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Estimation of Polymer Rigidity in Cell Walls of Growing and Nongrowing Celery Collenchyma by Solid-State Nuclear Magnetic Resonance in Vivo[1]

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When the growth of a plant cell ceases, its walls become more rigid and lose the capacity to extend. Nuclear magnetic resonance relaxation methods were used to determine the molecular mobility of cell wall polymers in growing and nongrowing live celery (*Apium graveolens* L.) collenchyma. To our knowledge, this is the first time this approach has been used in vivo. Decreased polymer mobility in nongrowing cell walls was detected through the ^{13}C-nuclear magnetic resonance spectrum by decreases in the proton spin-spin relaxation time constant and in the intensity of a subspectrum corresponding to highly mobile pectins, which was obtained by a spectral editing technique based on cross-polarization rates. Flexible, highly methyl-esterified pectins decreased in relative quantity when growth ceased. A parallel increase in the net longitudinal orientation of cellulose microfibrils was detected in isolated cell walls by polarized Fourier-transformed infrared spectrometry.

The growth of plants is driven by turgor but controlled by a poorly understood loosening phenomenon within the complex polymer structure of the cell walls. Cell wall loosening is an enzymic process (Carpita and Gibeaut, 1993; Cosgrove, 1993). It allows linear extension (creep) over some hours when the isolated cell walls are experimentally under tension (Cosgrove, 1993; McQueen-Mason, 1995). When growth ceases with maturity, the cell walls become measurably more rigid and incapable of creep in response to added enzymes (Goldberg and Prat, 1982; McQueen-Mason, 1995). To understand the control and eventual cessation of growth in a plant cell, we need to understand how the internal polymer structure of its walls sustains the stresses imposed by turgor pressure and deforms in response to them.

The mechanical properties of plant cell walls, like those of most complex biological solids, are largely unexplained at the molecular level. For composites of synthetic polymers in the solid state, such explanations can be provided by NMR experiments using magnetic relaxation phenomena as a probe of thermal molecular motion (Schaefer et al., 1977; Kenwright and Say, 1993; Stejskal and Memory, 1994), allowing the rigidity of each molecular component to be estimated and related to the rigidity of the material as a whole. Plant cell walls are amenable to this approach, and there is a growing body of data on NMR relaxation behavior (Atalla and VanderHart, 1984, Tekely and Vignon, 1987; MacKay et al., 1988, Newman, 1992; Newman et al., 1994, 1996; Ha et al., 1996; Jarvis et al., 1996).

Nuclear magnetism can be dissipated (relaxed) by a variety of processes that are influenced by thermal motion in different ways. After hydration the polymers of cell walls show wider variation in mobility than most solids, and we have found that the most useful proton relaxation processes are those defined by the time constants $T_{1\rho}$ and T_2 (Ha et al., 1996; Jarvis et al., 1996). Both can be measured in CP experiments in which magnetization is transferred from protons to ^{13}C, so that $T_{1\rho}$ and T_2 values can be associated with the protons surrounding any C atom giving an identifiable peak in the ^{13}C spectrum.

The proton T_2 is often more specific about molecular rigidity because it is averaged over smaller spatial distances (approximately 1–2 nm) than the proton $T_{1\rho}$ by the process of proton spin diffusion. The ^{13}C T_1 can also be readily measured and is not spatially averaged at all (Newman et al., 1996), but is influenced by thermal motion on a much shorter time scale (in microseconds rather than milliseconds), and is therefore more likely to be influenced by the motion of individual functional groups such as rotating hydroxyls or methyls. In a proton T_2 experiment on hydrated onion (*Allium sepa L.*) cell walls modified to eliminate most of the averaging by spin diffusion, we showed that all of the ring Cs within a given polysaccharide gave similar T_2 values, and therefore were responding to motions of whole monosaccharide units (twisting or undulation of the polymer chain) (M.A. Ha and M.C. Jarvis, unpublished data).

