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Lignification in relation to the biennial growth habit in brassicas

B.W. Evans[a,b], C.E. Snape[b,1], M.C. Jarvis[a,*]

[a]Chemistry Department, Glasgow University, Glasgow G12 8QQ, Scotland, UK
[b]Department of Pure & Applied Chemistry, Strathclyde University, Glasgow G1 1XL, Scotland, UK

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Abstract

The forage brassicas are a useful model system for the study of wood formation because the thickened cell walls of their vascular tissue can vary widely in lignin content. Solid-state ^{13}C NMR spectroscopy was used to quantify lignin, and determine features of its structure, in the vascular cell walls of forage rape (*Brassica napus* L.), and Thousandhead and marrowstem cultivars of kale (*Brassica oleracea* L. var. *acephala*). During the first season of vegetative growth, lignin levels in these cell walls remained low in the upper part of the stems despite the physical resemblance of this tissue to wood. The extended flowering stems produced in the following year were thinner and their vascular tissue contained much more strongly lignified cell walls. The structure of the lignin was typical of angiosperm wood. It showed only small variations in syringyl/guaiacyl ratio, but this ratio increased with lignin content and thus with the proportion of the lignin that was associated with secondary cell-wall layers.

Keywords: Lignin; *Brassica napus* L.; *Brassica oleracea* L. var. *acephala*; Cruciferae; Flowering; Development; Mobility; Solid-state NMR

1. Introduction

In their anatomy and developmental origins, lignified tissues in herbaceous plants differ greatly from wood in trees (Dengler, 2001; Jarvis and His, in press). For example, in elongating Arabidopsis hypocotyls the vascular strand is formed from initials at the apical meristem and normally only the helical secondary thickenings of its xylem vessel(s) are lignified; but under specialised culture conditions the hypocotyl can be induced to stop elongating and form a vascular cambium, from which differentiates heavily lignified secondary xylem tissue resembling typical dicotyledonous wood (Chaffey et al., 2002).

Between these two extremes, *Brassica* spp. show a particularly wide range of variants in xylem anatomy, which are instructive about the relationship between wood formation and growth (Alexander et al., 1987; Wilson et al., 1988; Femenia et al., 1999). Some of these *Brassica* xylem tissues also influence the economic value of crop products through their mechanical properties. For example, the vascular ring in the stems of forage rape and kale contains little lignin (Wilson et al., 1988; Wilman and Moghaddam, 1998) but is mechanically 'woody' enough to restrict the palatability of the crop to livestock (Wilson et al., 1989; Mtengeti et al., 1995), and this combination of characteristics has lead to interest in its potential as a raw material for papermaking (McDougall et al., 1993). The vascular ring in cauliflower stems is similar, becoming more lignified and accumulating secondary cell-wall layers with maturity (Femenia et al., 1998, 1999). The extent of its development is reflected in 'stringiness', a negative quality characteristic.

In dicotyledonous plants true wood, originating from the vascular cambium, is restricted to organs in which longitudinal growth has ceased. Indeed wood is often regarded as dead tissue, although there is evidence that this is not universally the case (Dumbroff and Elmore, 1977). To withstand the compressive element of bending stresses originating from the distal part of the plant, wood cells require stiffness and strong attachment to one another. These mechanical qualities derive respectively from the lignified secondary cell wall and from lignin deposition in the middle lamella (Jarvis and His, in press). It would appear that lignification and elongation growth are usually incompatible except in the

* Corresponding author. Tel. +44-141-330-4653; fax: +44-141-330-4888.
E-mail address: mikej@chem.gla.ac.uk (M.C. Jarvis).
[1] Present address: Fuel and Energy Centre, University of Nottingham, Nottingham, NG7 2RD, England, UK.

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primary xylem vessels of seedlings, where the helical geometry of the lignified thickenings of the cell wall allows axial extension while resisting the radial compressive stresses exerted by turgor in the surrounding cells (Esau, 1977; Esau and Charvat, 1978).

In the taller brassica crops like kale, rape and Brussels sprouts, the biennial habit of growth imposes unusual constraints on the formation of woody tissue. In the first season of growth, as is normal in dicots, the vascular ring extends to the top of the stem and reinforces it against the weight of the leaves and the stresses due to wind. When the stem is thin as in the Thousandhead kales and the winter forms of oilseed rape, the vascular ring is heavily lignified and comprises much of its cross-sectional area. In contrast the marrowstem kales, forage rapes and cauliflower have broad stems with only a thin vascular ring, in which the lignin content is low and the polysaccharide composition is intermediate between that of typical primary and secondary cell walls (Wilson et al., 1988; Femenia et al., 1999). These latter forms derive their mechanical stability from their greater stem diameter, a geometrical factor, and not primarily from the development of lignified tissues.

