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The relationship between *in vitro* enzymatic digestibility of cell walls of wheat internodes and compositional changes during maturation

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SUMMARY

Analyses of internodes from wheat taken at various times during maturation, and determination of their *in vitro* dry matter digestibility confirmed that the decrease in digestibility could be attributed both to a progressive loss in cell contents and also to a marked decrease in cell-wall digestibility. The latter was not easily explained by the changes in the proportion of the major wall components (cellulose, arabinoxylan and lignin), but may be explained in terms of the physical and chemical associations between wall polymers. Among these, covalent cross-linking and bridging by hydroxycinnamic acids are important candidates.

Key-words: cell walls, chemical composition, cross-linking, digestibility, ferulic acid bridge, internodes, *Triticum aestivum*

INTRODUCTION

It is well-known that the digestibility of walls of forage plants by rumen microorganisms decreases as they mature (Deinum 1976), the changes in their chemical composition during maturation are also well-investigated (Nevins *et al.* 1968; Deinum 1976; Cherney & Marten 1982; Nandra *et al.* 1983; Volenec *et al.* 1986). Analyses of the relationship between chemical composition and digestibility (see Minson 1976 for a summary) have led to the conclusion that the lignin (Richards 1976; Fahey & Jung 1983) and phenolic acid (Hartley 1972; Jung & Fahey 1983) constituents of the wall are most important in determining digestibility.

In this paper, changes in chemical composition and *in vitro* dry matter digestibility (IVDMD) of cell walls of internodes from two series of wheat plants are reported as a function of their maturity. Changes in the crystallinity of cellulose in the walls were also determined.

MATERIALS AND METHODS

Sample 1 (S-series)

First internodes from maturing wheat (*Triticum aestivum* L. cv. Millewa) plants grown at the Mt Derrimut Field Station of the University of Melbourne in the 1987 season were

This paper is dedicated to Professor Dr M. M. A. Sassen on the occasion of his retirement.

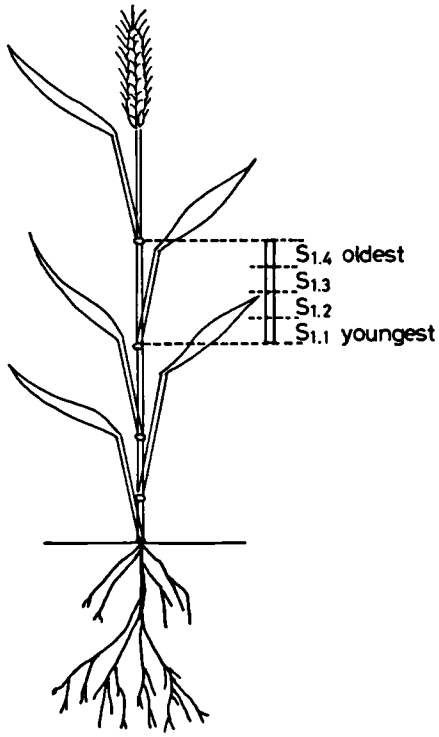

Fig. 1. Sampling parts from wheat plant for S-series.

harvested 138 days after sowing (just after anthesis) and cut into four segments as shown in Fig. 1. The pooled segments were ground in a Wiley mill to pass a 420 μm sieve ($SO_{1.1}$–$SO_{1.4}$), and extracted as described by Lam *et al.* (1990b) (Fig. 2). The extracted material was designated as ($S_{1.1}$–$S_{1.4}$). The residues after *in vitro* enzymatic digestion ($D_{1.1}$–$D_{1.4}$) were also analysed.

Sample 2 (H-series)

Second internodes of wheat plants were harvested at 25, 32, 49, 60 and 123 days after sowing at the Mt Derrimut Field Station of the University of Melbourne in the 1986 season. The internodes from each harvest were pooled, milled, and extracted as described above. The extracted materials were designated H_{25}, H_{32}, H_{49}, H_{60} and H_{123}, respectively.

Cell-wall fractions

Internode walls were fractionated as described by Lam *et al.* (1990b) to give, after extensive ball-milling, a 96% dioxane–water soluble fraction (Björkman lignin, ML), a water extractable fraction (MC) from the residue of the dioxane–water extracted ML fraction, and a residue (ER) obtained by treating the residue after extraction of the MC fraction with a polysaccharide hydrolase (Meicelase PS) mixture.