The results of such NMR relaxation experiments can be presented either as relaxation time constants for specific peaks in the ^{13}C spectrum, or as a number of spectra

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Abbreviations: CP, cross-polarization; FTIR, Fourier-transformed IR; MAS, magic-angle spinning; $T_{1\rho}$, spin-lattice relaxation time constant; T_2, spin-spin relaxation time constant.

derived from mobile and more rigid polymers. Indeed, the standard (CP-MAS) spectrum of hydrated cell walls is derived only from those polymers with medium and high rigidity, which excludes the highly mobile polysaccharides (Foster et al., 1996; Ha et al., 1996). We have shown that the most mobile polymers in hydrated cell walls, including pectic galactans and methyl-esterified galacturonans, cross-polarize so slowly that they are lost from the spectra obtained at normal CP contact times (Ha et al., 1996). They also do not appear in spectra recorded under conventional solution-state conditions (Foster et al., 1996).

The ^{13}C spectrum of these mobile polymers in hydrated onion cell walls was recovered (Ha et al., 1996) by extending the contact time into the region (1–10 ms) in which the signal intensity begins to decay by the proton $T_{1\rho}$ process. The apparent rate of $T_{1\rho}$ decay was slow for mobile polymers because CP was still progressing throughout this period. A parallel delayed-contact experiment was used to correct for true $T_{1\rho}$ decay so that the spectrum of the mobile components could be extracted (Ha et al., 1996). Independently, Foster et al. (1996) obtained very similar "mobile" spectra from onion cell walls by a quite different approach: CP was omitted and the rigid components were edited out by allowing insufficient time for ^{13}C T_1 relaxation. This confirms the validity of the method of Ha et al. (1996) that was adopted here.

Since growth is a characteristic of living plant cells, relaxation experiments to study its molecular basis by NMR would be best carried out in vivo. To our knowledge, nothing like this has previously been attempted on any living tissue because to identify polymer molecules through the ^{13}C-NMR spectrum it is necessary to use MAS (Stejskal and Memory, 1994), which destroys most living cells by centrifugal force. The collenchyma cells of celery (*Apium graveolens* L.), however, withstand MAS forces due to the unusual mechanical strength of their walls, and in vivo solid-state ^{13}C-NMR spectra of these cell walls have been obtained by Jarvis and Apperley (1990).

The anatomy of maturing celery collenchyma has been described by Vian et al. (1993). Much of its cell wall mass is concentrated in rib-like thickenings at the cell corners. These appear to be laid down in an ordered structure with helicoidal layering of the microfibrils, which is dissipated in the process of extension growth. When growth ceases, the helicoidal pattern of deposition continues so that the ribs become thicker and coalesce, retaining their helicoidal order in those layers formed after the cessation of growth (Vian et al., 1993). Like the structurally similar epidermal cell walls that control stem growth in young dicotyledonous plants, collenchyma cell walls are loosened by auxin or acid to permit cell extension (Pilet and Roland, 1974; Jaccard and Pilet, 1977). Here we compare the solid-state NMR relaxation properties of living collenchyma from growing and nongrowing celery petioles to find the molecular origin of the macroscopic mechanical changes at the end of the growing period. It is not known whether these changes involve the same polymers as the wall-loosening that permits growth.

MATERIALS AND METHODS

Collenchyma strands were excised with a razor blade from the top 50 mm (the growing region) of immature celery (*Apium graveolens* L.) petioles that were approximately 80 mm in length from the base to the first internode; and from the top 200 mm of mature, nongrowing petioles approximately 300 mm in length. For the NMR experiments, excess water was removed from the strands by pressing lightly between sheets of filter paper until approximately 70% moisture was reached. The cells regained turgor when rehydrated after the experiment. For FTIR spectrometry, longitudinal sections approximately 50 μm thick were cut with a microtome from fresh collenchyma strands and dehydrated completely between filter paper under pressure, giving dry transparent films from which spectra were recorded directly. Only the NMR experiments were conducted on living material.