In the second season of growth the upper part of the brassica stem extends rapidly until at flowering the height of the plant is more than doubled. This raises a number of biomechanical as well as developmental questions. How does the lower part of the stem withstand the bending stresses that result from the greatly increased stem height and weight at flowering? The diameter of the lower part of the stem does not increase during the second season, so does it become more lignified? Does the lignin change in composition? The forage brassicas are therefore a useful system in which to study the developmental interrelationship of lignification, mechanical strength and the potential for growth.

This paper describes the changes in lignin composition that accompany flowering in two fodder brassica species, kale and rape. Solid-state NMR methods were used to determine the composition of the cell walls. This approach is particularly well suited to lignified tissues because it allows lignin to be studied in situ, avoiding considerable problems that ensue from the difficulty of bringing it into solution.

2. Results

The ^{13}C CP-MAS (cross-polarisation, magic-angle spinning) NMR spectra of the vascular cell walls from forage rape, Thousandhead kale and marrowstem kale are shown in Fig. 1. Carbohydrates appear in the spectral region 60–110 ppm and the aromatic ring carbons of lignin in the region 110–160 ppm (Table 1). The carbohydrate spectra were not well resolved because the moisture content was kept a low level (ca. 10%) optimal for the

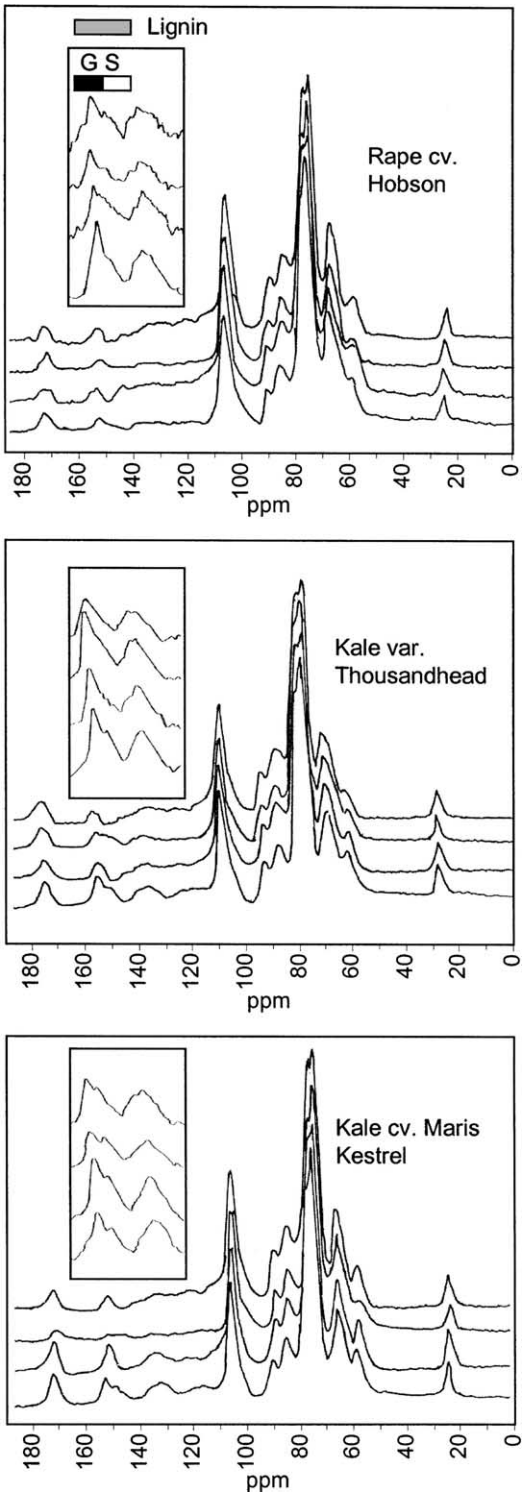

Fig. 1. CP-MAS ^{13}C spectra of vascular cell walls from forage rape, cv. Hobson; Thousandhead kale; and marrowstem kale, cv. Maris Kestrel. Top to bottom for each plant variety: (1) top of flowering stem, second season; (2) top of vegetative stem, first season; (3) base of stem, second season; (4) base of stem, first season. Inset: the aromatic region of the dipolar-dephased spectra from the same cell walls, in the same order top to bottom and on the same horizontal scale. Bars mark the regions of the spectrum used throughout to calculate lignin content (CP-MAS spectrum) and syringyl: guaiacyl (S/G) ratio (dipolar-dephased spectrum). For assignment of other peaks in the spectra see Table 1.

determination of lignin. In general they resembled the spectra previously published for *Brassica* primary cell walls (Jarvis, 1990, Müller et al., in press), but at the level of resolution obtained it is not possible to make deductions about non-cellulosic polysaccharide composition.

