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Severe Inhibition of Maize Wall Degradation by Synthetic Lignins Formed with Coniferaldehyde†

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Abstract: Although the enzymatic or ruminal degradability of plants deficient in cinnamyl alcohol dehydrogenase (CAD) is often greater than their normal counterparts, factors responsible for these degradability differences have not been identified. Since lignins in CAD deficient plants often contain elevated concentrations of aldehydes, we used a cell-wall model system to evaluate what effect aldehyde-containing lignins have on the hydrolysis of cell walls by fungal enzymes. Varying ratios of coniferaldehyde and coniferyl alcohol were polymerised into non-lignified primary walls of maize (*Zea mays* L) by wall-bound peroxidase and exogenously supplied H_2O_2. Coniferaldehyde lignins formed fewer cross-linked structures with other wall components, but they were much more inhibitory to cell wall degradation than lignins formed with coniferyl alcohol. This suggests that the improved degradability of CAD deficient plants is not related to the incorporation of *p*-hydroxycinnamaldehyde units into lignin. Degradability differences were diminished if enzyme loadings were increased and if hydrophobic aldehyde groups in lignins were reduced to their corresponding alcohols by ethanolic sodium borohydride. © 1998 Society of Chemical Industry.

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Key words: *Zea mays*; brown midrib; cinnamyl alcohol dehydrogenase; coniferyl alcohol; coniferaldehyde; quinone methide; cross-linking; lignin; cell wall; degradability

INTRODUCTION

CAD catalyses the reduction of *p*-hydroxycinnamaldehydes **1** to their corresponding alcohols **2**, the immediate precursors of lignin in plants (Fig 1). CAD-deficient plants (eg bm_1 maize, *bmr*-6 sorghum and CAD antisence tobacco) are of considerable interest because their enzymatic or ruminal cell-wall degradability can be up to 50% greater than their normal counterparts (Thorstensson *et al* 1992; Bernard-Vailhe *et al* 1996; Provan *et al* 1997). As may be expected, lignins in CAD deficient plants often contain higher concentrations of *p*-hydroxycinnamaldehyde units, but other modifications occur as well and these may include reduced deposition of *p*-coumarate lignin esters, reduced deposition of guaiacyl or syringyl lignins, increased deposition of unusual lignin units and deposition of unusual aldehydes into lignins or cell walls (Pillonel *et al* 1991; Halpin *et al* 1994; Hibino *et al* 1995; Provan *et al* 1997; Ralph *et al* 1997). Elucidation of factors contributing to improved cell-wall degradability of CAD deficient plants would enhance plant selection or molecular engineering efforts aimed at improving the utilization of fibrous crops as feedstuffs for livestock and as feedstocks for industrial purposes. Recent studies with dehydrogenation polymer–cell wall (DHP–CW) complexes revealed that the degradability of structural polysaccharides are not affected by altering the proportions of *p*-hydroxyphenyl, guaiacyl and syringyl units in lignin (Grabber *et al* 1997). In the present study, we used this model system to evaluate what effect incorporation of coniferaldehyde into lignin has on cell wall degradability and on other cell wall properties.

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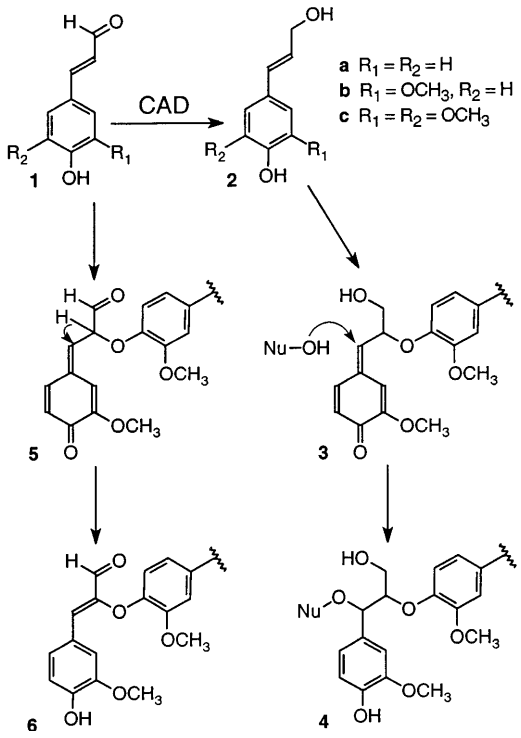