Tensile Testing

Cell walls were prepared by extraction of most of the cell contents without disruption of the collenchyma tissue (Jarvis et al., 1984). The tensile modulus was determined on wet cell wall strands 50 mm long (growing) or 100 mm long (nongrowing) (aspect ratio, approximately 300) on a home-built Instron-type test system with a draw rate of 40 $\mu m\ s^{-1}$.

NMR Spectrometry

All NMR experiments were carried out at 75 MHz for ^{13}C with MAS at 2 to 3 kHz. Both nongrowing and growing strands of celery collenchyma proved to be robust enough to remain alive at these relatively slow MAS rates, which were nevertheless sufficient to eliminate spinning side bands (except from the carboxyl resonances). A more serious problem was the absorption of proton radio-frequency energy by water and water-soluble constituents of the cytoplasm, particularly in the growing tissue, which had a higher ratio of cytoplasm to cell wall. Because of this problem it was exceptionally difficult to tune the MAS probe, and the following procedure had to be used to obtain a Hartmann-Hahn match.

The proton radio-frequency field was set to the maximum obtainable (approximately 70 kHz), and the ^{13}C field was adjusted until a maximum in signal intensity at 73 ppm indicated the Hartmann-Hahn condition. The initial proton 90° pulse was then readjusted for maximum signal intensity at 73 ppm. The optimum length of the proton 90° pulse was approximately 6 μs, which showed that more than one-half of the proton radio-frequency energy was being absorbed before reaching the cell wall polymers. For this reason and because the growing tissue contained less cell wall mass than the nongrowing tissue, signal to noise ratios were always lower in the growing tissue. A further restriction on the signal to noise ratio in the growing tissue resulted from the fact that the duration of each experiment had to be limited to 5 h because incubation of growing collenchyma strands from tissue for longer periods caused

the tensile modulus to increase, presumably by the same maturation mechanism responsible for the higher tensile modulus of the nongrowing tissue (McQueen-Mason, 1995).

Spectra labeled "rigid" were recorded under standard CP-MAS conditions. Resonance assignments are based on published data (Jarvis and Apperley, 1990; Ha et al., 1996). The proton T_2 was measured by inserting a variable delay of up to 20 μs after the initial 90° proton pulse (Tekely and Vignon, 1987; Newman, 1992). The proton $T_{1\rho}$ was measured by a standard delayed-contact pulse sequence, with proton spin-locking for 2.5 ms prior to the Hartmann-Hahn contact. CP rates were measured in a variable-contact experiment, with contact times ranging from 30 μs to 16 ms.

Spectra labeled "mobile" are difference spectra ($x - y$) between the 3-ms contact time (x) and the 0.5-ms contact time (y) after a 2.5-ms delay (Tekely et al., 1995; Ha et al., 1996). These experiments were repeated twice, with qualitatively identical results: the magnitude of the mobile spectrum for growing cell walls varied but was always at least three times the intensity of the mobile spectrum for the nongrowing cell walls when the data from growing and nongrowing cell walls were normalized to give equal total signal intensity to one another in the standard CP-MAS (rigid) spectra.

RESULTS AND DISCUSSION

Living collenchyma strands were excised from growing and mature, nongrowing celery petioles. The nongrowing collenchyma strands were wider than the growing strands (0.65 and 0.36 mm, respectively). This and the increase in cell wall thickness noted by Vian et al. (1993) meant that the mass of cell wall per unit of length was greater by a factor of 2.3 in the nongrowing collenchyma strands (Fig. 1). At the same time the tensile modulus of the cell walls increased by a factor of four (Fig. 1), implying internal changes in cell wall structure. The NMR experiments were designed to explain this increased rigidity of the nongrowing collenchyma cell walls.