Differences in the lignin content of the cell walls can be deduced from the intensity of the peak centred on 150 ppm in the CP-MAS spectra (Fig. 1) and the lignin contents are summarised in Table 2. In the first growth season, lignin contents were consistently lower at the top of the vegetative stem as shown previously (Wilson et al., 1988) by chemical estimation of acetyl bromide lignin. The flowering stem produced in the second growth season was significantly more heavily lignified than the upper part of the vegetative stem had been in the previous season.

The monomer composition of the lignin can be deduced more readily from the dipolar-dephased ^{13}C spectra (Fig. 1, inset) than from the CP-MAS spectra. The aromatic region (110–160 ppm) was typical of angiosperm lignins (Love et al., 1992; Boudet, 1998). The proportion of syringyl residues (or, more strictly, of syringyl with an ether linkage at C-4) was estimated from the intensity of the signal at 153 ppm. Syringyl/guaiacyl ratios were significantly lower ($P<0.05$) in marrowstem kale than in the other two forms.

3. Discussion

Solid-state NMR methods have been widely used to determine lignin composition in wood, with good results (Manders, 1987; Newman, 1992). NMR has been less commonly used for lignified cell walls in herbaceous plants, but we have shown that when the lignin content is low or its composition is unusual, solid-state NMR can give more accurate quantification than conventional chemical methods (Love et al., 1994). Unless the lignin content is high it is normally necessary to use cross-polarisation (CP) experiments to obtain signals of adequate intensity from lignin. Absolute quantification then depends on the assumption that lignin and carbohydrate cross-polarise with equal efficiency, which is not always the case. However a good approximation can be achieved using longer than normal CP times, and the conditions used here were optimised and validated on wood (Love et al., 1992). All methods of lignin determination are subject to interference from low-MW phenolic substances insolubilised by adsorption on cell walls. These interferences were minimised by the detergent and phenol extraction steps during the preparation of the cell walls (Renard et al., 2001). The neutral extraction conditions are mild enough to avoid any structural rearrangements in the lignin polymer or loss of lignin fragments by acid hydrolysis.

The maximum lignin contents observed in the flowering stems, over 30%, were as great as in hardwood cell walls. The lignin structure appeared to be similar to that found in angiosperm wood. Low syringyl/guaiacyl ratios in the cell walls from the base of the marrowstem kale stems can be related to previous histological observations (Wilson et al., 1988): the small amount of lignin in these cells was largely restricted to the cell corners and middle lamellae. It is known that guaiacyl lignins are typical of the middle lamella in wood (Fergus and Goring, 1970).

Table 1
Spectral assignments

Chemical shift (ppm)	Assignment
173	Acetyl carboxyl
153	Syringyl OR, C-3/C-5
149	Guaiacyl OR, C-3
147	Guaiacyl OR and OH, C-4; guaiacyl OH C-3; syringyl OH, C-3/C-5.
136	Guaiacyl OR C-1; syringyl C-4
133	Guaiacyl and syringyl OH, C-1
119	Guaiacyl C-5
116	Guaiacyl C-4
112	Guaiacyl C-2
105	Cellulose C-1, syringyl C-2,C-5
102	Xylan C-1, galacturonan C-1
89	Crystal-interior cellulose, C-4
84	Crystal-surface cellulose, C-4
73	General carbohydrate, C-2/C-3/C-5
65	Crystal-interior cellulose, C-6
62	Crystal-surface cellulose, C-6, xylan C-5
56	Guaiacyl and syringyl methoxyl
21	Acetyl methyl.

Lignin monomer residues with aryl ether linkages are denoted by OR, and residues with the phenolic hydroxyl free are denoted by OH.

Table 2
Lignin content in isolated cell walls and syringyl:guaiacyl (S/G) ratio in the lignin, estimated by integration of the spectral regions shown in Fig. 1

Stem section	Age, Months	% Lignin	S/G ratio
Rape, cv Hobson			
Upper	6	12	1.5
Upper	12	18	2.1
Lower	6	14	2.3
Lower	12	12	1.8
Kale, var. Thousandhead			
Upper	6	13	2.3
Upper	12	32	2.7
Lower	6	19	1.5
Lower	12	20	2.3
Kale, var. Maris Kestrel			
Upper	6	9	1.0
Upper	12	16	1.5
Lower	6	21	1.5
Lower	12	34	1.5

Data were analysed by 2-way ANOVA showing that the upper part of the stem had greater lignin content ($P<0.05$) at age 12 than at age 6 months, while the S/G ratio was lower ($P<0.05$) in kale of cv. Maris Kestrel than in the other two varieties.

