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Degradability of parenchyma and sclerenchyma cell walls isolated at different developmental stages from a newly extended maize internode

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SUMMARY

Parenchyma and sclerenchyma cell walls were mechanically isolated from sections of a newly extended maize internode containing cells at different stages of maturity. Internodes were divided into four equal segments (A[top]–D[bottom]) and preparations of both cell types obtained from each segment. The lignin content of sclerenchyma (57·5–108·3 g kg^{-1}) was greater than that of parenchyma (48·0–86·8 g kg^{-1}) at all stages of development. The extent of lignification increased with age in both cell types but was similar in cells taken from segments A and B. The ratio of the saponifiable phenolic acids differed with age with levels of (*E*+*Z*)-ferulic acid remaining essentially constant while the (*E*+*Z*)-*p*-coumaric acid content increased in parallel with lignification. The extent to which commercial 'cellulase' was able to degrade both cell types decreased with age and extent of lignification. Sclerenchyma walls were less degradable than parenchyma walls isolated from the same segment. The fitted rate constant calculated for the period 2–72 h, however, was independent of both age and cell type. The mean thickness of sclerenchyma walls increased with age because of secondary wall formation (confirmed by image analysis of sections prepared for microscopy) while parenchyma remained thin walled. The consequences of lignification appeared to be essentially the same for both cell types and, by implication, for both primary and secondary wall layers.

Key-words: cell wall, degradability, image analysis, lignification, parenchyma, sclerenchyma.

INTRODUCTION

Microscopic evidence suggests that during secondary thickening and lignification the primary cell wall (and middle lamella) of forage plants becomes extensively lignified and highly resistant to degradation by gut micro-organisms (Akin 1988). The secondary wall layers, in contrast, appear to remain potentially degradable (Chesson *et al.* 1986; Engels

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1989; Wilson *et al.* 1991) although they may be protected from microbial attack in cells which retain structural integrity.

Use of a newly extended maize internode model to study the process of lignification and its consequences has shown that the xylan–cellulose network which forms the bulk of the secondary wall layers is laid down independently and partly in advance of lignification (Scobbie *et al.* 1993). At this early stage of development the secondary wall does not stain positively or stains only weakly with acid phloroglucinol (AP) suggesting that it is largely free of lignin (Engels & Schuurmans 1992). Later in development the secondary layers become increasingly AP-positive implying a substantially increased degree of lignification, but apparently remain highly degradable. Lignified primary walls, including those which form the primary wall/middle lamella layer of secondary-thickened cell walls, stain heavily with AP from an early developmental stage and, unlike older secondary layers, retain the response to AP after treatment with sodium hydroxide or potassium permanganate (Morrison 1988; Engels 1989). The apparent resistance shown by the primary layer to degradation has led some to suggest that the progressive lignification of the primary wall–middle lamella is the key factor responsible for limitations to cell-wall degradation by micro-organisms (Cone & Engels 1990; Engels & Schuurmans 1992).

Although the walls of both parenchyma and sclerenchyma cells become progressively lignified, parenchyma cells remain thin-walled with little evidence of the deposition of the secondary wall layer that is clearly evident in mature sclerenchyma. The potentially degradable secondary wall thus makes an increasing contribution to sclerenchyma dry matter during development but not to parenchyma walls. These developmental differences would be expected to be reflected in the degradability characteristics of the two cell types.

This work takes advantage of the spatial separation of the temporal events associated with lignification which occur in newly extended Gramineae internodes (Barnoud *et al.* 1973; Joseleau & Barnoud 1976; Lam *et al.* 1990), including those of maize (Scobbie *et al.* 1993). Sufficient isolated parenchyma and sclerenchyma cell walls at various developmental stages were obtained from single maize internodes to examine directly their degradability and lignin content before and after degradation.

