23	11	24	30
	Decrease (nm h^{-1})	71	62	38	40	64	54
	0–24 h						
	Mirror (µm)	4.1	3.3	2.3	4.2	3.2	2.2
	After 24 h (µm)	2.4	2.1	1.2	2.7	2.0	1.5
	Difference (µm)	1.7	1.2	1.1	1.5	1.2	0.7
	% decrease	41	36	49	35	37	33
	Decrease (nm h^{-1})	70	49	47	62	49	31
	0–48 h						
	Mirror (µm)	3.9	3.3	2.1	3.7	3.1	2.2
	After 48 h (µm)	2.2	1.6	1.0	2.4	2.0	1.5
	Difference (µm)	1.7	1.7	1.1	1.4	1.0	0.7
	% decrease	44	52	52	37	34	32
	Decrease (nm h^{-1})	36	36	23	29	21	14
2000	*0–12 h*						
	Mirror (µm)	4.6	4.0	2.3	4.3	3.5	2.6
	After 12 h (µm)	3.5	3.3	1.7	3.6	3.2	1.9
	Difference (µm)	1.1	0.7	0.6	0.8	0.3	0.7
	% decrease	23	17	25	18	9	27
	Decrease (nm h^{-1})	89	57	47	66	25	57
	0–24 h						
	Mirror (µm)	4.8	4.3	2.5	4.3	3.5	2.6
	After 24 h (µm)	2.6	2.1	1.4	3.0	2.1	1.6
	Difference (µm)	2.2	2.1	1.1	1.2	1.4	1.0
	% decrease	46	50	44	29	40	39
	Decrease (nm h^{-1})	92	88	45	52	58	43
	0–48 h						
	Mirror (µm)	4.9	4.1	2.4	3.9	3.8	2.7
	After 48 h (µm)	2.1	1.9	1.1	2.6	2.0	1.6
	Difference (µm)	2.8	2.2	1.3	1.4	1.7	1.1
	% decrease	56	54	53	35	46	42
	Decrease (nm h^{-1})	57	47	27	28	36	24

[1] See Materials and methods.

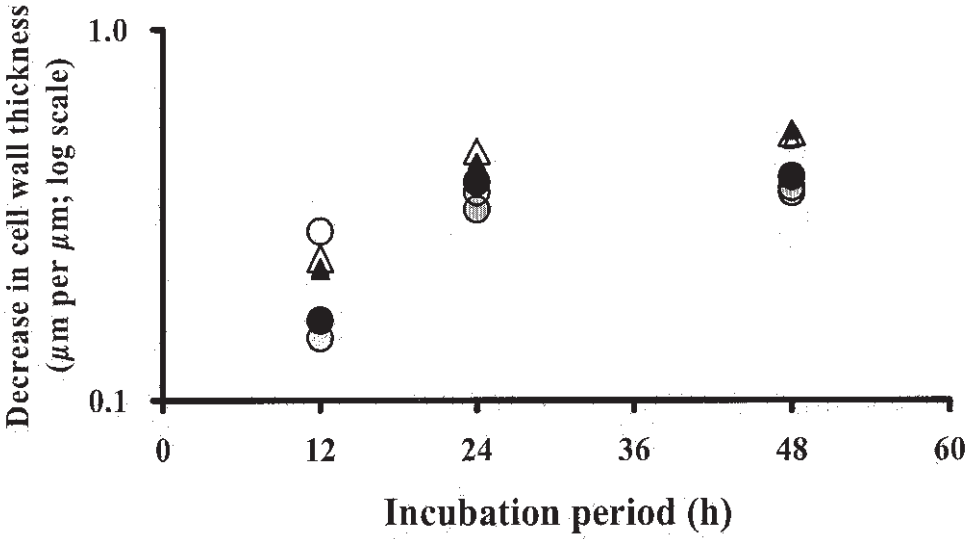

Figure 7. Decrease in cell wall thickness of segments B (shaded symbols), C (open symbols) and D (black symbols) of internode 7 of the maize cultivars Vitaro (△) and Volens (○) at anthesis. Data averaged over 1999 and 2000.

Discussion

Differences between 1999 and 2000

Year effects were not unexpected, as the weather was unusually dry and hot in the first year, and rather wet and cool in the second year. For example, although anthesis in 1999 and 2000 only differed one day, irradiation on the day anthesis started was 24.7 MJ m^{-2} d^{-1} in 1999 against 13.8 MJ m^{-2} d^{-1} in 2000. The higher photosynthesis rate in 1999 was expected to have caused a higher sugar level in that year, which did not appear to be the case. Averaged over the different internode sections, sugar content in 1999 was the same as in 2000. But in 1999 it was slightly higher in sections B through D, and slightly lower in sections A and E. However, when NDF, crude protein, ash and sugar contents of the 1999 samples are added, about 65 g per kg dry matter is unaccounted for, whereas in the 2000 samples only 8 g per kg dry matter is unaccounted for. The 1999 samples were analysed later than the 2000 samples, and the 1999 samples had been subject to a (short) period of defrosting due to a technical defect of the freezer in which they were stored. It is speculated that during this period of defrosting, sugar metabolism may have taken place, and that oligomeric sugars were formed that could not be detected with the method that was used for determining sugar content. Another possibility is that part of the sugars may have been metabolized into organic acids. Therefore, sugar content in the 1999 samples may have been underestimated.