The plasticity in composition of the brassica vascular tissue is clearly evident from the results shown here. Lignin was present only at low levels, less than half of those typical of wood, in the upper vegetative stems where the capacity for renewal of growth was retained. The gradient of decreasing lignin content towards the top of the vegetative stem was not maintained, however, in the new growth in the flowering season. The tall, thin flowering stems were much more strongly lignified. Thus the plants demonstrated a mixture of two strategies to resist the compressive element of bending stresses on the stems: lignification of the vascular ring (flowering stems, all stems of Thousandhead kale) and large stem diameter (vegetative stems of marrowstem kale). The balance between these strategies appears to be controlled at the level of the switch between vegetative growth and flowering. Understanding what happens at the interface between biomechanics and vascular development will allow the ontogeny of wood and the potential for new materials of plant origin to be explored.

4. Experimental

4.1. Materials

Kale (cv. Thousandhead and cv. Maris Kestrel) and forage rape (cv. Hobson) plants were grown under standard commercial conditions at the Scottish Crop Research Institute, Invergowrie and either harvested after 6 months from planting, or transplanted at that stage for a further 6 months growth during which they produced flowering stems. Segments 150 mm long were removed from the top and bottom of the main stems, cut into 1 cm lengths and stored at −20 °C.

Isolation of the cell walls was based on the method of McCluskey et al. (1984) with minor modifications. The frozen samples were mixed with ice and homogenised in aqueous Triton X-100, with octan-1-ol as a anti-foaming agent, for 15-s periods until all the material was broken down. The homogenate was filtered through 2 mm, 1 mm and 50 μm sieves. The material from the 1 and 2 mm sieves was extracted with saturated aqueous phenol for at least 1 h, filtered and washed with water until all traces of the detergent were removed. The cell walls were then washed in acetone and left to dry in normal atmospheric conditions.

4.2. NMR methods

NMR experiments were carried out on a Bruker MSL-100 spectrometer at 25 MHz for ^{13}C, with magic-angle spinning (MAS) at 4.95 kHz in a 7 mm ceramic rotor. In this low-field spectrometer the dissipation of lignin spectral intensity into spinning side-bands is minimised (Love et al., 1992). Because the rate of cross-polarisation (CP) is less for non-protonated aromatic carbons than for carbohydrate, the CP contact time cannot be optimised simultaneously for both carbohydrate and lignin (Stejskal and Memory, 1994). The CP conditions used (2 ms contact time) lay between the optima for carbohydrate and lignin and have been shown (B.W. Evans, D.C. Apperley, M.C. Jarvis and C.E. Snape, unpublished) to give somewhat reduced but approximately equal CP efficiency for both, so that the lignin:carbohydrate ratio could be estimated from the CP-MAS spectra (Love et al., 1992). In principle more accurate lignin quantification could be achieved by single-pulse ^{13}C excitation as described by Love et al. (1992) but for these materials the ^{13}C spin-lattice relaxation time is so long (ca. 1 min) that sufficient scans for adequate signal/noise ratios could not be obtained. Details of the calibration of this procedure against other methods of determining lignin, and the assumptions involved, are given by Manders (1987) and Love et al. (1992, 1994).

To examine the aromatic part of the spectrum with less interference from carbohydrate and other aliphatic signals, dipolar-dephased spectra were obtained by adding a short delay (ca. 40 μs) without proton decoupling to the CP-MAS pulse sequence before acquisition of the ^{13}C signal (Opella and Frey, 1979; Newman, 1990). This delay allows most protonated carbons to relax through dipolar coupling to the attached protons. The length of the delay was optimised individually for each sample so that most of the signal intensity from protonated carbons had decayed leaving a spectrum derived principally from the non-protonated aromatic and carboxyl carbons.

4.3. Calculation of lignin content and composition

The lignin content of the cell walls was calculated from the proportion of the total spectral intensity in the range 143–160 ppm, on the assumption that this spectral region contained 19 mol% of the carbon in the lignin [two carbons out of 10 for guaiacyl (G) units or 11 for syringyl (S) units] (Love et al., 1992). The S/G ratio (strictly, the percentage of etherified syringyl units) was calculated from the relative intensity of the 153 ppm signal within the 143–160 ppm region of the spectrum. This procedure is similar to, but simpler than, the spectral decomposition procedure of Manders (1987).

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