MATERIALS AND METHODS

Plant material

Maize (*Zea mays* L. cell line Co125), grown under glass by ICI Seed SES NV at Tienen, Belgium under a lighting regime of 16-h daylight (minimum temperature 20°C) and 8-h night (minimum temperature 15°C), was harvested 5 days after anthesis. Sample plants (stems only) were transported to this laboratory under dry ice and maintained frozen until required. Internode 1, numbering from the top of the plant, was retained for microscopy and was dissected into ten equal segments labelled A–J (Fig. 1). Sections I and J were further divided into three parts I_1–I_3 and J_1–J_3. The second internode was used for the preparation of sclerenchyma and parenchyma cell walls. These internodes were cut into four equal segments labelled 2A–2D, each corresponding to 2·5 segments of internode 1 (Fig. 1).

Microscopy: sectioning and image analysis

After fixation in 2·5% glutaraldehyde in 0·1 M pH 7·3 phosphate buffer, internode segments were washed with buffer, dehydrated in a series of increasing ethanol

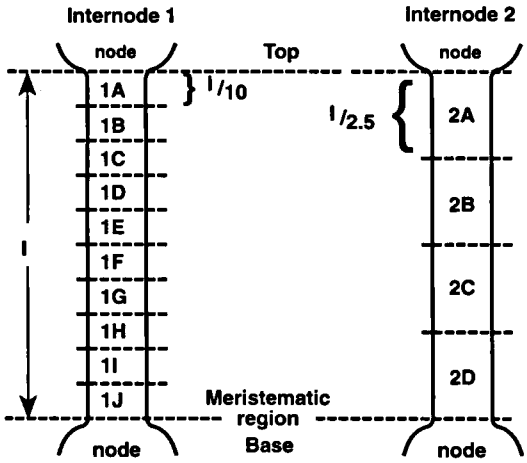

Fig. 1. Sectioning and labelling of maize internodes showing the relationship between sections taken for image analysis (internode 1) and those from which parenchyma and sclerenchyma cells were isolated (internode 2).

concentrations and embedded in LKB Historesin (Cambridge Instruments, Cambridge, UK). Transverse sections (3 μm thickness) were cut from the resin-embedded stems on a microtome (Reichert Autocut supplied by Cambridge Instruments, Cambridge, UK) using a glass knife. Sections were then dried onto ethanol-cleaned slides using a hot plate at 70°C, stained with toluidine blue (2·5 g sodium tetraborate +2·5 g toluidine blue in 100 ml distilled water) and mounted in 'Histomount' (Cambridge Instruments, Cambridge, UK).

Sections were examined by light microscopy and cell-wall thickness, mean cell size and percentage of tissue area occupied by cell wall (density) were measured using the image skeletonization methods described by Travis *et al.* (1993). A Torch QS2000 Unix workstation (CubeIT, Cambridge, UK) fitted with a 'Virtuoso' video frame-grabber (Prima Graphics, Royston, UK) was used to capture and analyse images from a Sony AVC-D5CE video camera (Sony Corporation, Tokyo, Japan).

Preparation of parenchyma and sclerenchyma cell walls

Cell walls from anatomically defined cell types free from cell contents were obtained using only mechanical methods as described previously for a number of plant materials (McCluskey *et al.* 1984; Gordon *et al.* 1985; Grabber & Jung 1991).

Each of the four segments from each of four plants were separately dissected into three parts: a central pith, a 'middle' layer and an outer rind (epidermis plus sclerenchyma). The pith was obtained by scraping the inner surface of the opened segment with the round end of a spatula. The material removed consisted mainly of parenchyma with some vascular tissue. Since parenchyma was more readily disrupted than the vascular tissue, the pith was manually ground in a mortar and pestle and then washed through a sieve (300 μm mesh). Retained material, largely vascular material and adherent parenchyma, was returned to the mortar and reground, the process being repeated 5–7 times. Material passing through the 300 μm mesh was pooled, passed again through the 300 μm mesh and collected on a 20 μm mesh. After microscopic examination for homogeneity the parenchyma fraction was freeze-dried.