Cell-wall analyses

Lignin was determined by the acetyl bromide procedure (Iiyama & Wallis 1990) using 20·0 g^{-1} litre cm^{-1} as the value of the specific absorption coefficient of lignin. The extracted internodes (S and H series) were hydrolysed with sulphuric acid by the method

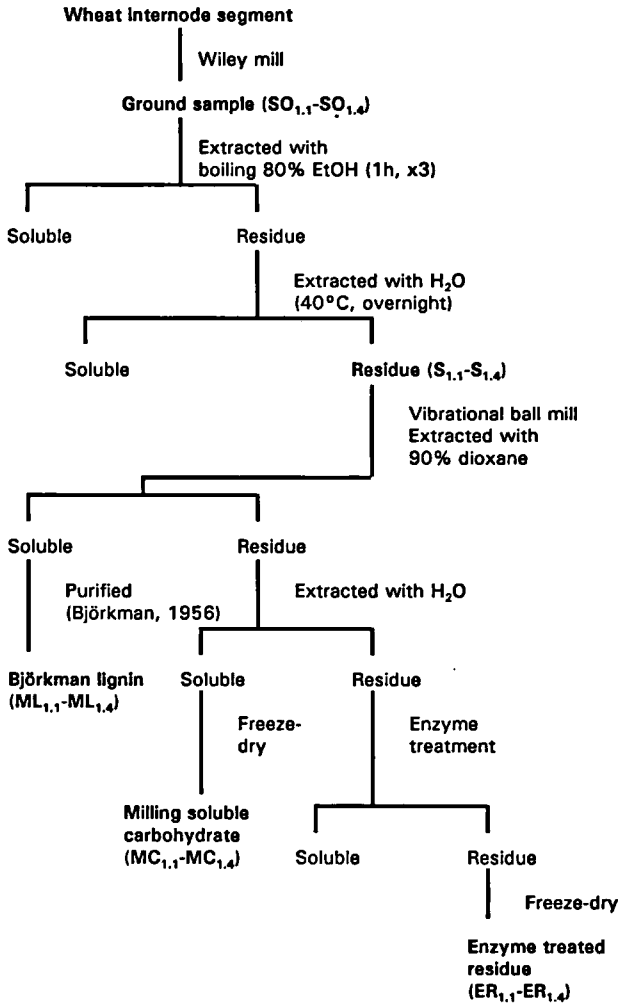

Fig. 2. Fractionation of first internode segments (S-series) of wheat.

of Blakeney *et al.* (1983). Neutral sugars in the boiling 5% sulphuric acid hydrolysate were analysed as their alditol acetates (Blakeney *et al.* 1983). The uronic acids in the sulphuric acid hydrolysate were determined by the method of Ralph (1979). Alkaline nitrobenzene oxidations (Iiyama & Lam 1990) and analyses of both esterified (Lam *et al.* 1990c) and etherified (Iiyama *et al.* 1990) hydroxycinnamic acids were performed using the procedures described in the papers cited. Protein content was calculated as 6·25 × percentage of nitrogen determined by elementary analysis. All analyses were duplicated.

Digestibility determination

The *in vitro* dry matter digestibility of the samples was determined by the procedure of McLeod & Minson (1978) using a porcine pepsin (Sigma Chemicals Co. Ltd, USA) and a commercial broad spectrum cellulase (Onozuka-SS (P1500), from *Trichoderma viride*, Yakult Co. Ltd, Japan).

Table 1. Chemical composition of fractions of first inernode segments (S-series) of wheat plants