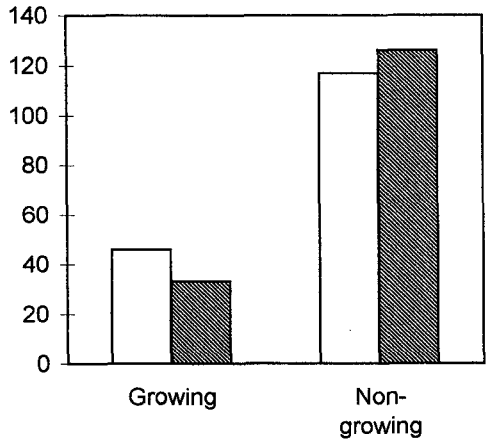

Figure 1. Tensile modulus of cell walls (shaded columns; in N mm^{-2}) and mass of hydrated cell wall per unit length in excised celery collenchyma strands (open columns; $\mu g\ mm^{-1}$).

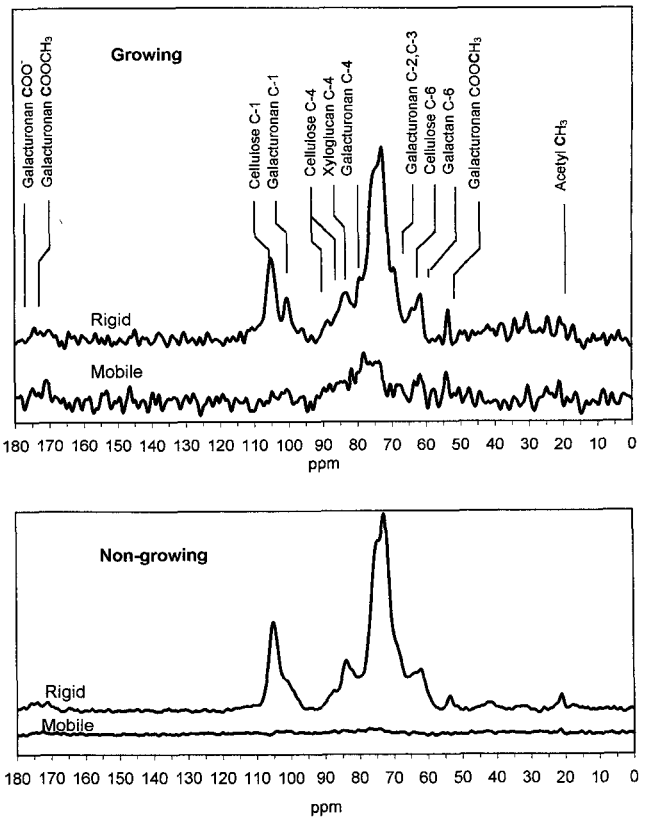

Figure 2. ^{13}C-NMR spectra for living collenchyma tissue from growing and nongrowing celery petioles. Spectra labeled "Rigid" were obtained under standard CP-MAS conditions; spectra labeled "Mobile" were derived from a minor group of polysaccharides that were undergoing so much thermal motion that CP from protons to ^{13}C was disrupted and had to be continued for contact times of >2 ms to obtain measurable signal intensity. These mobile spectra are difference spectra between a 3-ms contact experiment and a delayed-contact experiment; see "Materials and Methods" for details. For each tissue the same vertical scale is used for the rigid and mobile spectra.

The CP-MAS ^{13}C spectra of living collenchyma strands (Fig. 2, spectra labeled "rigid") were derived almost entirely from the cell wall polysaccharides: cytoplasmic proteins and other constituents remained in solution and did not contribute to the CP-MAS spectra. The CP-MAS spectra of the nongrowing cells included a smaller contribution from pectins (in particular, from methyl-esterified pectic galacturonan) than those of the growing cells.

However, these CP-MAS ^{13}C spectra, recorded with the relatively short CP contact time of 0.5 ms, do not represent the entire cell wall. Foster et al. (1996) and Ha et al. (1996) have shown independently that onion and other cell walls contain highly mobile methyl-esterified galacturonans and β-(1,4)-linked galactans that cross-polarize too slowly to be represented in conventional CP-MAS spectra. For these pectic polymers CP proceeds over a longer period (1–10 ms) simultaneously with the decay of the spectrum by the proton spin-lattice relaxation process, which is characterized by the time constant $T_{1\rho}$.

