agronomie (1996) 16, 123-130

agronomie: plant genetics and breeding

Genotypic variation in phenolic components of cell-walls in relation to the digestibility of maize stalks

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(Received 21 September 1995; accepted 28 December 1995)

Summary — Stalks of several normal maize hybrids, displaying a broad range in cell-wall digestibility, were examined for lignin content and monomeric composition and for ester-linked and ether-linked p-coumaric and ferulic acids. Genotypic variation was found in all of these components. Changes in cell-wall digestibility were associated with several phenolic component characteristics. It was therefore difficult to attribute a clear causal effect on degradability to a single cell-wall component, and to distinguish direct from indirect effects. However, amongst normal hybrids, it seemed that the lignin content, together with the content of etherified ferulic acid, which probably acts as a bridge between lignins and hemicelluloses, had a strong influence on the inhibition of the cell-wall digestibility.

***Zea mays* L = maize / digestibility / cell-wall / hydroxycinnamic acid / lignin**

Résumé — Variabilité génotypique pour les composés phénoliques des parois de tiges de maïs en relation avec leur digestibilité. *La teneur en lignine et sa composition monomérique, les teneurs en acide férulique et acide p-coumarique estérifiés et éthérifiés ont été étudiées dans les tiges de plusieurs hybrides de maïs présentant une gamme de variation importante pour la digestibilité des parois. Des différences entre les hybrides ont été mises en évidence pour tous ces caractères. Les variations de digestibilité des parois sont liées à des variations au niveau de plusieurs caractéristiques des composés phénoliques. Il est donc difficile d'expliquer par un unique caractère le niveau de dégradabilité des parois, et de distinguer les effets directs des effets indirects. Il semble cependant que la teneur en lignine et la teneur en acide férulique éthérifié, qui sert probablement de pont entre lignines et hémicelluloses, affectent négativement la digestibilité des parois.*

Zea mays *L = maïs / digestibilité / parois / acides hydroxycinnamiques / lignines*

INTRODUCTION

Silage maize is an important forage crop, providing roughage with a high energy content. Improvement in maize feeding value will result primarily from an improvement in the cell-wall digestibility (Deinum and Struik, 1985; Dolstra and Medema, 1990; Barrière et al, 1992; Argillier et al, 1995a). Lignins have been widely reported as limiting components in cell-wall digestibility, but lignin content is not always a good predictor of degradability. Thus, other factors such as lignin composition and the nature of bound hydroxycinnamic acids may also influence cell-

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wall degradation (Hatfield, 1993; Jung and Deetz, 1993; Besle et al, 1994).

Cell-walls consist mainly of a cellulosic, fibrillar phase, embedded in a matrix phase composed of phenolic components and hemicelluloses, which are partly covalently linked. Linkages between cell-wall constituents have been recently reviewed by Hatfield (1993), Ralph and Helm (1993), and Iiyama et al (1994). Grass lignins mainly comprise guaiacyl (G) and syringyl (S) units, interconnected by labile ether bonds (referred to as 'β-O-4' linkages) and/or by resistant carbon–carbon bonds (referred to as 'condensed bonds'). Hydroxycinnamic acids, mainly ferulic acid (FA) and *p*-coumaric acid (PA) may be covalently linked to hemicellulose carbohydrates through ester linkages. PA may be covalently linked to lignins mainly through ester bonds but also through ether bonds and FA may be covalently bound to lignins through ether linkages. Lam et al (1992b) showed that, in wheat and phalaris internodes, FA which was ether-linked was also ester-linked, forming bridges between lignins and hemicelluloses. In contrast, PA does not appear to be involved in cross-linked structures (Lam et al, 1992b; Ralph and Helm, 1993).