Sample	Yield % of ODM	Neutral sugar, % of sample					Protein % of sample	Uronic acid % of sample	Lignin % of sample
		Ara	Xyl	Gal	Glc	Total			
Extracted segment									
$S_{1\cdot1}$	41·0	3·1	13·9	—	24·7	41·7	37·6	0·9	11·5
$S_{1\cdot2}$	56·6	2·4	22·6	—	34·7	59·7	11·5	1·7	13·4
$S_{1\cdot3}$	61·3	2·1	25·2	—	36·9	64·2	8·7	1·1	14·7
$S_{1\cdot4}$	67·2	2·2	25·5	—	34·1	61·8	10·2	1·3	16·2
Björkman lignin fraction									
$ML_{1\cdot1}$	0·1	0·9	7·6	—	1·0	9·5	15·6	nd	59·0
$ML_{1\cdot2}$	0·6	1·2	14·2	—	0·9	16·3	4·6	nd	69·3
$ML_{1\cdot4}$	0·7	0·9	9·4	—	0·3	10·6	2·4	nd	79·8
Water soluble fractions of finely ground meal after Björkman lignin extraction									
$MC_{1\cdot1}$	15·3	6·4	30·3	1·0	16·7	54·4	27·5	nd	13·3
$MC_{1\cdot2}$	11·8	4·6	38·3	2·0	22·5	67·4	8·8	nd	15·4
$MC_{1\cdot3}$	7·4	4·7	36·0	1·2	19·7	61·6	8·1	nd	15·3
$MC_{1\cdot4}$	7·9	4·9	41·9	1·2	16·7	64·7	7·4	nd	14·2
Cellulase hydrolysis residue of finely ground meal after MC fraction extraction									
$ER_{1\cdot1}$	4·8	0·6	1·6	—	2·1	4·3	70·8	nd	17·2
$ER_{1\cdot2}$	6·6	0·7	6·8	—	5·5	13·0	37·6	nd	33·4
$ER_{1\cdot3}$	12·5	1·5	9·5	—	5·8	16·8	28·5	nd	36·6
$ER_{1\cdot4}$	14·0	1·4	9·7	—	5·4	16·5	27·1	nd	39·7
Pepsin–cellulase digestion residue of extracted first internode meal ($S_{1\cdot1}$–$S_{1\cdot4}$)									
$D_{1\cdot1}$	6·2*	nd	nd	nd	nd	nd	nd	nd	nd
$D_{1\cdot2}$	23·5*	1·1	26·3	—	41·7	70·0	nd	1·3	19·9
$D_{1\cdot3}$	41·9*	1·2	28·4	—	40·5	70·7	nd	1·2	21·1
$D_{1\cdot4}$	44·2*	1·1	26·7	—	45·1	73·4	nd	1·7	22·1

nd: Not determined, —: not detected. All data are averages of duplicate runs. *: Yield based on original dry matter (ODM).

Crystallinity determination

The X-ray diffractograms of pellets of extracted internode segments, ground to pass a 420 μm sieve, were recorded on a JOEL X-ray Diffractometer DX-CRSC2 (Tokyo, Japan) equipped with a reflection type goniometer, using Ni-filtered CuK_{α} radiation (Akishima *et al.* 1992). The crystallinity was calculated after correcting for scattering from amorphous cellulose (Segal *et al.* 1959). Amorphous cellulose was prepared by vibrational ball milling of cotton linters.

RESULTS AND DISCUSSION

Changes in chemical composition of wheat internode walls during plant maturation

The chemical compositions of the extracted residues of maturing first internode segments (S-series), their fractions (ML-, MC- and ER-series) and the residues after digestion with pepsin-'cellulase' (D-series), are shown in Table 1. Similar analyses for second internodes

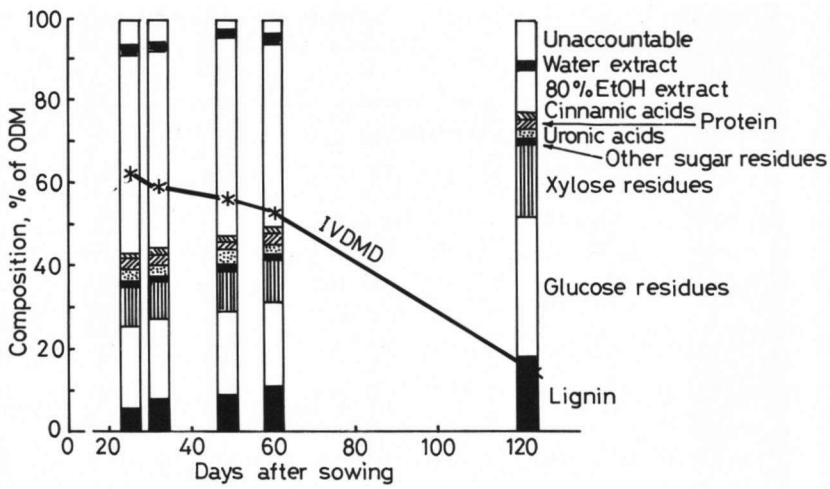

Fig. 3. Chemical composition and *in vitro* dry matter digestibility of second internodes harvested at different stages of development of wheat plants (H-series).

harvested at different stages of development (H-series) are shown in Fig. 3. The percentage recovery of organic material was between 82% and 100%. Ash was not determined. It has been shown previously (Lam *et al.* 1990b) that the increase in lignin of the S-series was smaller than that reported by Stone *et al.* (1951) and Brice & Morrison (1982) for maturing wheat plants and Italian ryegrass, respectively. The increase in lignin content in the H-series was also smaller than observed in the earlier studies, but larger than those of the S-series.

