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Polymer nanoscale morphology in *Chara australis* Brown cell walls studied by advanced solid state techniques

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Abstract — The cell wall of the green algae *Chara australis* Brown (Charophyta, Algae) has been examined with solid state techniques (suitable for characterising the nanoscale arrangement of polymers) and solid state chemistry. The chemical composition of a bulk dried sample of *Chara australis* was examined using cross-polarisation magic angle spinning (CP-MAS) ^{13}C nuclear magnetic resonance (NMR). The presence of phenyl-propane subunits typical of the cross linked polymer, lignin, is not revealed. The arrangement of cellulose in the cell wall was probed by examining the wide angle diffraction pattern of a single fibre oriented with respect to an x-ray beam. The pattern produced was typical of a textured crystalline lattice embedded in an amorphous matrix. The x-ray reflections are much broader than most found in higher plants and it is difficult to resolve texture. Polarised Raman scattering from a similar sample provided clearer evidence of a textured cellulose matrix embedded in an amorphous matrix. It is found that the charophyte cell wall has many similarities in chemical composition and nanoscale arrangement of cell wall polymers to that of the higher plant, including the presence and arrangement, of cellulose and various polysaccharides.

Charophyte / cell wall / solid-state NMR / x-ray diffraction / Raman spectroscopy

Résumé — **Morphologie en nano-échelle des polymères pariétaux de *Chara australis* Brown étudiée grâce à des techniques de pointe en phase solide.** La paroi de l'algue verte *Chara australis* Brown (Charophyta) a été examinée en utilisant des techniques d'observation à l'état solide (adaptées à la caractérisation de la disposition des polymères en nano-échelle) et d'analyse chimique en phase solide. La composition chimique d'un échantillon brut de *Chara australis* a été examiné en utilisant la résonance magnétique nucléaire (NMR) du ^{13}C par rotation d'angle magique en polarisation croisée (CP-MAS). La présence de sous-unités phényl-propane typiques de la lignine qui est un polymère de cohésion n'a pas été mise en évidence. La disposition de la cellulose dans la paroi a été explorée par un examen du vaste patron de diffraction angulaire d'une fibre isolée orientée

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par rapport à un faisceau de rayons X. Le modèle obtenu est typique d'un entrelacs de fibres cristallines baignant dans une matrice amorphe. Les rayons X diffusent plus que chez les plantes supérieures et il est difficile d'obtenir une bonne résolution. La dispersion polarisée Raman à partir d'un échantillon similaire a démontré plus clairement cette structure cellulosique plongée dans une matrice amorphe. La paroi des charophytes possède de nombreuses similarités de composition chimique et de disposition des polymères en nano-échelle avec celle des plantes supérieures, en particulier quant à la présence et à la disposition de la cellulose et de divers autres polysaccharides.

Charophyte / paroi cellulaire / RMN en phase solide / diffraction en rayons X / spectroscopie Raman

INTRODUCTION

On the basis of phylogenetic evidence the charophycean algae have been proposed as the closest living relatives of land plants (Karol *et al.*, 2001) and as such could be considered representative of the adaptations in cell wall polymers that resulted in successful land plants. At the macroscale they have spiral phyllotaxis (Alder *et al.*, 1994; Soulié-Märsche, 1999) and similar creep and stress relaxation properties as the primary cell walls of higher plants (Kamiya *et al.*, 1963; Cleland, 1971; Toole *et al.*, 2002). The elongation and growth of the internodal cells of charophytes have been used as a macroscale model (Green *et al.*, 1970) by which to understand the microscale growth and elongation of cells in higher plants (Cosgrove, 1993). The chemical composition and biosynthesis of the charophyte cell wall cellulose (Hotchkiss & Brown, 1988) and other polysaccharides (Popper & Fry, 2003) have already been studied in relation to the evolution of the vascular cell wall. The composition of the monosaccharides is within the normal range of higher plant tissues.