The year had an effect on several of the characteristics investigated. Not all of these

effects were statistically significant, but nevertheless a year effect was visible. Interactions between the factors year, cultivar and position were frequently found, and complicated interpretation of the results (this also applies to the paragraphs on cultivar and year effects).

Year effects in 2000 included a lower number of Scl layers, higher NDF, ADF, ADL, crude protein and ash contents, a slightly lower sugar content, and a lower GP72 than in 1999. The lower GP72 might be attributed to the higher ADL and to a slightly lower sugar content, although the number of Scl layers could have had some influence on gas production. Judging from the higher total decrease in cell wall thickness, section fermentation was more complete in 2000 than in 1999, which is in contrast to the lower GP72 in 2000. However, mirror cell wall thickness values were higher for samples from 2000 (Table 5). This is not confirmed by the values in Table 2. This suggests variability in cell wall thickness, which made it difficult to ascertain a possible difference in cell wall thickness between cultivars. Because mirror sections were used, the variability in cell wall thickness should not pose a problem for measurements of cell wall thickness decrease, considering that cells from exactly the same vascular bundles, and in many cases exactly the same cells were measured before and after fermentation.

Differences between Vitaro and Volens

Vitaro is known to have a higher whole stem digestibility and therefore was also expected to have a higher fermentability of stem components. Gas production was indeed higher for Vitaro: not only GP72, but also GP3 (cell contents) and GP20–GP3 (cell wall) were higher. The anatomy of Vitaro stems did not differ significantly from the anatomy of Volens stems, but there was a tendency towards slightly more Scl layers and slightly thicker cell walls in Vitaro. Vitaro had a higher hemicellulose content and a lower lignin content than Volens. Fermentation of sections showed that Vitaro Scl cell walls were broken down to a further extent than Volens cell walls, and that the rate of cell wall disappearance in Vitaro, although initially the same as in Volens, was higher in later stages of fermentation. Within a year and over cultivars there was a significant negative correlation between NDF and decrease in cell wall thickness.

It appeared that the percentage fermentable cell wall was higher for Vitaro than for Volens (52% against 38% after 48 hours; calculated from Table 5). The higher fermentability of Vitaro could be the result of a higher amount of available fermentable secondary cell wall. The rate of fermentation appeared to drop considerably after 24 hours of fermentation. From data of Wilman *et al.* (1996a) it could be calculated that the fermentation rate of grass silage was highest during the periods 3–8 and 8–16 hours of fermentation, that it decreased in the period 16–24 hours, and then dropped considerably during the period 24–45 hours of fermentation. Their data are consistent with the data presented here on fermented sections of maize stem (Table 5).

Although sections were only fermented for 48 hours in the experiments presented in Table 5, cell wall decrease after 24 hours was 70–100% of that after 48 hours. This indicates either that cell walls did not (or hardly) decrease in thickness after 24 hours,

or that the measuring method was not accurate enough to establish a further decrease. Wilman *et al.* (1996b) found that degradation of maize silage after 24 hours, expressed as a percentage of 72 hours fermentation, was around 70%. From our data, combined with literature, it can be concluded that the largest extent of cell wall fermentation takes place during the first 24 hours of fermentation, associated with a high rate of fermentation during this period. Nevertheless, cultivar differences are better expressed after a long period of fermentation.

When the different parameters in Tables 4 and 5 are compared it becomes clear that the cultivar differences were much more apparent in secondary cell wall degradation and in GP20–GP3 of NDF than in GP72 or T&T. This also suggests that especially the rate of cell wall degradation differed between the two cultivars.

Position effects

A distinct anatomical difference was observed between the top and the base of the internode, namely an increase in the number of Scl layers and a decrease in the thickness of the cell walls. The thinner cell walls at the base were fermented to the same extent as the thicker cell walls at the top (as a percentage of total cell wall thickness).

The number of Scl layers in the subepidermal layer was low, and mostly varied from 1 to 4 layers, with incidental counts of 0 or 5. It is therefore unlikely that natural variation in the number of Scl layers below the epidermis will have a significant influence on the fermentation process. However, in some cases subepidermal Scl was associated with Scl surrounding vascular bundles in the rind, thereby forming larger structures that could lead to larger particle size *in situ* (Wilson, 1990).

Layers of Scl near vascular bundles in the rind took up a considerable area of the section. Although the number of Scl layers increased from top to base, and the cell wall thickness of the Scl decreased, the total area taken up by Scl tissue appeared to remain constant. An increased number of layers is thought to enlarge the total cell wall area available for fermentation (assuming equal particle size). Thinner cell walls could mean a lower amount of fermentable cell wall material per wall. An increase in the number of cells, combined with thinner cell walls, could result in a similar fermentability.