Xylose and glucose, were the major neutral monosaccharides released from the extracted internodes by acid hydrolysis. In the S-series their concentration increased gradually with maturation. However, no significant changes were observed during maturation in the H-series. As reported by Buchala & Wilkie (1973), galactose was always a minor component. Arabinose was present in small but significant amounts. The ratio of arabinose to xylose decreased during maturation. Similar observations have been made for wheat straw (Buchala & Wilkie 1973), for ryegrass (Morrison 1974), and for sorghum (Nandra *et al.* 1981).

Monosaccharides in the ML (Björkman lignin fraction) and the water-extractable (MC) fractions

Ball-milling of the 80% ethanol and water extracted internodes enables dioxane–water to extract a fraction of the wall lignin (ML). Treatment of the dioxane–water extract by successive precipitation and solution in non-polar and polar solvents, respectively, yields a purified Björkman lignin. This lignin accounts for only 0·1% of the extracted wall and is associated with some polysaccharide in which xylose is the predominant monosaccharide. Because the Björkman lignin is soluble in the polar 1,2-dichloroethane–ethanol and is then precipitated by diethyl ether, the polysaccharide may be assumed to be covalently associated with the lignin. Ball-milling also allows the extraction by water of a quantitatively important fraction (MC) which is rich in polysaccharide (again with xylose as the predominant monosaccharide) and also contains a significant amount (10–43%) of the

Table 2. Alkaline nitrobenzene oxidation of extracted second internodes of wheat harvested after different periods of development (H-series)

Sample	Total yield of products: Based on sample, %	Total yield of products: Based on lignin, %	*S/V**	*H/V*‡
H_{25}	2·55	19·2	0·68	0·00
H_{32}	3·75	21·0	0·71	0·00
H_{49}	4·25	21·4	0·82	0·00
H_{60}	4·55	22·0	1·02	0·00
H_{123}	5·69	26·5	1·13	0·00

* and ‡: The ratios of syringyl nuclei to guaiacyl nuclei and *p*-hydroxyphenyl nuclei to guaiacyl nuclei, respectively, are corrected for oxidation products from hydroxycinnamic acids by the previously described procedure (Iiyama & Lam 1990). All data are averages of duplicates.

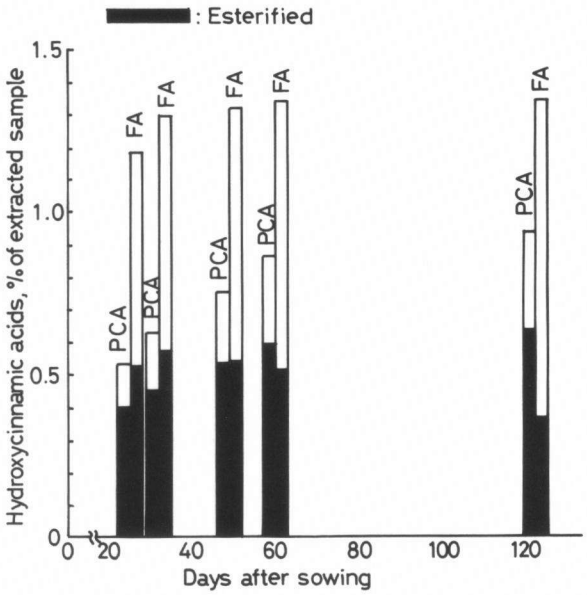

Fig. 4. Total and esterified (black) hydroxycinnamic acids of second internodes harvested at different stages of development of wheat plants (H-series).

wall lignin (Lam *et al.* 1990b). Again this suggests that the hydrophobic lignin is being brought into solution through its association with the hydrophilic polysaccharide.