Fig 1. During lignin biosynthesis, *p*-hydroxycinnamaldehydes **1** are normally reduced by CAD to *p*-hydroxycinnamyl alcohols **2**, which are subsequently polymerised into lignin. *p*-Hydroxycinnamaldehydes may also become a major component in lignin if CAD activity is depressed. During lignification, coniferyl alcohol **2b** (and other *p*-hydroxycinnamyl alcohols) are frequently coupled by *β-O*-4 linkages to form quinone methide intermediates **3**. These intermediates are stabilised by the addition of hydroxyl groups from nucleophiles (Nu), such as water, uronic acids, amino acids, ferulate esters, and neutral sugars to form structures **4** which are substituted with α-hydroxyl groups or cross-linked by α-ester and α-ether linkages to cell wall proteins and polysaccharides. During *β-O*-4 coupling of coniferaldehyde **1b** (and other *p*-hydroxycinnamaldehydes), quinone methide intermediates **5** rapidly undergo loss of *β*-H and re-aromatisation to form an α,*β*-enone structure **6**. This reaction prevents the formation of α-hydroxyl groups and cross-links involving the α-position of aldehyde sidechains.

EXPERIMENTAL

DHP–CW complexes were formed by adding H_2O_2 and varying ratios of coniferaldehyde **1b** and coniferyl alcohol **2b** to non-lignified primary walls isolated from cell suspensions of maize (Grabber *et al* 1996). After lignification, cell walls were thoroughly washed with water followed by acetone to remove unreacted monolignols and non-bound dehydrogenation polymers. Lignification experiments were replicated four times.

DHP–CWs were hydrolysed with H_2SO_4 and insoluble residues were collected to estimate Klason lignin (Hatfield *et al* 1994). The chemical composition of DHP–CWs from one replicate were then studied in greater detail. Sulphuric acid hydrolysates were analysed for uronic acids and neutral sugars (Blumenkrantz and Asboe-Hansen 1973; Hatfield and Weimer 1995). DHP–CWs were saponified at room temperature for 20 h with 2 M aq NaOH to release alkali-labile ferulates and lignin. Ferulates were analysed by GC-FID (Ralph *et al* 1994). A subsample of the alkaline hydrolysate was diluted with water, acidified to pH 2 with HCl, and its absorbance was read at 278 nm to estimate the quantity of alkaline-soluble lignin. Dehydrogenation polymers of coniferyl alcohol and coniferaldehyde, prepared according to the methods of Ralph *et al* (1992), were used as references. Absorbance due to alkali-labile ferulates was subtracted prior to calculation of alkaline soluble lignin.

DHP–CWs (1% w/v in 20 mM acetate buffer, pH 4·8, 40°C) were hydrolysed for 6 and 72 h with a mixture of Celluclast 1.5 L and Viscozyme L, each added at 40 μl g^{-1} of cell wall. The hydrolysate was clarified by centrifugation (10 min, 2500 × *g*) and an aliquot was analysed for total carbohydrate (Dubois *et al* 1956) to estimate cell wall degradation. Aliquots from one replicate were also analysed for uronic acids (Blumenkrantz and Asboe-Hansen 1973) and for neutral sugars following TFA hydrolysis (Hatfield and Weimer 1995). We also determined the release of total carbohydrate from two replicates hydrolysed for 72 h with a 1-, 5-, or 25-fold concentration of Celluclast and Viscozyme enzymes.

Aldehyde groups in lignin were reduced by suspending 500 mg of P_2O_5-dried DHP–CWs from one replicate in 100 ml of absolute ethanol with 500 mg of sodium borohydride. Suspensions were mixed for 6 days without precautions to exclude atmospheric moisture (reductions were less effective if strict anhydrous conditions were maintained). DHP–CWs were then pelleted by centrifugation (10 min, 2500 × *g*) and the supernatant was discarded. The pellet was resuspended in 50 ml of ethanol and 10 ml of 1 M acetic acid was added to destroy excess borohydride. DHP–CWs were collected on glassfibre filters (1·2 μm retention), washed repeatedly with 80% ethanol followed by acetone and then air dried. Borohydride reduction of aldehyde groups in DHP–CWs was evaluated by recording the absorbance spectra (240–540 nm) of alkaline-soluble lignin at pH 12. Spectra were corrected for absorbance due to alkali-labile ferulates. Normal and reduced DHP–CWs were analysed for Klason lignin, ferulates and cell-wall degradability as described earlier and for methanol (Kim and Carpita 1992).