The variability in such cell-wall characteristics in relation to the degradability was studied in maize: at various maturity stages, and i) when comparing normal genotypes ii) in brown-midrib mutants. A higher lignin content, an enhanced *p*-coumaric esterification (Lopez et al, 1993; Scobbie et al, 1993) and a preferential deposition of syringyl units in the lignin polymer (Chabbert et al, 1994) are associated with an increase in the stage of maturity of maize internodes, and with a decrease in digestibility. From biochemical studies, the *bm3* gene is characterised by a reduction in the lignin content, a lower content in syringyl units in β-O-4 lignin structures and the presence of unusual 5-hydroxyguaiacyl units (Lapierre et al, 1988; Chabbert et al, 1994). It is also typified by a decrease in ester-bound *p*-coumaric acid content (Chabbert et al, 1994) and a reduction in etherified hydroxycinnamic acid contents (Goto et al, 1994).

For plant breeding purposes, brown-midrib genes have drastic effects on maize silage quality (Barrière and Argillier, 1994). However, if the digestibility of *bm3* mutants is improved, the agronomical value of these *bm3* genotypes is distinctly lower than that of their isogenic counterparts (Barrière et al, 1994). A large variation in in vivo crude fiber digestibility (Barrière et al, 1992) and in vitro cell-wall digestibility (Dolstra and Medema, 1990; Argillier et al, 1995a) was found among normal hybrids, whereas phenolic components were scarcely investigated. Among normal hybrids, it seems conceivable to simultaneously improve biomass productivity, root and stalk lodging resistance, and feeding value (Argillier et al, 1995b).

It is, therefore, of great importance for silage maize breeding to study, between normal hybrids, the genotypic variation in cell-wall biochemical characteristics in relation to the digestibility. We also attempt to establish whether or not high digestibility in normal hybrids is built on the same biochemical patterns as in *bm3* plants. The final objective of the current study is to investigate the possibility of identifying biochemical criteria useful for the understanding and breeding of quality traits.

MATERIALS AND METHODS

Plant material

Six maize hybrids of similar earliness, W117 x EP1, F7 x W117, F2 x F113, F2 x F288, MBS847 x Co125 and LH74 x F271, were chosen on the basis of differences in the digestibility of their cell walls, when harvested for silage, which were observed by a large multilocal network during a two year study in many climatically diverse locations (Argillier et al, 1995a). The parental lines originated from a broad genetic germplasm basis (Canadian, French, Spanish and US lines or ecotypes; Argillier et al, 1995a). Plants were cropped in the Station d'amélioration des plantes fourragères (INRA Lusignan, France). Each plot (corresponding to one hybrid) consisted of one 4 m long row: the row spacing was 0.80 m, and the density was 115 000 plants per hectare. At the ensiling stage, five plants per plot were sampled and segments, 45 cm long, were collected from the bottom of each stalk. The samples were dried in an oven (at 70 °C) and ground with a hammer mill to pass through a 1 mm screen.

Chemical analysis

Neutral detergent fiber (NDF) was determined by the Goering and Van Soest method (1970). The lignin content of the NDF was estimated by the modified Klason procedure (Effland, 1977). Acid detergent fiber (ADF) was also determined by the Goering and Van Soest method (1970) in order to estimate the hemicellulose content as NDF – ADF. Soluble carbohydrates were also analysed (Lila, 1977). These analyses were performed in duplicate.

Treatments of NDF fractions with NaOH were performed according to Morrison et al (1993). NDF samples (100 mg) were mechanically shaken under nitrogen with 2 M NaOH (5 mL) at 25 °C for 20 h. Other NDF samples (100 mg) were placed in a Teflon screw-capped vial containing 4 M NaOH (5 mL) and purged with nitrogen. This vial was then sealed in a stainless steel reaction vessel. The vessel was placed in an oven at 170 °C for 2 h. After these two different methods of alkaline hydrolysis, the samples were treated in the same way. An internal standard (3,4,5-trimetoxycinnamic acid) was added to the suspensions prior to centrifugation (380 *g*, 10 min). The supernatants were acidified to pH 2 with 3 M HCl, kept at 4 °C overnight and centrifuged again (380 *g*, 10 min).