Further scraping to remove the 'middle' layer, which also consisted of vascular tissue and parenchyma but with a substantially greater proportion of the former, left an epidermis and sclerenchyma-enriched material. A sclerenchyma-enriched fraction was obtained from this material by scraping the inner surface of the rind with a scalpel blade. The material recovered in this way was homogenized in a blender for 30 s in iced water and passed through a 300 μm mesh. Material passing through the mesh was discarded. Retained material was transferred to a mortar and pestle, hand-ground for 2 min and again passed though the 300 μm mesh. The process was repeated until microscopic examination showed that the material retained was virtually a pure preparation of sclerenchyma fibres. Samples were then freeze-dried.

The yield of sclerenchyma and parenchyma from the younger (lower) two segments of individual plants was judged to be too low to meet the needs of all of the intended assays. Accordingly cell types from corresponding sections of two plants were pooled.

Chemical analysis

The total phenolic content of the cell-wall preparations and their digested residues was determined by the acetyl bromide method (Morrison 1972) using ferulic acid as the standard. Saponifiable phenolic acids released by treatment with 1 M NaOH overnight at room temperature were separated, identified and measured by HPLC on a C_{18} RP silica column as described by Hartley & Buchan (1979) except that the peaks were eluted with water:acetic acid:*n*-butanol (983:12:5 ml litre^{-1}) at a flow rate of 1·5 ml min^{-1}. The lignin content of the walls was calculated by subtracting the total amount of saponifiable phenolic acids and phenolic acid dimers from the total phenolic value.

Measurement of degradability

A modified 'cellulase' enzyme assay system based on that originally described by Dowman & Collins (1982) but adapted to handle the very small amounts of cell wall material available was used to measure degradability of cell-wall samples. The major difference between the present method and those previously described was that degradation was assessed by measuring the amount of cell-wall carbohydrate released rather than the loss of dry matter.

A buffered cellulase solution was prepared by dissolving 750 mg of a commercial enzyme preparation (Fluka cellulase ex *Trichoderma reesei*) in water, dialysing overnight against running water in the cold (4°C) and making up to 250 ml. This was mixed with an equal volume of a pH 4·8 buffer solution (2·72 g sodium acetate trihydrate, 1·2 ml glacial acetic acid) containing 200 mg chloramphenicol litre^{-1}. The cellulase solution (5 ml) was added to a known weight of cell walls (approximately 5 mg) in a stoppered tube. To ensure rapid hydration of the samples, each tube was centrifuged at 3000 ***g*** for 5 min and then mixed to resuspend the pellet. The tubes were then incubated in a water-bath at 40°C for 72 h. Tubes were sampled after 2, 4, 6, 10, 14, 18, 24, 48 and 72 h. At each sampling time tubes were removed from the bath, centrifuged at 3000 ***g*** to pellet the residual cell wall and 0·5 ml of supernatant were removed for assay. Fresh cellulase solution (0·5 ml) was added to replace that removed and the tubes were then mixed to resuspend the solid material and returned to the water-bath. The total sugar content of the samples or appropriate dilutions was determined by the phenol–sulphuric acid method (Dubois *et al.* 1956) using a standard sugar solution containing glucose and xylose in approximately the same proportion (7:3 w/w) as that found in maize cell walls (Scobbie *et al.* 1993). Carbohydrate released within a given time was calculated to include that contained in the supernatant at