Monomeric constitution of lignin and wall hydroxycinnamic acids

We have reported previously on the change, during maturation, in the monomeric constitution of internode lignin as determined by alkaline nitrobenzene oxidation (Lam *et al.* 1990b) and on the hydroxycinnamic acid content of the internodes (Lam *et al.* 1990c) of the S-series. The data for the H-series are shown in Table 2 and Fig. 4, respectively. The

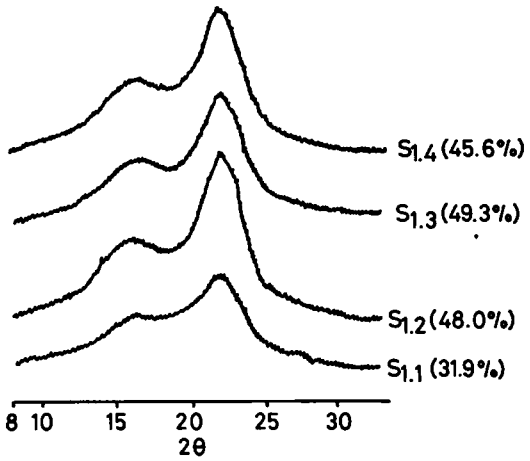

Fig. 5. Change in cellulose crystallinity of first internode segments during the maturation of wheat plant. Data in parentheses: % crystallinity.

total yield of alkaline nitrobenzene oxidation products, based on lignin, and the ratio of syringyl to guaiacyl nuclei (*S/V* value) in the H-series internodes increased significantly during maturation (Table 2). This is presumably a consequence of the changes in the monomeric composition of the lignin deposited during maturation as reported and discussed by Terashima and Fukushima (1988). The changes in hydroxycinnamic acid composition in the H-series (Fig. 4) were similar to those for the S-series (Lam *et al.* 1990c). The total ferulic acid, determined by alkaline hydrolysis at 170°C, remained constant during plant maturation, however the concentration of ferulic acid released by mild alkaline treatment decreased significantly, which is consistent with an increase in the proportion of ether–ester ferulic acid (Lam *et al.* 1992a). On the other hand the total concentration of *p*-coumaric acid and its esterified form increased steadily during maturation.

Relationship between IVDMD, chemical composition and crystallinity of cellulose in maturing internodes of wheat plants

The IVDMD of the original unextracted wheat internode segments (SO-series) decreased from 94% ($SO_{1\cdot1}$, youngest) to 56% ($SO_{1\cdot4}$, oldest) during the maturation of the wheat plant. This change is closely paralleled by the decrease in the protein and total extract contents of the sample. A similar result was observed in the H-series (Fig. 3). Pearce (1984) reported that decreases in the *in vitro* digestibility of wheat internodes taken 5 weeks before and 5 weeks after grain harvest, are correlated with decreases in the proportion of cell contents. In the pasture plants, Italian ryegrass (*Lolium multiflorum*), perennial ryegrass (*L. perenne*), white clover (*Trifolium repens*), red clover (*T. pratense*) and lucerne (*Medicago sativa*) the digestibility of plant parts was strongly correlated with the proportion of cell contents (Wilman & Altimimi 1982, 1984).

Although the IVDMD of the extracted residue (S-series) decreased dramatically between $S_{1\cdot2}$ (76%) and $S_{1\cdot3}$ (58%) (Table 3), smaller changes were observed in the same period in the content of lignin ($S_{1\cdot2}$ 13·4%–$S_{1\cdot3}$ 14·7%), neutral sugars from polysaccharides (Table 1), hydroxycinnamic acid content (Lam *et al.* 1990c) and the monomeric

Table 3. IVDMD of original dry matter and wall polysaccharides, and lignin-free, xylan-free and lignin–xylan-free digestibilities of first internode segment (S-series) of wheat

	Segment			
	$S_{1\cdot1}$	$S_{1\cdot2}$	$S_{1\cdot3}$	$S_{1\cdot4}$
IVDMD of original dry matter	94	76	58	56
IVDMD of neutral sugars				
Arabinose	—	89	75	74
Xylose	—	73	53	51
Glucose	—	72	54	52
Total	—	72	54	52
IVDMD of uronic acid	—	79	58	55
Lignin-free digestibility	—	62	37	36

'Lignin-free' digestibility was calculated on the basis of the loss in weight of the components in the original 'S-' and 'D-' series internode material other than lignin. 'Xylan-free' and 'lignin-xylan-free' digestibilities were similarly calculated.

constituent of lignin (Lam *et al.* 1990b). The small, but significantly lower 'digestibility' of xylose residues, the main sugar residues in the non-cellulosic polysaccharides, than the IVDMD of the original dry matter (Table 3) may relate to their high degree of substitution with acetyl groups and hydroxycinnamic acids. However, this cannot account for the large change in the IVDMD of original dry matter between the $S_{1\cdot2}$ and $S_{1\cdot3}$ stages since there is only a small increase in xylose content between $S_{1\cdot2}$ (22·6%) and $S_{1\cdot3}$ (25·2%) (Table 1). Similar observations were made for the H-series (Figs 3 and 4).