Hydroxycinnamic acids released by alkaline hydrolysis at ambient temperature and at 170 °C were extracted with ethyl acetate and analysed by high-performance liquid chromatography (HPLC), according to Chabbert et al (1994). A reverse-phase column (LiChrospher C18 column, 250 x 4 mm, 5 µm) was used for chromatographical separation with a linear gradient of methanol (30–53%). The column was operated at 30 °C at a flow rate of 1.2 mL min^{-1}. Hydroxycinnamic acids were detected at 280 nm with a L-4250 UV VIS MERCK detector. All assays were made with three replicates.

Thioacidolysis was performed on 20 mg NDF, in a 10 mL mixture of dioxane/ethanethiol (9:1, v/v) containing 0.2 M boron trifluoride etherate for 4 h in an oil bath at 100 °C (Lapierre et al, 1986). Monomeric products were recovered from the mixture by dichloromethane extraction. Their trimethylsilyl derivates were analysed by capillary column gas chromatography (SPB1 column, 30 m x 0.32 mm, 0.25 µm; vector gas: He; pressure: 5.5 bar; column temperature rate: 2 °C min^{-1}; detector: FID). Amounts were calculated with reference to an internal standard (tetracosane). All assays were made with eight replicates.

Digestibility measurement

The in vitro dry matter digestibility (IVDMD) of the stalk samples was determined by the Libramont-Limagrain enzymatic solubility (Ronsin, 1990). The measures were made with five replicates. The in vitro digestibility of the non-soluble carbohydrate part (IVDNC) was also computed, assuming that soluble carbohydrates are completely digestible:

$$\text{IVDNC} = \frac{100 \times (\text{IVDMD} - \text{soluble carbohydrate content})}{(100 - \text{soluble carbohydrate content})}$$

This trait, like the previously described IVDNSC (in vitro digestibility of the non-starch and non-soluble carbohydrate part, which was measured on whole plant samples; Argillier et al, 1995a), gives an estimation of the cell-wall digestibility, and seems to be a good criterion for improving the nutritive value of forage maize.

RESULTS AND DISCUSSION

Variation in digestibility, lignin and hemicellulose contents

The IVDNC ranged from 36.37–50.84% (table I). Three of the hybrids tested in this experiment (W117 x EP1, F7 x W117 and LH74 x F271) have also been evaluated with in vivo sheep measurements at INRA Lusignan for several years; the ranking of these hybrids for their IVDNC was the same as for the crude fibre digestibility estimated with the sheep (table I). The Klason lignin and hemicellulose contents in the cell-wall ranged from 17.5–22.5% and 37.9–43.7% NDF respectively (table I).

Hydroxycinnamic acid contents

Differences in hydroxycinnamic acid contents were observed between the genotypes (table II). Alkaline hydrolysis at ambient temperature (2 M NaOH, 25 °C) is the conventional method to determine hydroxycinnamic esters. The ranges for esterified *p*-coumaric and ferulic acid contents were 27.72–33.58 and 5.31–6.44 mg g^{-1} NDF respectively (table II).

In order to measure the total amount of hydroxycinnamic acids, including the ether-bound fraction, we applied a direct treatment of the cell-walls with 4 M NaOH at 170 °C for 2 h. This method, proposed by Iiyama et al (1990), proceeds by cleavage of both ester and ether bonds. For ferulic acid, values greater than those obtained with 2M NaOH at 25 °C were found (table II). This allowed a calculation of the ether-bound FA content. The mean relative percentage of ferulic ethers was 23%. The amount of etherified FA varied among the hybrids (table II). As reported by Morrison et al (1993) for pearl millet, the values obtained for *p*-coumaric acid with 4M NaOH at 170 °C were lower than those obtained with 2 M NaOH at 25 °C. These results could partly be explained as follows. When authentic PA and FA are subjected to alkaline hydrolysis at elevated temperature, PA is recovered in lower yield than FA (Lam et al, 1992a). The content in *p*-coumaric ethers assessed by alkaline hydrolysis at 170 °C is thus more severely underestimated than that of ferulic ethers.