In this work we examine the solid state architecture of cell wall polymers in *Chara australis* Brown and compare with literature observations from the cell walls of higher plants. For highly anisotropic structures, such as the cell walls of higher plants, it is not only the chemical composition of the cell wall that is important. The degree of mixing, or phase separation between the various chemical components must be considered, but also the orientation of fibrous polysaccharide fractions (Preston, 1974). The effect of the nanoscale arrangement, or formation of separate polymer phases, is to modulate the nature of the load bearing structures within the cell wall (Toole *et al.*, 2002; Garvey *et al.*, 2004), and allow differential accessibility to the mediators of cell wall elongation and growth (Cosgrove, 1993; Fenwick *et al.*, 1999).

The chemical composition of charophytes has been examined in terms of individual sugars (Hough *et al.*, 1952; Hotchkiss & Brown, 1988) as well as inorganic materials removed during various extraction procedures (Anderson & King, 1961a; Anderson & King, 1961b). To study the chemical linkages between the various polysaccharides, enzymatic action has been used to selectively remove sugar units (Popper & Fry, 2003). The chemical composition is not different from the range found in land plant cell walls (Hotchkiss & Brown, 1988), but due to the nature of the degradation of the cell wall while pursuing the analysis, much of the important information about the spatial arrangement of the constituent monomeric sugars is lost.

A basic model for polymer arrangement, based on that found in higher plants and charophytes, is of a stiff framework of cellulose surrounded by a softer phase (Preston, 1974). Cellulose is a linear polymer of β-1,4-linked D-glucan-pyranose (Figs 1, 2). Cellulose is synthesised from precursors into elementary crystallites called microfibrils of the order of nanometres in size by synthetic complexes in the plasma membrane (Hotchkis & Brown, 1987). The size and number of cellulose chains per microfibril, of these elementary crystallites appears to be a function of the species (Davies & Harris, 2003; Newman, 1999) and structure of the synthetic complex (Hotchkis & Brown, 1987; Brown *et al.*, 1996). Preston (1974) showed that the microfibrils in the cell wall are usually not arranged isotropically but in a helix around the fibre axis (Fig. 1). The arrangement of these microfibrils is an important mechanism by which the cell wall of higher plants may modulate its mechanical functioning (Probine & Preston, 1961; Lichtenegger *et al.*, 1999). The preferential alignment of microfibrils in elongating *Nitella* cells has been observed during the growth and elongation of the cell (Green 1958; Green *et al.*, 1970; Green *et al.*, 1971) with the transverse alignment of the microfibril gradually giving way to a more longitudinal alignment at the growing ends of the inter-node cell

A further feature of the cellulose phase is the presence of two allomorphs, a triclinic form cellulose I_β, and the monoclinic form I_α (Attala & VanderHart, 1984). While certainly different amounts of each allomorph is associated with different sources of cellulose (Attala & VanderHart, 1984; Belton *et al.* 1989), it has been suggested that these differences may be due to different arrangements of the cellulose in the cell walls. Mechanical stress, or bending, the stiff polymer chain induces phase transitions (Jarvis, 2000).

Around this crystalline phase there is a less ordered layer consisting of a complex gel called pectin (Ridely *et al.*, 2001) and other polysaccharides which may be referred to collectively as hemicelluloses (Heredia *et al.*, 1995). The overwhelming preponderance of polysaccharide chemistry and the strongly hydrated nature of the cell wall are indicative of the importance of hydrogen bonding, which as well as the covalent bonding is responsible for the mechanical properties of the cell walls. The former may be modified without degradation of cell wall polymers, by displacing hydrogen bonding during elongation and growth (Cosgrove, 1993). The cellulose provides a stiff framework but the less ordered phase has an important impact on the overall mechanical properties of the cell wall (Toole *et al.*, 2002).

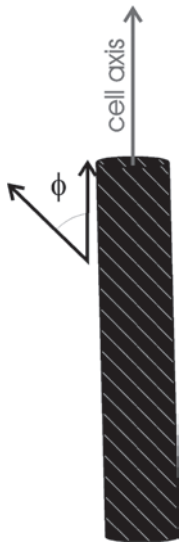

Fig. 1. Proposed helical arrange of cellulose microfibrils around the axis of a single mature internodal cell with an angle, ϕ, defined between the helical angle and the cell axis.