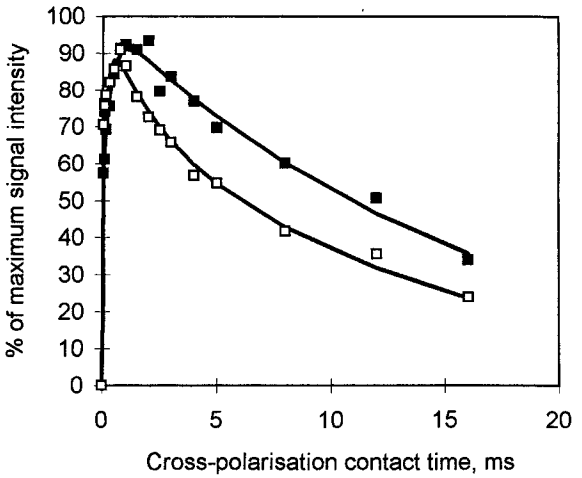

Figure 3. Kinetics of CP and $T_{1\rho}$ decay of the signals from C-4 of cellulose (□; 84 ppm) and C-2, C-3, and C-5 of pectic galacturonans (■; 69 ppm) in live, nongrowing celery collenchyma. Varying contact times were allowed for CP. The intensity of both signals increased during the first 1 ms, but the galacturonan signal decayed more slowly thereafter, as CP continued in highly mobile galacturonan components. The fitted curves are based on the model of Fenwick et al. (1996).

Normally, in the absence of such mobile components, a plot of signal intensity against contact time increases in the 1st ms as CP is completed and then decreases as $T_{1\rho}$ decay proceeds (Fig. 3, 84 ppm). For a signal derived from a highly mobile component alone, the initial increase is much slower (Ha et al., 1996). The pectic peaks in the ^{13}C spectrum are derived from a mixture of highly mobile and more rigid components. The intensity of these peaks increases rapidly with increasing contact time as the rigid component cross-polarizes, but the subsequent decrease is slower than would be expected for the rigid component alone, because the signal intensity from the mobile component is still increasing (Fig. 3, 69 ppm).

Therefore, the apparent proton $T_{1\rho}$ in this experiment was longer for pectic than for cellulosic resonances, whereas the true values showed the reverse behavior: 3.9 ms for the pectic signal at 69 ppm and 5.4 ms for the cellulose signal at 84 ppm, in a delayed-contact experiment in which the CP duration was held constant (Fig. 4). The same characteristics were shown by hydrated cell walls from onion bulbs (Ha et al., 1996), citrus peel (Jarvis et al., 1996), tomato pericarp (Fenwick et al., 1996), and potato tubers (R.J. Vietor and M.C. Jarvis, unpublished data), and appear to be diagnostic for the presence of highly mobile pectic polymers.

The spectrum of the highly mobile material in the celery tissue was recovered by the method of Ha et al. (1996) and is shown in Figure 2. The spectra labeled "mobile" in Figure 2 represent polymer fractions cross-polarizing up to 3 ms after the commencement of the CP contact. The spectral intensity associated with the mobile fraction was approximately 25% of that of the rigid fraction of the walls from growing cells in the experiment shown, but less than 10% for the walls of nongrowing cells. These percentages are subject to considerable uncertainty for two reasons: the low signal to noise ratio of the mobile spectra and the assumption that they represent a fixed fraction of the highly mobile polymer component (i.e. in different samples its signal intensity appears and decays in a similar fashion with increasing contact time).