RESULTS AND DISCUSSION

Wall-bound peroxidases and exogenously supplied hydrogen peroxide were used to polymerise coniferaldehyde **1b** and coniferyl alcohol **2b** into non-lignified primary walls of maize. Lignin content was increased from 8 mg g^{-1} in non-lignified walls to an average of

138 mg g^{-1} in DHP–CW complexes. Previous work has demonstrated that lignins formed in these complexes are structurally similar to natural lignins formed in grasses (Grabber *et al* 1996). Non-lignified walls were rapidly and extensively degraded by fungal enzymes with 440 mg g^{-1} of total sugars released after 6 h and 766 mg g^{-1} of total sugars released after 72 h of incubation. Lignification significantly reduced cell wall hydrolysis but lignins formed with coniferaldehyde were much more inhibitory to degradation than lignins formed with coniferyl alcohol (Table 1). Degradability differences between coniferyl alcohol and coniferaldehyde DHP–CWs were only partly overcome by a 5- or 25-fold greater concentration of hydrolytic enzymes (Fig 2). Digestion of DHP–CWs by mixed rumen microorganisms was also severely restricted by lignins formed with coniferaldehyde (Grabber J H unpublished). Although cell walls in CAD deficient plants are enriched in aldehyde groups, several studies indicate that only a portion of these aldehydes are incorporated into lignin (Baucher *et al* 1996; Bernard-Vailhe *et al* 1996; Provan *et al* 1997; Ralph *et al* 1997). Therefore our results suggest that improved degradability of CAD deficient plants is due to some factor other than the incorporation of *p*-hydroxycinnamaldehyde units into lignin. Some caution, however, must be used in extrapolating results from our primary wall model system to plants because relationships between lignin composition and degradability may differ in secondary cell walls (Grabber *et al* 1996).

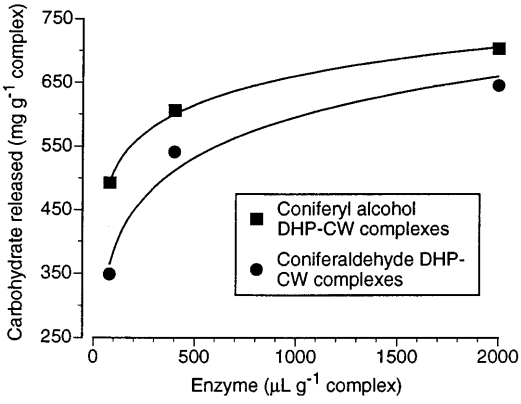

Fig 2. Degradability of DHP-CW complexes incubated for 72 h with a 1-, 5- and 25-fold quantity of Celluclast and Viscozyme (1 : 1).

Additional studies with selected DHP–CW complexes were conducted in an attempt to elucidate the mechanism by which aldehyde-containing lignins inhibit cell wall degradation. As expected, coniferyl alcohol and coniferaldehyde DHP–CWs formed from the same batch of non-lignified walls had identical monosaccharide compositions (Table 2). The release of these sugars was rather uniformly depressed by coniferaldehyde lignin, suggesting that the inhibition was general in nature, not involving interactions with specific structural polysaccharides.

The concentrations of lignin and *p*-hydroxycinnamates were also quite similar for both types of complexes (Table 3). Lignin structure was not characterized. However, previous work with DHPs suggests that homocoupling reactions of coniferaldehyde are similar to that of coniferyl alcohol, but fewer *β-O*-4 structures are probably formed (Higuchi *et al* 1994). The abundance of *β-O*-4 structures in lignin has no effect on the degradability of DHP–CWs (Grabber J H unpublished). Coniferaldehyde lignins were much more extractable in aqueous NaOH than coniferyl alcohol lignins (93 vs 56%). Differences in extractability are probably related to the propensity of lignins to form cross-linked structures with other wall constituents via quinone methide intermediates. Quinone methide intermediates are formed by *β-O*-4 coupling of monolignols to lignin (Fig 1). Normally, water adds to quinone methide intermediates of coniferyl alcohol **3**, but uronic acids, acidic amino acids, ferulate esters and neutral sugars may also be incorporated to form benzyl ester and benzyl ether structures **4** which cross-link lignin to cell wall proteins and structural polysaccharides