Many higher plant cell walls may contain varying amounts of the cross-linked phenyl-propane polymer, lignin, which imparts compressive strength to wood (Mark, 1967). As such the development of this compound represents a significant adaptation for land plants. On the basis of various extractions Anderson & King (1961a; 1961b) proposed a measurable content of lignin in the cell walls of *N. translucens* and *C. australis*. However, there are no other observations of lignin

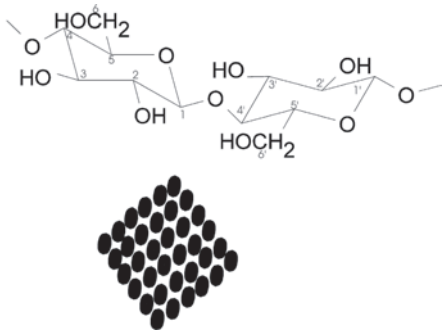

Fig. 2. The assignment of the cellulose peaks for ^{13}C CP-MAS NMR spectra to different carbons (top) in the cellobiose monomer and the interior and exterior of the unitary cellulose crystallite (bottom). The ovals represent glucose ring and the arrangement of a single crystallite or microfibril is shown.

in the cell wall of charophytes. The precursors for biochemical synthesis of lignin are present in sporopollenin, which is present oospores. Thus the precursors of lignin are certainly present. For this reason it is impossible to discern between the degradation products of sporopollenin and lignin even though the solid state chemistry is quite distinct.

The techniques discussed here have been used with success in the study of the cell walls of higher plants. Solid state ^{13}C cross polarisation magic angle spinning (CP-MAS) NMR (Jarvis & Apperly, 1990), Raman spectroscopy (Attala, 1976) and x-ray scattering (Preston, 1974) are less sensitive than conventional means of chemical analysis, but allow insight into the nanoscale arrangement of cell wall polymers. In this way they may avoid the artefacts which may occur in the preparation and dehydration of samples for electron microscopy and can be used to analyse the structure of the cell wall *in situ*.

METHODS AND MATERIALS

Chara *and cellulosic materials*

Mature internode cells of *Chara australis* were used for all experiments. For x-ray diffraction and Raman spectroscopy a single internode cell in a full hydrated state was used after removal of both ends and syringing out the cell contents with distilled water. NMR spectroscopy was carried out on bulk washed and dried material. A sample of Whatman number 4 filter paper was used as a cellulosic standard. Tunicin is extracted from the mantle of *Halocynthia spp.* and is a pure form of cellulose I_β, the monoclinic form of cellulose (Belton *et al.*, 1989).

X-ray diffraction

X-ray diffraction patterns were accumulated from midway along a single dried internode cell on the ID01 beam line at the European Synchrotron Research Facility using X-rays of wavelength 0.81566 Å. Operational details of the beam line can be found on the website http://www.esrf.fr/exp_facilities/ID1/user_guide/.

^{13}C CP-MAS NMR Spectroscopy

All spectra were obtained on a Bruker DRX 300-MHz instrument using the cross-polarisation (CP) and delayed contact methods. Samples were packed

into 5-mm zirconia rotors with Kel-F caps and spun at speeds of 4 kHz. This spinning frequency is sufficient to eliminate the dipolar couplings and anisotropic chemical shift broadening from the ^{13}C spectra and to remove the spinning side bands from proximity to the peaks (Hill *et al.* 1994). The CP experiments required up to 3.000 transients with a contact time of 2 ms and recycle delay of 2 s. The decoupler was turned on only during acquisition. Blanks were run of rotors to ensure no artefacts in the spectra. The data were collected in 1 K of memory, zero-filled to 4 K, and then Fourier transformed using line-broadening factors of 10-20 Hz. All spectra were referenced to TMS (0 ppm). The low-field peak of adamantane was employed as a secondary reference (38.3 ppm).