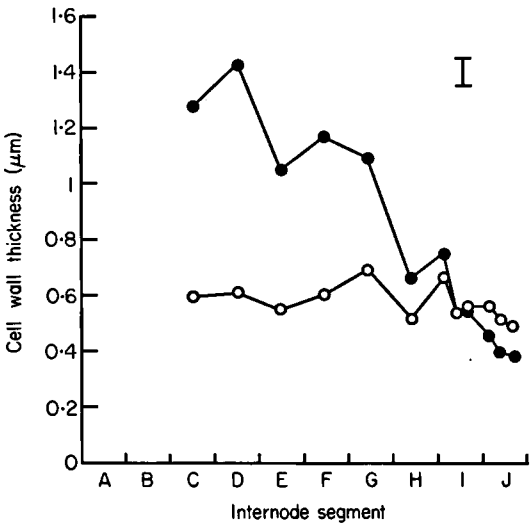

Fig. 2. Changes in the mean thickness of parenchyma (○) and sclerenchyma (●) cell walls with age measured by image analysis of light microscopic sections cut from a newly extended maize internode. The vertical bar represents the least significant difference (LSD, $P=0{\cdot}05$, df = 46).

the time of sampling plus the sugar contained in all previous samples minus the sugar content of 5 ml of cellulase solution. Total released sugar was expressed as a percentage of cell-wall dry matter and the rate and extent of degradation calculated by fitting an exponential curve of the form:

$$y=a+be^{-ct}$$

where the release of sugar (y) decreases exponentially over time (t). A separate curve was fitted for each sample and analysis of variance was used to investigate differences in the asymptote (a), which represents maximum degradability, and the rate constant (c) for each tissue type at different positions along the internode.

Experimental design

Measurements were made using a randomized block experimental design in which blocks represented individual plants. Analysis of variance was used to assess differences between the tissue types and where appropriate, the residual mean square from the analysis of variance was used to calculate the least significant difference (LSD) between sample means.

RESULTS

Image analysis applied to the sections cut from various points along internode 1 confirmed that the mean cell-wall thickness of parenchyma cells remained unchanged as the tissue aged (Fig. 2). As expected, the mean thickness of sclerenchyma walls increased with age from approximately 0·4 μm near the intercalary meristem (segment J1) to 1·4–1·5 μm in sections from the upper quarter of the internode. Estimates of mean cell radius made from the skeletonized image showed that, while the mean sclerenchyma cell diameter did not increase with age, there was a substantial increase in the area occupied by the cell wall

Table 1. The total phenolic, saponifiable phenolic acid and lignin content (g kg^{-1} dry matter) of parenchyma and sclerenchyma cell walls isolated from segments of a newly-extended maize internode representing different developmental stages. Internodes were divided into four equal lengths with segment 2A being the oldest (top) part. The residual mean square obtained by analysis of variance was used to calculate the least significant difference (LSD, $P = 0{\cdot}05$, df = 7)

	Total phenolics	*p*-Coumaric acid	Ferulic acid	Total phenolic acids	Lignin
Parenchyma					
2A	122·2	30·7	4·6	35·3	86·9
2B	113·9	29·4	4·4	33·8	80·0
2C	86·8	20·8	4·3	25·2	61·7
2D	64·1	10·2	5·8	16·0	48·1
Sclerenchyma					
2A	138·2	24·7	5·1	29·9	108·4
2B	134·9	24·6	5·5	30·2	104·7
2C	111·9	20·7	5·9	26·6	85·3
2D	76·4	12·4	6·5	18·9	57·4
(LSD)	(6·53)	(4·09)	(0·64)	(4·49)	(10·19)