TABLE 1
Lignin content and degradability (mg g^{-1} cell wall) of DHP-CW complexes hydrolysed with a mixture of Celluclast and Viscozyme, each added at 40 µl g^{-1} of cell wall[a]

Complex type	*Klason lignin*	*Carbohydrate released*[b]	
		6 h	*72 h*
Coniferyl alcohol	135a	212a	471a
Coniferyl alcohol + coniferaldehyde (1 : 1 wt ratio)	139a	144b	401b
Coniferaldehyde	139a	115c	334c
SEM	4·7	3·4	10·4

[a] Least-squares means within a column not sharing a common following letter are different ($P < 0{\cdot}01$) based on the PDIFF option of the GLM procedure (SAS 1990).
[b] Carbohydrate released into the hydrolysate was estimated by a colorimetric method (Dubois *et al* 1956). Klason lignin content was used as covariate to adjust means and to increase precision.

TABLE 2
Composition (mg g^{-1} cell wall ± standard deviation) and degradability[a] (% ± SD) of monosaccharides in DHP-CW complexes

Complex type	*Arabinose*	*Xylose*	*Glucose*	*Galactose*	*Uronate*
Composition					
Coniferyl alcohol	167 ± 2·0	144 ± 1·5	286 ± 9·4	74 ± 0·8	82 ± 3·8
Coniferaldehyde	168 ± 2·0	141 ± 1·3	286 ± 10·8	76 ± 0·9	84 ± 1·4
Loss after 6 h of hydrolysis					
Coniferyl alcohol	20·6 ± 0·01	13·8 ± 0·01	27·7 ± 0·04	26·2 ± 0·03	49·0 ± 0·01
Coniferaldehyde	11·8 ± 0·01	6·2 ± 0·01	17·4 ± 0·01	14·2 ± 0·01	26·8 ± 0·02
Loss after 72 h of hydrolysis					
Coniferyl alcohol	59·1 ± 0·04	49·7 ± 0·03	58·5 ± 0·04	63·7 ± 0·05	88·2 ± 0·01
Coniferaldehyde	44·2 ± 0·01	27·3 ± 0·01	42.9 ± 0·02	54·6 ± 0·03	72·9 ± 0·01

[a] Monosaccharides released into the hydrolysate during incubation with Celluclast and Viscozyme. Uronic acids were determined by a colorimetric procedure (Blumenkrantz and Asboe-Hansen 1973) and neutral sugars by HPLC following TFA hydrolysis (Hatfield and Weimer 1995).

(Brunow *et al* 1989; Sipilä and Brunow 1991a,b,c; Quideau and Ralph 1994; Li and Helm 1995). In contrast, cell wall constituents cannot add to quinone methide intermediates of coniferaldehyde **5** because an α,β-enone structure **6** is formed by loss of the β-H and re-aromatisation (Connors *et al* 1970) thereby preventing cross-linking of lignin to other wall polymers. As a result, cross-linking in coniferaldehyde DHP–CWs is probably restricted to copolymerisation of coniferaldehyde with ferulate polysaccharide esters (Ralph *et al* 1992; Grabber *et al* 1995; Ralph *et al* 1995) and tyrosine residues in proteins (McDougall *et al* 1996). The former structures are readily cleaved by saponification, allowing for extensive solubilisation of coniferaldehyde lignins. Ferulate polysaccharide esters would also be cleaved in coniferyl alcohol DHP–CWs but alkali-stable bonds (possibly benzyl ethers formed via quinone methide intermediates) apparently prevent complete solubilisation of lignin. Elimination of cross-links formed via quinone methide intermediates should improve cell-wall degradability, but this benefit is not realised because of another overriding property of coniferaldehyde lignins—a property probably related to lignin hydrophobicity.