Polarised Raman Spectroscopy

The sample consisted of a single wet *Chara* internodal cell mounted on a microscope slide placed in sample position of the spectrometer. The Raman spectra were acquired on a T64000 Jobin Yvon confocal micro-Raman spectrometer from a position mid-way along the internodal cell. The angle ϕ is defined by the rotation of the sample with respect to the vertical polarisation of the excitation laser (514.4 nm) and an analyser with its polarization axis placed in the vertical plane in front of the spectrometer slit. Spectra were collected over an integration time of one minute.

RESULTS AND DISCUSSION

The ^{13}C CP-MAS spectrum of the dried internode cells is shown in Fig. 3 with the spectra from a sample consisting largely of pure cellulose, Whatman Number 4 filter paper, for comparison. It is clear, although the spectral peaks are somewhat broadened, that the typical spectrum of cellulose is present in this material (Attala & VanderHart, 1984; Attala & VanderHart, 1999). These are labelled according to the cellulose hexose ring shown in Fig. 2. Unfortunately the resonances of many other hexoses in important polysaccharides are found in this spectral region (Jarvis 1990; Jarvis & Apperley 1990) and it is difficult to obtain information about the other polysaccharide components of the cell wall (Bootten *et al.*, 2004). However, it is possible to deduce some ultra-structural aspects of the cellulose microfibrils from the relative intensities of some of the peaks.

The regions labelled C_4 and C_6 consist of two peaks due to cellulose chains on the interior and exterior of the microfibril. In this way the ratio of the integral intensity of the two peaks reflects the lateral dimensions of the cellulose micro fibril (Newman, 1999; Heux *et al.*, 1999). These workers, using certain assumptions about the nature of the cross polarisation process (Hill *et al.*, 1994), have quantified this dimension. There is some disagreement on how exactly this may be done to yield quantified results (Attala & VanderHart, 1999). Using the model of Larsson *et al.* (1997) we suggest a microfibril with a lateral dimension (see Fig. 2) of the order of 1 nm, which is smaller than those found for a range of tissues from other vascular plants (Newman, 1999). This is consistent with observations of the size of the cellulose synthesizing rosette-complex in charophytes (Hotchkiss & Brown, 1988; Okuda & Brown, 1992). However there are a number of uncertainties in this measurement, namely the ability to

accurately subtract the non-cellulosic baseline and the effect of the dynamics of the cross-polarisation on signal integral intensities (Garvey *et al.*, 1998).

Two regions of the cellulose spectra have been enlarged in Figs 4 and 5. These two regions contain information about the nature of the cellulose lattice. For comparison, the spectra of filter paper (a mixed form of cellulose allomorphs — Attala & VanderHart, 1984) and tunicin (pure crystalline cellulose I_β — Belton *et al.*, 1989) have been included. Compared to the spectra of tunicin and filter paper, the spectrum of *C. australis* is of much lower resolution and consists of broad over lapping peaks. The peaks of cellulose I_β are clearly resolved. The two allomorphs do not differ in the conformation of the glucose ring, but rather the pattern of hydrogen bonds between neighbouring cellulose chains in the microfibril (Jarvis, 2000).

Additional spectral features have been assigned according to Jarvis (1990) and Jarvis & Apperley (1990). The broad feature centred at about 175 ppm (Fig. 3) is assigned to various carboxyl groups. In terms of the known chemistry of the cell walls these are most likely to belong to non-esterified and esterified galacturonan groups as well as acetyl groups on various polysaccharides predominantly pectins. The pectin phase is probably not so important in a mechanical sense but is important for its ion-exchange properties (Gillet & Liners, 1996), and may serve as an important nucleation site for the polymerisation of phenyl-propane units to lignin in higher plants (Lairez *et al.*, 2005).

Fig. 3 shows a broad feature centred at a chemical shift of 25 ppm. The feature is likely to comprise of a number of overlapping resonances, most probably due to CH_3– groups in ethyl pectic esters. Another broad feature at ~53 ppm, on the edge of the cellulose surface C_6 crystallite peak at 62 ppm, is due to pectic methyl esters.