In contrast to the results of studies undertaken through the maturity stages, whatever the species (Lam et al, 1993; Lopez et al, 1993;

Table I. Hybrid means for digestibility, lignin and hemicellulose content.

Hybrid	*IVDNC* [a]	*CFD* [b]	*KL* [c]	*HEM* [d]
MBS847 x Co125	36.4 ± 1.4	–	21.3 ± 0.1	39.6 ± 0.1
LH74 x F271	39.2 ± 0.7	48.9 ± 1.3	22.5 ± 0.2	37.9 ± 0.3
F2 x F113	44.4 ± 1.3	–	21.4 ± 0.7	42.4 ± 0.2
F2 x F288	47.3 ± 1.3	–	20.9 ± 0.8	43.7 ± 0.1
F7 x W117	48.9 ± 1.7	54.2 ± 2.1	17.5 ± 0.6	43.1 ± 0.3
W117 x EP1	50.8 ± 1.6	56.6 ± 2.1	19.4 ± 0.9	43.1 ± 0.6

[a] IVDNC: digestibility of the non soluble carbohydrate part, expressed as % ± standard deviation. [b] CFD: crude fibre digestibility, evaluated with in vivo sheep measurements, expressed as % ± standard deviation. [c] KL: Klason lignin content, expressed as %NDF ± standard deviation. [d] HEM: hemicellulose content, expressed as %NDF ± standard deviation.

Table II. Hybrid means for the hydroxycinnamic acid content, determined by alkaline hydrolysis.

Hybrid	*Esterified phenolic acids* [a]		*Total phenolic acids* [b]		*Etherified ferulic acid* [c]
	PA	*FA*	*PA*	*FA*	*FA*
MBS847 x Co125	33.58 ± 0.18	5.76 ± 0.03	28.31 ± 1.02	7.99 ± 0.26	2.23 ± 0.26
LH74 x F271	29.69 ± 0.15	5.31 ± 0.02	25.13 ± 0.35	7.42 ± 0.11	2.11 ± 0.11
F2 x F113	27.72 ± 0.81	6.31 ± 0.08	24.04 ± 0.99	8.21 ± 0.19	1.90 ± 0.20
F2 x F288	27.74 ± 0.45	6.44 ± 0.05	22.83 ± 0.36	8.49 ± 0.04	2.05 ± 0.06
F7 x W117	31.35 ± 0.87	6.31 ± 0.12	26.60 ± 0.07	7.65 ± 0.03	1.34 ± 0.12
W117 x EP1	28.87 ± 0.65	6.24 ± 0.10	24.43 ± 0.22	7.72 ± 0.09	1.48 ± 0.13

PA: *p*-coumeric acid; FA: ferulic acid; content expressed as mg g^{-1} NDF ± standard deviation; [a] recovered by alkaline hydrolysis with 2M NaOH at 25 °C for 20 h; [b] recovered by alkaline hydrolysis with 4M NaOH at 170 °C for 2 h; [c] calculated as the difference between total and esterified FA.

Scobbie et al, 1993), or comparing normal maize genotypes with the *bm3* mutants (Chabbert et al, 1994), or studying several maize inbred lines of different earliness (Jung and Buxton, 1994), we found no positive correlation, among normal maize hybrids of the same earliness group, between the Klason lignin content and the esterified PA content (fig 1a). The esterified FA content was positively related to hemicellulose content (fig 1b) and the etherified FA content calculated was positively related to Klason lignin content (fig 1c). Moreover the amounts of esterified and etherified FA tended to be negatively associated (fig 1d). These results are in line with the hypothesis that ferulic acid may first be esterified to hemicelluloses and then, during lignification, the ferulic acid esters form cross-links through the etherification of the phenolic hydroxyl group to lignin polymers (Scalbert et al, 1985; Kondo et al, 1990; Iiyama et al, 1990; Lam et al, 1992b). This was confirmed by Lam et al (1992b) who found, in wheat and phalaris internodes, that all the etherified ferulic acid was also ester-linked and therefore formed ester–ether bridges between lignins and hemicelluloses. Thus, the esterified FA detected in our experiments represented the ester-linked FA which was not ether-bridged to phenylpropenoid units, whereas the etherified FA measured probably corresponded to the ester–ether bridges between hemicelluloses and lignins.