The crystallinity of cellulose in extracted residues of internodes of the S-series changed in the $S_{1\cdot1}$–$S_{1\cdot2}$ stage, but thereafter remained fairly constant (Fig. 5). The glucose content increased in the $S_{1\cdot1}$–$S_{1\cdot2}$ period suggesting that crystalline cellulose was being deposited presumably in secondary walls. Although the crystallinity of the cellulose remained virtually constant after the $S_{1\cdot2}$ stage, the IVDMD changed continuously in that period (see Table 1 and Fig. 3), supporting the suggestion (Beveridge & Richards 1975; Puri & Pearce 1986) that the crystalline form of cellulose in the wall is not a factor in determining IVDMD. However, other workers have suggested that the effect of cellulose content on digestibility may be related to its crystallinity (Dekker & Richards 1972; van Soest *et al.* 1978; Morrison 1974; Ford 1983; Burritt *et al.* 1985).

Thus digestibility of whole plants is a function of the ratio of easily digestible cell contents to cell wall and this decreases as the plants mature and senesce. When lignin was excluded from the components by calculation as an indigestible material, the digestibility of the components other than lignin ('lignin-free' digestibility) was lower than IVDMD of whole wheat internode segments (Table 3), suggesting that the changes in digestibility of the walls themselves are not easily accounted for simply in terms of changes in the proportion of the major polymeric components. The chemical associations and physical inter-relationships of these polymers also becomes important. Among the proposed

interactions are cross-linking between polysaccharides by oxidative dimerization of ferulic acid residues that are esterified to polysaccharides to form dehydrodiferulic acid bridges (Hartley & Jones 1976; Markwalder & Neukom 1976; Fry 1986), direct lignin–polysaccharide linkages (see Lam *et al.* 1990a) and physical protection by lignin (Ford 1983). Phenolic acids are also good candidates for bridging between wall polymers since they are bifunctional molecules having both a phenolic hydroxyl group and a carboxyl group (Karr & Albersheim 1970; Brice & Morrison 1982). The presence of phenolic acids in the ether linkage to lignin was detected in wheat straw (Scalbert *et al.* 1985; Iiyama *et al.* 1990), subsequently ether-linked ferulic acid was found in rice stems (Sharma *et al.* 1986) and phalaris (*Phalaris aquatica*) (Lam *et al.* 1992a), supporting the possibility of a bridge between lignin and polysaccharides through ester- and ether-linked ferulic acid. Recently we have demonstrated directly the presence of ferulic acid bridges between wall polymers in dioxane-soluble fractions of wheat and phalaris (Lam *et al.* 1992a), and that dehydrodiferulic acid also occurs in the diester–ether linkage in wheat and phalaris internode walls (Lam *et al.* 1992b).

Increases in the physical and chemical associations between wall polymers especially between polysaccharides and lignin may provide the best explanation of observed changes in IVDMD with maturity. Such explanations need to be able to account for observations such as the significant improvement of IVDMD with alkaline treatment of grasses (Morrison 1991). In this respect the ferulic acid bridges between wall polymers involving alkali-labile ester linkages would be expected to be one of the most important associations affecting rumen digestibility. The observed improvement of digestibility of forage grasses after treatment with alkaline or alkaliogenic reagents (Morrison 1991) is easily reconciled with the presence of alkali-labile ferulic acid bridges.

The current model for lignified cell walls (Northcote 1972) suggests that lignin is infiltrated into the spaces, previously occupied by water, i.e. between the molecules of non-cellulosic polysaccharides, in the middle lamella, primary and secondary walls. As suggested by Lam *et al.* (1985) for beech (*Fagus crenata*) wood walls and by Ford (1983) for pangola grass (*Digitaria decumbens*) stems, the infiltrated, hydrophobic lignin overlying these polysaccharides, would be an effective barrier to the approach of hydrolytic enzymes to their polysaccharide substrates. If in addition, lignin is covalently bridged to wall polysaccharides, then the interfacial association between the lignin and polysaccharides will make the association resistant to the swelling action of aqueous solvents. Thus, the ferulic acid bridges and other covalent associations will be important stabilizing forces in the three-dimensional lignified wall and contribute to its resistance to microbial attack.

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