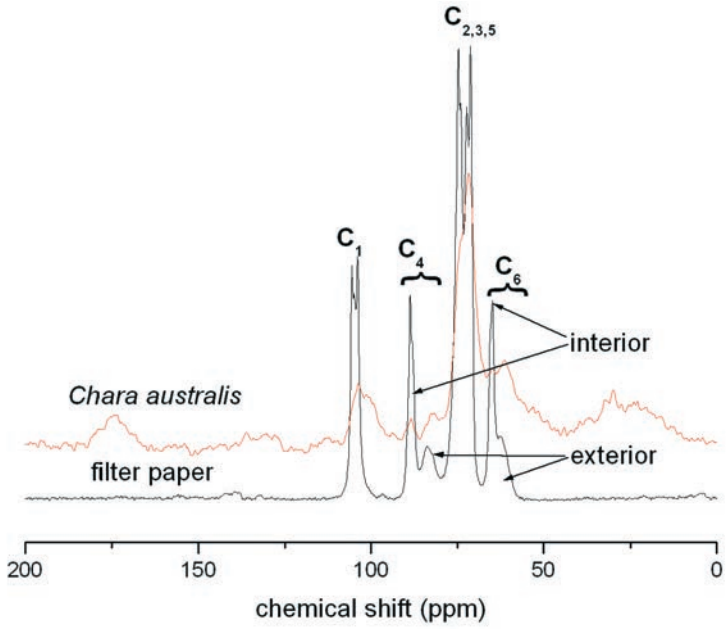

Fig. 3. Comparison between ^{13}C delayed contact CP-MAS NMR spectra of dried *Chara australis* internode cells.

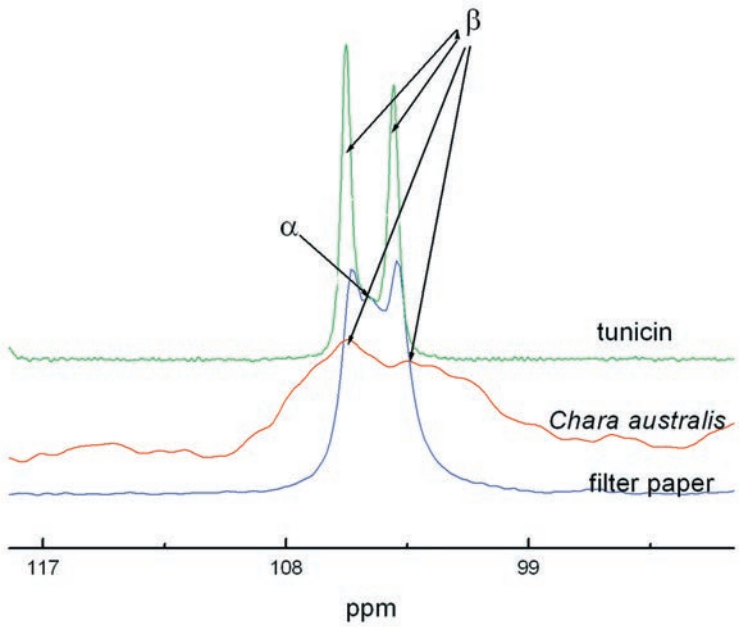

Fig. 4. The C_1 crystallite interior region of the ^{13}C delayed contact CP-MAS spectra of filter paper, tunicin, and dried *Chara australis* internode cells.