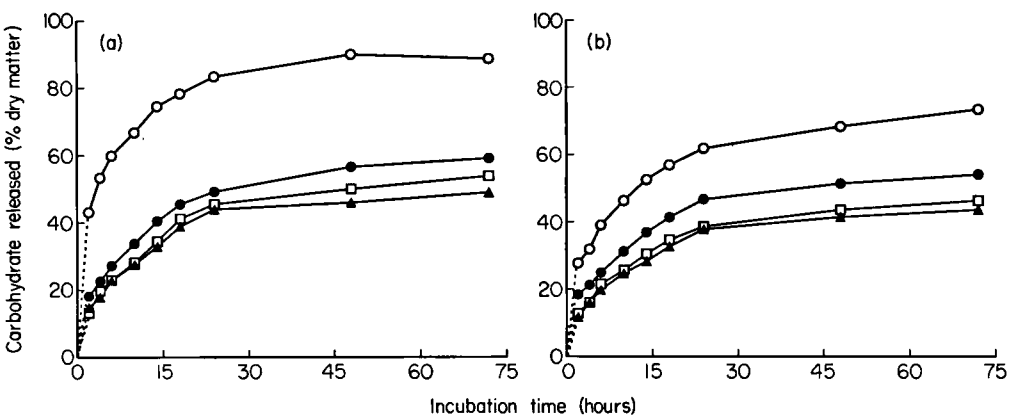

Fig. 3. The degradability of parenchyma (a) and sclerenchyma (b) cell walls isolated at different developmental stages from segments A (top, ▲), B (□), C (●) and D (base, ○) of a newly extended internode. Degradability was measured using a 'cellulase' enzyme preparation. Released carbohydrate is expressed as a percentage of cell-wall dry matter.

within the tissue area. This was consistent with cell-wall expansion occurring at the expense of the cell lumen which steadily decreased in area as the width of the wall increased.

The total phenolic and lignin content of the sclerenchyma walls was greater than that of the parenchyma walls at all stages of development (Table 1). However even in parenchyma cells isolated from the youngest part of the internode (segment 2D) there was an appreciable amount of lignin present. The extent of lignification increased with age in both cell types reaching a maximum within segment 2B. Thereafter there was little detectable

Table 2. The maximum degradability (% total dry matter) and degradation rate of parenchyma and sclerenchyma cells isolated from segments of a newly extended maize internode. A separate exponential curve was fitted for each of two replicate samples from segment 2A (top) to 2D (bottom) of the internodes. The residual mean square obtained by analysis of variance was used to calculate the least significant difference (LSD, $P=0{\cdot}05$, df = 7)

Segment	Degradability (%)	Degradation rate
Parenchyma		
2A	49·0	0·0728
2B	53·9	0·0683
2C	59·1	0·0650
2D	89·3	0·0931
Sclerenchyma		
2A	43·7	0·0673
2B	46·7	0·0667
2C	54·6	0·0646
2D	72·2	0·0669
(LSD)	(7·00)	(0·0244)

Table 3. The total phenolic content of parenchyma and sclerenchyma cell-wall residues recovered after incubation for 72 h with a 'cellulase' enzyme preparation

	Total phenolics ($g\ kg^{-1}$ dry matter)	
Segment	Parenchyma	Sclerenchyma
2A	129·5	135·0
2B	129·5	126·6
2C	120·6	113·2

change in the amount or nature of the phenolics present. Cells from segments 2B and 2A did not differ significantly in their total phenolic or lignin content. The saponifiable phenolic acids, (*E*+*Z*)-*p*-coumaric and (*E*+*Z*)-ferulic acid were present in all cell walls examined although the ratio of the two acids differed with age. Levels of ferulate were essentially constant in all samples while the level of (*E*)-*p*-coumaric acid increased 2–3-fold with age and in parallel with increased lignification.

The degradability of both cell types was reduced with age and increasing lignification, with sclerenchyma walls exhibiting a lower extent of degradation than parenchyma cell walls isolated from corresponding sections (Fig. 3a and b). Lignin content provided a guide to the extent to which cells were degraded by the cellulase preparations with the response of both cell types being statistically indistinguishable. Of particular significance

was the observation that, with a single exception, the rate constant for cell-wall degradation, calculated from curves fitted to the data from 2 to 72 h, was independent of both age and cell type. Only parenchyma cell walls prepared from the youngest part of the internode (segment 2D) deviated from this pattern. The rate constant of the fitted degradation curve for young parenchyma walls was significantly different ($P > 0{\cdot}10$) from other samples. Maximum degradability and degradation rates are summarized in Table 2. The total phenolic content of the residues remaining after cellulase digestion of the more extensively lignified cells prepared from segments 2A and 2B was little changed from that originally present (Table 3). Only the residue from parenchyma cells from segment 2C, whose phenolic content approached that of the residues of cells from the older segments, had an increased phenolic content. Insufficient material was recovered from digests of the youngest cells (ex segment D) to allow their phenolic content to be determined.