Most of the increase in the number of Scl layers took place from the middle to the base of the internode, where also most of the decrease in cell wall thickness took place. Chemical composition and fermentability did not show such a large change from the middle to the base of the internode.

Chemical analysis revealed that the outer parts of the internode (A and E, the parts including the nodes) had a higher hemicellulose and lower cellulose content, and section E also had a lower lignin content than any other section. Gas production of the cell wall slightly increased from section B (top) to D (base), but in most cases sections A and E produced more gas during fermentation than the adjacent section. This is likely related to the difference in chemical composition. The anatomy of the area close to the node was not studied in sufficient depth to draw any conclusions as to the influence of node anatomy on fermentation rate and fermentation extent.

Lumen area and lumen perimeter

Preliminary measurements indicated that lumen area and lumen perimeter increased by 50 and 100%, respectively, over the fermentation period of 48 hours (data not shown). These figures were estimated by rather crude measurements and only give an indication of two-dimensional changes as they were performed on fermented sections. Furthermore, the surface of the sections was readily accessible to micro-organisms from the very start of fermentation.

The increase in available cell wall surface was not substantial. Although twice as many micro-organisms could in theory adhere to the wall (and even many more if translated to three dimensions), it was not likely that lumen area was the limiting factor to cell wall access. As the rate of cell wall disappearance decreased rather than increased as fermentation time increased, the chemical and/or physical structure of the cell wall seemed of greater influence.

However, the variation in cell lumen area was considerable. It cannot therefore be ruled out that some cells were initially difficult to access for micro-organisms. Especially the larger protozoa might be prevented from entering certain cells, but this would likely have only minor consequences for cell wall disappearance as protozoa make a small contribution to *in vitro* cell wall disappearance (Lee *et al.*, 2000).

Division of the internode

The division of the internode into five sections was initially made with a possibly lower fermentability of the nodes in mind. The node itself is tougher than the surrounding internode material, and was speculated to have a higher lignin content, more vascular bundle material and less highly fermentable parenchyma. Although the node itself is not longer than about 1 cm, a length of 2 cm was chosen for ease of division. In fermentation tests with the five sections it was found that fermentation characteristics of the upper (A) and lower (E) sections differed to a certain degree from the other sections. Initial expectations were of a slight decrease of maximum gas production (at least of the insoluble fraction) from the top to the base of the internode, based on the physiological age of the tissues: older at the top than at the base. Correlated with this, a slight decrease in lignin content from the top to the base of the internode was expected. On anatomical examination of section A it was found that the original meristem of the internode was included in this section. Therefore, the youngest tissue was located in this section, which presupposes a higher maximum gas production in this section than in the sections above it. These expectations were confirmed by the gas production data (Table 4), with the exception of Volens samples in 2000 when there was little difference between samples. Note that, contrary to expectation, maximum gas production of the insoluble phase of section A was always equal to or higher than that of section B. A possible explanation for this could be that the division of the internodes was sometimes difficult, and some material from the base of internode 8 may have been included in the A sections of internode 7. It is also possible that the fermentation of node material is not less or slower than that of internode material. Ramanzin *et al.* (1986) found a higher degradability for nodes than for internodes in

barley. Little information on the anatomical structure of the node and its relation to fermentability is available.

It was expected that fermentability of the insoluble fraction of top sections would be less than that of middle sections, and that the fermentability of the insoluble fraction of middle sections would in turn be less than that of base sections. Fermentability at the base of the internode would be highest, because of a large supply of well digestible secondary cell wall (large surface area of Scl), and a relatively large lumen size of the Scl cells. But this was not observed. In fact, in several cases the opposite appeared to be the case. This could be because of a larger supply of potentially fermentable secondary cell wall material (thicker Scl cell walls). Apparently, in this case lumen size was not a limiting factor.

Cell wall thickness measurement method

A practical obstacle in the measurement of cell wall thickness was the thickness of the sections themselves. Not embedded material could not be easily sectioned thinner than 100 µm, and embedding would most likely influence fermentation. When sectioning, the cell walls are not always cut at a straight angle. Therefore, when measuring the cell wall thickness on the 100 µm-thick sections that were used here, the thickness of the wall was likely overestimated. Previously found values for cell wall thickness of sclerenchyma were around 2.0 to 2.5 µm (Travis *et al.*, 1993; Ahmad & Wilman, 2001). Although all measurements were done on sections with the same section thickness (which implies that measurement error should be similar), cell walls were much thinner after 48 hours of fermentation and this might have led to a higher overestimation of cell wall thickness. If this reasoning is correct, then the actual rates of cell wall degradation are even higher than reported in Table 5.

Particles incubated in buffered rumen fluid did still produce gas after 48 hours of fermentation.

Conclusions

The weak relationship between secondary cell wall disappearance on the one hand and lignin content and gas production on the other, was a strong indication of the importance of other factors in the fermentation process. Anatomical factors influencing the accessibility of the tissues to micro-organisms and chemical and/or physical factors such as lignin composition, lignin localization within the cell wall and nature and extent of cross-linking lignin to other cell wall components may play an important role as well.

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