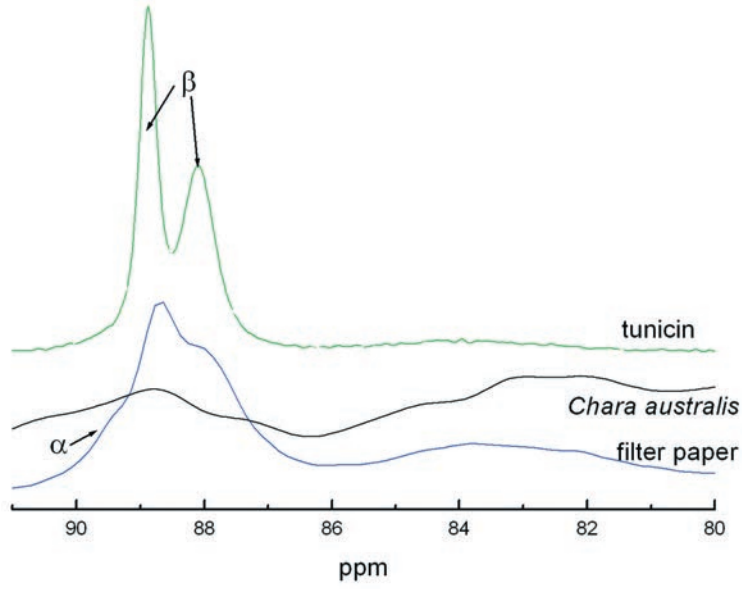

Fig. 5. The C_4 crystallite interior region of the ^{13}C delayed contact CP-MAS spectra of filter paper, tunicin, and dried *Chara australis* internode cells.

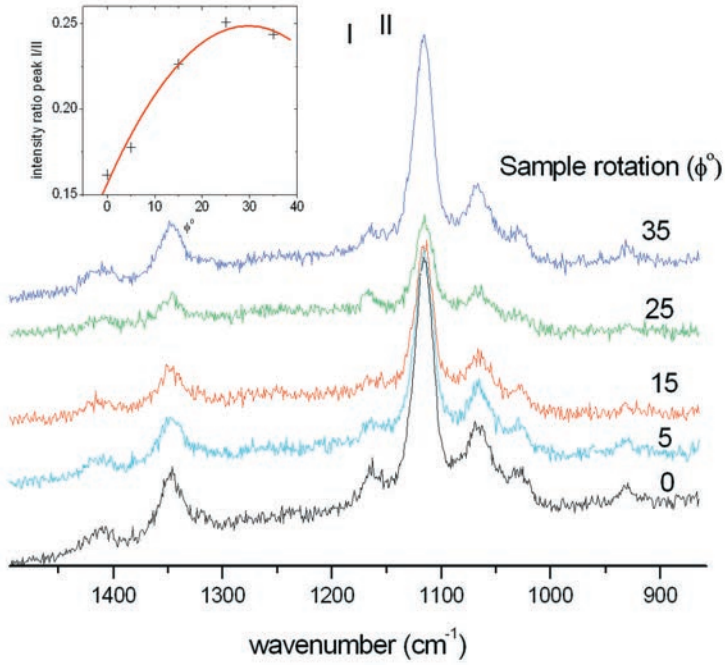

Fig. 6. Raman spectra from a single wet *Chara australis* internode cells as a function of rotation angle. The inset shows fitted (second order polynomial) intensity ratios as a function of sample rotation.

Fig. 6 shows the Raman spectra obtained from a single wet internode cell as a function of sample rotation. Our aim has been to determine the average micro-fibril angle, or orientation of cellulose molecules, in the volume illuminated by the spectrometer laser using the method of Pleasants *et al.* (1998). This method compares the relative intensity of the peaks belonging to the typical cellulose spectrum (Attala, 1976), labelled I and II in Fig. 6. Compared to spectra reported for cellulose in other works (Attala, 1976; Sénè *et al.*, 1994) they are of low signal to noise ratio. The cell walls were quite thin, and while higher resolution spectra could be acquired using longer accumulation times, it was found that the spectrometer's laser would burn a hole through the sample before an adequate number of sample rotations could be performed. Thus, the signal to noise of the spectrum represents a compromise between resolving peaks and covering an adequate rotation.

After subtraction of a base line, and fitting of Gaussian functions to the peaks, it can be shown that the relative intensities of each peak changes as a function of sample rotation. Although there are limited data points with low signal to noise, limiting the precision of the measurement, the ratio of peak I to II passes through a maximum in the angular range 25 to 35 degrees (Fig. 6, inset). Thus for a single fibre, and the region of the cell wall sampled in the experiment by the laser beam, the microfibril angle is in the range 25-35 degrees (Fig. 1). The schematic of the proposed helical arrangement of cellulose microfibrils in a single mature internode cell is shown in Fig. 1. The Raman measurement constitutes a measurement on a single sample volume within Fig. 1.