Re-aromatisation of quinone methide intermediates of coniferaldehyde prevents addition of the water to form an α-hydroxyl groups. This, in combination with γ-aldehyde groups, probably makes coniferaldehyde lignins much more hydrophobic than coniferyl alcohol lignins (Higuchi *et al* 1994), exacerbating binding of celluloytic enzymes to lignin and further restricting hydration and penetration of hydrolytic enzymes into cell walls (Sewalt *et al* 1997). These effects should be lessened if γ-aldehyde groups on lignin are chemically reduced to alcohols. To test this hypothesis, DHP–CWs were incubated with ethanolic sodium borohydride

TABLE 3
Concentration of lignin, alkaline-soluble lignin[a], and *p*-hydroxycinnamates[b] (mg g^{-1} cell wall ± SD) in DHP-CW complexes

Complex type	*Klason lignin*	*Alkaline-soluble lignin*	*p-Hydroxycinnamates*			
			Total ferulates		*p-Coumarates*	
			Alkali labile	*Copolymerised*	*Alkali labile*	*Copolymerised*
Coniferyl alcohol	117 ± 0·5	65·5 (0·23)	1·8 ± 0·15	16·1 ± 0·06	0·32 ± 0·006	0·12 ± 0·019
Coniferaldehyde	128 ± 1·8	118·5 (0·06)	2·8 ± 0·02	15·1 ± 0·21	0·28 ± 0·001	0·17 ± 0·010

[a] Lignin solubilised by aqueous 2 M NaOH was estimated by comparing the absorbance of extracts (278 nm) to that of dehydrogenation polymers formed with coniferyl alcohol or coniferaldehyde.
[b] Total ferulates equals ferulate monomers plus dehydrodimers. Alkali labile *p*-hydroxycinnamates esters are ester-linked to xylans and released by saponification. Copolymerised *p*-hydroxycinnamates are ester linked to xylans and ether or C–C linked to lignin. These cross-linked *p*-hydroxycinnamates are not released by saponification. Copolymerised *p*-hydroxycinnamates were calculated as the difference in *p*-hydroxycinnamates recovered following saponification of non-lignified walls and DHP-CW complexes.

(March 1968) in an attempt reduce aldehyde groups on lignins without causing other major changes in cell wall chemistry. Ethanolic sodium borohydride will also reduce ketone groups in lignins and aldehydic end-groups of polysaccharides but these changes are not expected to affect cell wall degradability. More importantly, used of ethanolic sodium borohydride should minimise cleavage of ester-linked constituents such as ferulates which mediate xylan and xylan–lignin cross-linking in grass cell walls (Grabber *et al* 1995). Complexes were bleached considerably by borohydride treatment due to reduction of aldehyde and ketone groups in lignin (Table 4). The effectiveness of borohydride reduction was revealed by changes in the absorbance spectrum of alkaline-soluble lignin released from DHP–CWs (Fig 3). Absorbance maxima at 350 nm (due to aldehyde groups), and at 420 nm (due to extended conjugation of the aldehydic polymer), diminished greatly in intensity following borohydride reduction of coniferaldehyde DHP–CWs. Borohydride reduction of coniferyl alcohol DHP–CWs caused a small reduction in absorbance at 350 nm. The overall absorbance profile of coniferaldehyde DHP–CWs following reduction was quite similar to that of coniferyl alcohol DHP–CWs but the former complexes had greater absorbance at 280 nm due to the higher inherent solubility of coniferaldehyde lignins in alkali. Borohydride treatment did not appreciably change Klason lignin concentrations but alkali-labile methanol (from pectin methyl esters) and ferulate concentrations were reduced *c* 42% and 22%, respectively, in both types of complexes indicating that some cleavage of ester groups had occurred (Table 4). Reduction by sodium borohydride was slightly enhanced if DHP–CWs were suspended in dioxane/ethanol or THF/ethanol solutions, but cleavage of ester groups was much more severe (Grabber J H unpublished).