DISCUSSION

Lignification of sclerenchyma cells was accompanied by the deposition of secondary cell wall with a consequent threefold increase in cell-wall thickness. In mature sclerenchyma, the secondary wall layer represented approximately two-thirds of the mass of the wall and was the only layer exposed to microbial (enzymatic) attack. The walls of parenchyma cells, in contrast, exhibited a similar thickness at all stages of development and, even when fully mature, were only marginally thicker than the walls of the meristem cells from which they were derived. Deposition of secondary wall was not evident at a microscopic level and could only have made a minor contribution to the mass of the mature parenchyma wall. As a result, the substrate for microbial (enzymatic) attack in mature parenchyma was the lignified primary wall.

It has been argued on the basis of histological observations that, in mature tissue, the lignified primary wall is inherently more resistant to microbial (enzymatic) attack than the secondary wall layer and that this resistance is a major factor determining the overall digestibility of forages and crop residues fed to ruminants (Cone & Engels 1990; Engels & Schuurmans 1992). On this basis mature parenchymatous walls would be expected to be substantially less degradable than secondary-thickened sclerenchyma walls with a comparable lignin content. This was not found to be the case. With a single exception the rate of degradation did not significantly differ with age or cell type (Table 2) and the extent of degradation was commonly determined by the overall lignin content. Only parenchyma cells isolated from segment 2D, the youngest portion of the internode, deviated from this pattern. Segment 2D contains the intercalary meristem and is the region of greatest developmental change (Scobbie *et al.* 1993). As a result cells taken from this segment were probably far more heterogenous with respect to their lignin contents than cells taken from the upper (older) segments. The extended initial linear rate of degradation shown in Fig. 3a, indicative of the presence of a substantial amount of highly degradable unlignified primary walls (Chesson *et al.* 1986), would support this view.

Comparison of the total phenolic content of the walls before and after extended incubation with the 'cellulase' enzyme preparation (Tables 1 and 3) showed that there was little or no accumulation of phenolic material during degradation of the more mature cells from segments A and B. This observation does not support the hypothesis that degradation occurs against a lignin concentration gradient (Jung & Deetz 1993) or, in the case of sclerenchyma, that degradation is halted by a highly lignified primary wall layer once an overlying but less lignified secondary wall layer has been removed (Engels & Schuurmans

1992). It is, however consistent with the concept of the development of a lignin-enriched inert layer at the cell-wall surface during attack as previously suggested (Chesson 1988; 1993). This predicts that walls have a common rate of degradation since attack is restricted to the cell-wall surface, that the *overall* lignin content (as opposed to the lignin content of the cell-wall surface) would be little increased during degradation and that the amount of lignin initially present in the wall would determine the rate at which the inert surface develops and thus the ultimate extent of degradation.

Histological evidence suggests that lignification of the primary wall occurs concurrently with secondary wall formation but in advance of secondary wall lignification. At this stage of wall development the secondary wall would be readily degradable and any material recovered after extended degradation would consist of residues from the more extensively lignified primary wall layer as observed by Engels & Schuurmans (1992). However, once lignification of the secondary wall layer is complete, the concept of a selectively resistant primary wall/wall layer becomes much more difficult to sustain in the light of the present results. A more plausible explanation of the microscopic observations, which does not have to invoke differences in the lignification process, is that lignified primary walls are partially degradable unless protected by a lignified secondary wall layer which is rarely, if ever, fully degraded. This explanation allows for a lignified primary wall layer embedded in a secondary-thickened sclerenchyma wall to remain intact after digestion since it is not exposed to microbial attack. In contrast, lignified primary walls from parenchyma cells which have not undergone secondary-thickening are exposed to attack and are degraded to an extent determined by their lignin content.

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