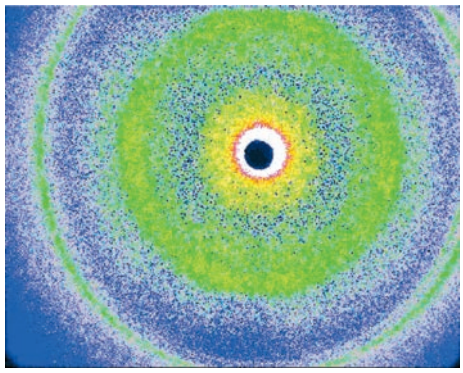

Fig. 7. 2-D x-ray pattern from single orient dried internode.

The x-ray pattern was acquired from a single dried internode cell. The pattern, or counts of x-rays per pixel on the CCD detector behind the sample, is shown in Fig. 7. Around the beam centre are several rings of intensity, which represent the small angle scattering and diffraction peaks respectively. A discussion of the crystallographic origin of the outer two rings (diffraction peaks) in terms of the cellulose lattice can be found in Garvey *et al.* (2005) and references therein. The inner intense region around the direction of the primary beam is due to small angle scattering. Orientational effects, or the microfibril angle, will be apparent in the loss of radial symmetry of the rings around the centre of the pattern (Lichtenegger *et al.*, 1999). Azimuthal integration around each of the outer rings yields a slight asymmetry. It is difficult to quantify this asymmetry and calculate the microfibril angle due to the low number of counts above a broad background. The small size of the microfibrils broadens each x-ray reflection and the amorphous materials each contribute a broad background (Garvey *et al.*, 2005). These observations are consistent with scattering from a material containing narrow cellulose microfibrils embedded in a largely amorphous matrix.

CONCLUSIONS

The organisation of cell wall polymers in *C. australis* shows a number of important similarities to the arrangement in the cell walls of higher plants. The microfibril, the unitary crystal form of cellulose, is a mixture of the monoclinic and triclinic forms which is characteristic of higher plants. This is unlikely to be a consequence of biosynthesis, but rather, as suggest by Jarvis (2000), related to the bending and distortion of the stiff cellulose microfibril in the cell wall.

The size of the microfibril in *C. australis* is small, and contains a smaller number of cellulose chains per microfibril than in higher plants. Since cellulose and microfibrils are generally accepted to simultaneously synthesised (Brown *et al.*, 1996), the observation of microfibrils with small lateral dimension is consistent with the relative size of rosette complexes observed in various charophytes by electron microscopy (Okuda & Brown, 1992; Hotchkis & Brown, 1986).

The understanding of the non-cellulosic component of the cell wall, mainly pectins and hemicelluloses, was hampered by the overlapping and relatively ill-defined nature of peaks in the ^{13}C CP-MAS NMR spectrum. A number of broad peaks were identified which most probably belong to cell wall pectins. However the use of selective degradation of the cell wall by chemical and enzymatic means (Popper & Fry, 2003) combined with the use solid state ^{13}C CP-MAS NMR spectroscopy has the potential to answer important outstanding questions about the composition of the cell wall by allowing the resolution of various important spectral peaks, particularly those belonging to the xyloglucans (Popper & Fry, 2003; Thimm *et al.*, 2002; Bootten *et al.*, 2004). A key question, as

to if the cell wall contains a polymer of phenyl-propane units such as lignin, remains unanswered.

In common with previous work we have found that cellulose in the cell wall is arranged locally in a textured (non-isotropic) fashion. Thus although this is the first measurement of cellulose orientation by Raman spectroscopy, more interesting scientific questions lie in the variation of microfibril angle both within single cells during growth (Probine & Preston, 1961; Probine & Preston, 1962), and between mature cells in different environmental conditions where mechanical demands are quite different (Lichtenegger *et al.*, 1999).

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