Lignin monomeric composition

Differences between genotypes were observed for lignin monomeric composition, determined by thioacidolysis. The main monomers recovered from G and S units involved only in labile ether bonds were analyzed (table III). Their relative ratio S/G varied from 1.09–1.85 (table III). Moreover, their total amount (S + G) was nega-

tively related to the lignin content in carbon–carbon ('condensed') linkages (Rolando et al, 1992).

While *bm3* maize hybrids while which have a lower lignin content than normal maize, also have a smaller proportion of syringyl units (Chabbert et al, 1994), we did not observe, among our normal hybrids, a relationship between lignin content and the preferential deposition of syringyl units in lignins (ratio S/G) (fig 1e). The lignin content tended to be positively associated with the sum (S + G) (fig 1f).

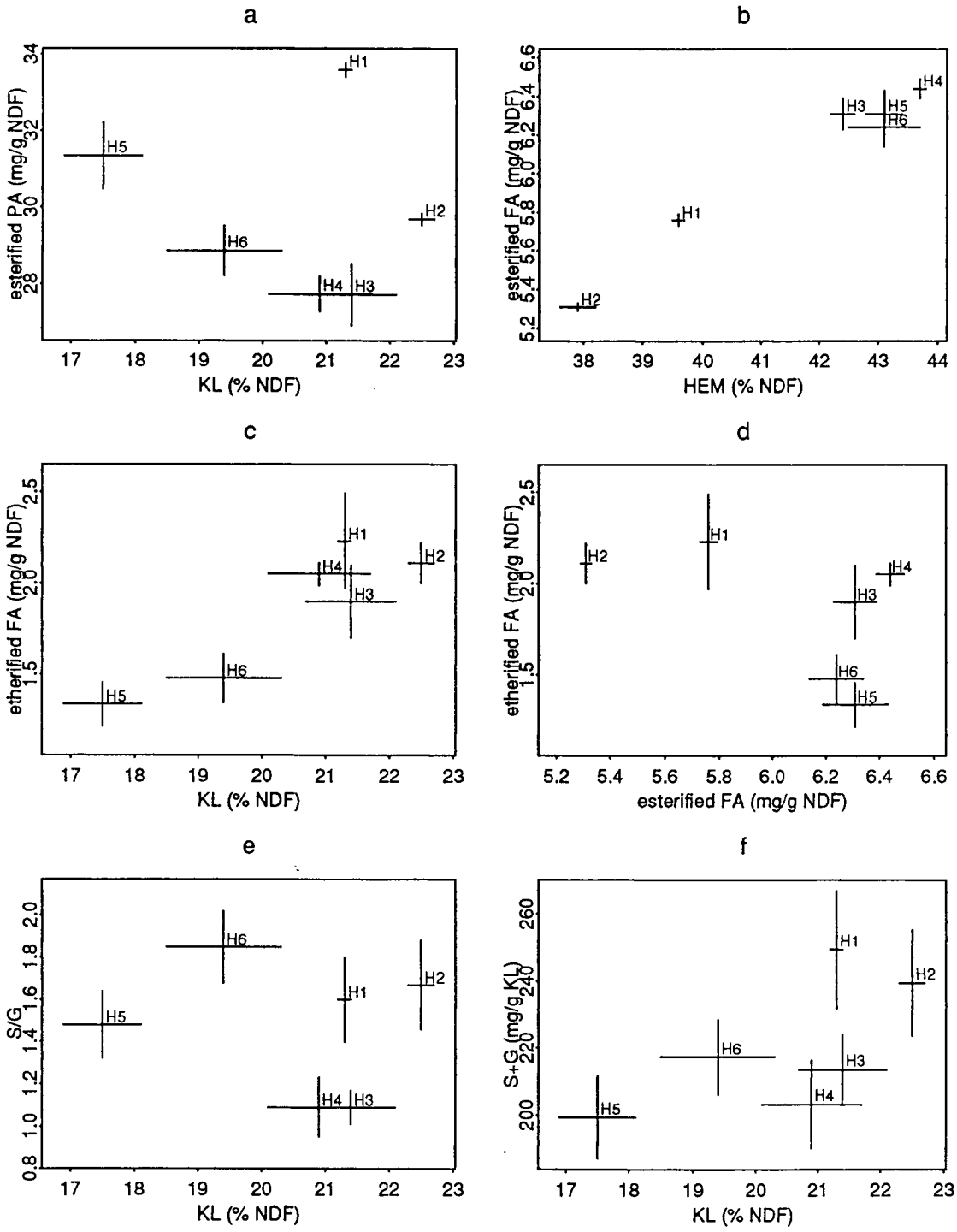

Fig 1. Relationships between a) Klason lignin (KL) content and esterified *p*-coumaric acid (PA) content, b) hemicellulose (HEM) content and esterified ferulic acid (FA) content, c) KL content and etherified FA content, d) esterified FA content and etherified FA content, e) KL content and syringyl/guaiacyl (S/G) ratio, f) KL content and (S+G) yield. The hybrids MBS847 x Co125, LH74 x F271, F2 x F113, F2 x F288, F7 x W117, and W117 x EP1 are represented by H1, H2, H3, H4, H5 and H6 respectively. For each hybrid the bars around the mean values are represent standard deviations of the considered traits.

Relationships between cell-wall digestibility and the biochemical characteristics of the phenolic components