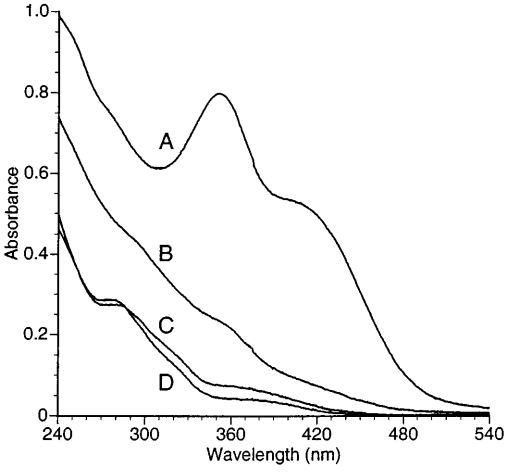

Fig 3. Absorption spectra of lignins solubilised from DHP-CWs by 2 M aq NaOH: (A) coniferaldehyde DHP-CWs; (B) coniferaldehyde DHP-CWs reduced with ethanolic $NaBH_4$; (C) coniferyl alcohol DHP-CWs; (D) coniferyl alcohol DHP-CWs reduced with ethanolic $NaBH_4$.

Borohydride treatment increased the hydrolase degradability of both coniferyl alcohol and coniferaldehyde DHP–CWs but, more importantly, degradability differences between complexes were lessened after 6 h and eliminated after 72 h of hydrolysis (Table 4). Degradability of complexes was probably enhanced by cleavage of ester linkages as has been observed with aqueous borohydride treatments (Ford 1989). Some improvement in degradability was probably due to the removal of acetyl esters from xylans (Bacon and Gordon 1980; Wood and McCrae 1986; Ford 1989) and methyl esters from pectin (Pressey and Avants 1982). Recently, we observed that reductions in diferulate ester and lignin–ferulate ester cross-links enhanced the degradability of coniferyl alcohol DHP–CWs by fungal hydrolases (Grabber J H unpublished). In that study, the quantity of diferulate and lignin–ferulate cross-links formed in DHP–CWs was reduced by using cell walls isolated from maize cell suspensions grown with 2-aminoindan-2-phosphonic acid (AIP). AIP is a specific inhibitor of a phenylalanine–ammonia lyase that reduces ferulate deposition into cell walls (Grabber *et al* 1995, 1998). Using this approach, we found that a

TABLE 4

Lignin, methanol and total ferulate content and degradability (mg g^{-1} cell wall $\pm$ SD) of DHP-CW complexes before and after reduction of aldehyde groups with ethanolic sodium borohydride

Complex type (*treatment*)	*Colour*	*Klason lignin*	*Methanol*[a]	*Total ferulates*[a]	*Carbohydrate released*[b]	
					6 h	*72 h*
Coniferyl alcohol (none)	Light beige	122 ± 1	6·5	3·0	272 ± 4	541 ± 10
Coniferaldehyde (none)	Light orange	137 ± 2	7·2	3·9	141 ± 2	383 ± 0
Coniferyl alcohol ($NaBH_4$)	White	128 ± 6	3·9	2·5	420 ± 1	615 ± 7
Coniferaldehyde ($NaBH_4$)	Light beige	127 ± 3	4·0	2·9	335 ± 1	593 ± 13

[a] Methanol and total ferulates (monomers plus dimers) released by saponification of DHP-CWs (value from single analysis).

[b] Carbohydrate released into the hydrolysate by Celluclast and Viscozyme was estimated by a colorimetic method (Dubois *et al* 1956).

70% reduction in ferulate cross-linking increased the carbohydrate degradability of both coniferyl alcohol and coniferaldehyde DHP–CWs by *c* 110 mg g^{-1} (Grabber J H unpublished), suggesting that cleavage of ferulate esters by borohydride should affect the degradability of these complexes in a similar manner.

CONCLUSIONS

This study provides compelling evidence that improved enzymatic degradability of CAD-deficient plants is not due to the accumulation of aldehyde enriched lignins. In fact, highly hydrophobic aldehyde lignins caused a severe depression in cell wall degradability in a model system where other cell wall properties remained constant. Presumably then, improved degradation of CAD-deficient plants must be attributed to other associative changes in cell wall biosynthesis and structure. Although incorporation of aldehyde-containing lignins is not desirable from a nutritional standpoint, the high alkaline solubility of these lignins should enhance the delignification of plants. Delignification is a crucial step in pulp production for papermaking and in the bioconversion of cellulosic materials into ethanol or other chemicals. The poor enzymatic degradability of walls containing aldehyde lignins may improve plant resistance against pathogenic wall degrading fungi. Aldehyde groups in lignin may also possess biocidal properties, providing additional protection against plant pests.

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