The differences observed between the hybrids in the digestibility of their cell-walls were partly, but not entirely, explained by the differences in their lignin contents. For example, F2 x F113 and MBS847 x Co125, with similar lignin contents, displayed a difference of 8 points for IVDNC (table I). However, they also differed in their hydroxycinnamic acid characteristics and lignin monomeric compositions. MBS847 x Co125 had significantly more *p*-coumaric acid, less ferulic esters and more ferulic ethers than F2 x F113 (table II). Furthermore, MBS847 x Co125 had a greater S/G ratio than F2 x F113 (table III). F2 x F288 exhibited a high cell-wall digestibility with, in general, an intermediate position regarding the contents of phenolic components. The two most digestible hybrids (F7 x W117 and W117 x EP1) had the lowest lignin contents, the lowest contents of hydroxycinnamic ethers and lignins enriched in carbon–carbon bonds, as shown by low thioacidolysis yield (tables I, II and III).

PA is predominantly linked to lignins and its influence on polysaccharide degradation is probably indirect and through the negative effect of lignins. We did not find an obvious effect of esterified FA on the cell-wall digestibility, which suggests that ferulic units ester-linked only to polysaccharides do not affect their degradation, which is in agreement with the literature (Jung and Deetz, 1993). On the contrary, ferulic ethers, which probably act as cross-links between hemicelluloses and lignins, seemed partly to explain, together with the lignin content, the differences in cell-wall digestibility between the hybrids, in agreement with Jung and Buxton (1994). The great difference in IVDNC between F2 x F113 and MBS847 x Co125, although they had similar lignin contents, could possibly be explained by the higher content in etherified FA in MBS847 x Co125. Ester–ether ferulic bridges between lignins and xylans may inhibit xylan accessibility and degradability by enzymes (Jung and Deetz, 1993).

Both hybrids F2 x F113 and F2 x F288, which exhibited moderately high cell-wall degradabilities and intermediate lignin contents, were characterized by the lowest S/G ratio (table III). Both hybrids with the weakest cell-wall digestibility, which also had a common genetic origin, displayed lignin enriched in labile ether bonds (as shown by high thioacidolysis yield), and a high proportion of S compared to G units in the uncondensed fraction (table III). Several aspects of lignin structure, such as the monomeric composition and the types of inter-monomeric linkages, may be related to cell-wall degradability. The molecular mechanisms of inhibition of polysaccharide degradation are not yet known, even if lignin structure probably has an influence on cell-wall degradability via its role as a physical or chemical barrier between carbohydrates and microbial enzymes.

CONCLUSION

Genotypic variation was found among a range of normal maize hybrids of a similar earliness, in cell-wall digestibility, lignin content and composition, and esterified and etherified hydroxycinnamic acid contents. We also observed that changes in cell-wall degradability were associated with modifications in several phenolic component characteristics. As these factors often developed jointly, it was not possible to attribute a clear causal effect on digestibility to any one of these

Table III. Hybrid means for the monomeric composition of the lignin, determined by thioacidolysis.

Hybrid	*G* [a]	*S* [a]	*S + G* [a]	*S/G* [b]
MBS847 x Co125	95.8 ± 6.5	153.7 ± 16.3	249.4 ± 17.5	1.60 ± 0.20
LH74 x F271	89.4 ± 7.6	150.2 ± 13.9	239.5 ± 15.8	1.67 ± 0.21
F2 x F113	101.8 ± 6.2	111.8 ± 8.4	213.6 ± 10.5	1.09 ± 0.08
F2 x F288	96.6 ± 6.5	106.8 ± 11.4	203.3 ± 13.1	1.09 ± 0.14
F7 x W117	81.8 ± 5.2	117.5 ± 11.0	199.3 ± 12.2	1.48 ± 0.16
W117 x EP1	75.9 ± 4.1	141.4 ± 10.5	217.3 ± 11.2	1.85 ± 0.17

[a] Guaiacyl (G), syringyl (S), and (S + G) yields determined by thioacidolysis, expressed as mg g^{-1} Klason lignin ± standard deviation.
[b] ratio S/G ± standard deviation.

factors, or to determine the respective influence of associated and direct effects. Furthermore, high or low digestibility is probably not due to a single mechanism. However, it seems that the lignin and etherified ferulic acid contents, which probably play a role in the cell-wall structure by acting as bridges between lignins and hemicelluloses, explain much of the cell-wall degradability. Lignin structure may also have an influence on cell-wall digestibility.

Between normal hybrids, we did not find the same biochemical pattern (esterified hydroxycinnamic acids and lignin monomeric composition), associated with high or low degradation, as was found in the comparison between normal and *bm3* mutant plants, except for the lignin content (although the range was lower among normal genotypes than among the *bm3* ones) and the etherified hydroxycinnamic acid contents. This confirmed that there is probably no unique mechanism which explains the digestibility level.

To distinguish the effect of lignin content from that of lignin structure, future research could utilize normal maize genotypes, differing in cell-wall digestibility and with similar lignin contents, to control this factor and therefore observe the other phenolic constituent characteristics (eg, hydroxycinnamic acid contents, lignin monomeric composition). It would also be interesting to investigate hybrids whose feeding values (digestibility and ingestibility) are already known from sheep, fattening bull and dairy cow experiments.

Plant breeders can improve the digestibility of forage maize using a criterion such as IVDNSC. However, an understanding of the biochemical patterns providing a higher cell-wall digestibility is necessary when breeding simultaneously for digestibility, ingestibility and lodging resistance.

ACKNOWLEDGMENTS

The authors are indebted to B Chabbert, MT Tollier and JM Besle for their help with biochemical techniques and for useful discussions and thank V Furstoss for his help in analysis measurements. We thank the reviewer for her helpful criticisms We also acknowledge A de Pourtalès for linguistic revision of the manuscript.

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