Figure 3 shows this relationship of signal intensity to contact time for the nongrowing collenchyma, but we were unable to obtain enough of a signal to noise ratio to construct comparable curves for the growing tissue. Data qualitatively similar to those in Figure 3, however, have been obtained from cell walls of a variety of species (Fenwick et al., 1996; Ha et al., 1996; Jarvis et al., 1996; R.J. Vietor and M.C. Jarvis, unpublished data). The relative magnitudes of the mobile spectrum from growing and nongrowing celery collenchyma may, therefore, suggest that the cell walls of the growing tissue were richer in these highly mobile polysaccharides.

The mobility of the polymers in the rigid fraction of the cell walls was estimated from the proton T_2, which increases with molecular motion in the kilohertz frequency range (Ha et al., 1996), and from the rotating-frame proton $T_{1\rho}$, which decreases with molecular motion in hydrated cell walls (Fenwick et al., 1996). The proton T_2 was consistently longer, and the $T_{1\rho}$ shorter, in those parts of the rigid spectrum corresponding to pectins (Fig. 3), confirming the increased mobility of the pectic fraction of the cell wall. The thermal motions concerned may include hydroxyl rotations and exchanges (Reynhardt and Latanowicz, 1996), but are dominated by the twisting and undulation of chain segments, motions that are more likely to be relevant to the mechanical properties of the cell wall as a whole.

Evidence for this comes from a proton T_2 experiment (M.A. Ha, M.C. Jarvis, and D.C. Apperley, unpublished data) using a contact time of only 25 ms, too short for

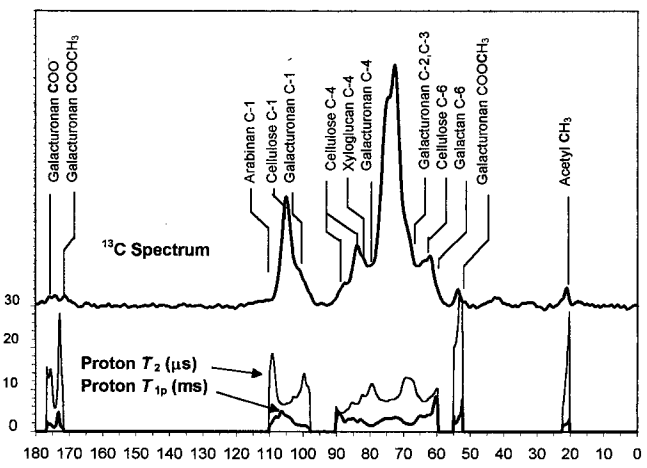

Figure 4. Proton T_2 and $T_{1\rho}$ plotted across the ^{13}C spectrum of cell walls of living, nongrowing celery collenchyma. Under the conditions used the proton T_2 increases and the $T_{1\rho}$ decreases with increasing polymer mobility (Ha et al., 1996; Newman et al., 1996). Therefore, peaks in the proton T_2 plot and minima in the proton $T_{1\rho}$ plot correspond to mobile polymers. Gaps in the T_2 and $T_{1\rho}$ plots indicate spectral regions where there was insufficient signal to determine either time constant.

appreciable spatial averaging of the T_2. Similar proton T_2 values were recorded for all resonances derived from the ring Cs of any one polysaccharide. Similar spectral plots of the proton T_2 and $T_{1\rho}$ could not be constructed for the rigid fraction of the growing cell walls because of inadequate signal to noise ratios, but the mean T_2 value for the 72- to 74-ppm region corresponding to ring Cs of all cell wall polysaccharides was 30 μs compared with 16 μs in the nongrowing cell walls. This indicates slightly greater overall mobility than in the nongrowing cell walls.

The principal effect detected by NMR was therefore a decrease in the proportion of the very mobile, methyl-esterified pectin in the cell walls when growth ceased. Decreased methyl-esterification of pectins has been found in other maturing plant tissues (Goldberg et al., 1994), although its significance is uncertain. In collenchyma cell walls pectins control the spacing between the layers of cross-linked microfibrils, but do not appear to be major contributors to the mechanical integrity of each layer (Jarvis, 1992; M.C. Jarvis, unpublished data). If the interlayer pectins are fluid enough, they may permit a degree of shear between the layers, allowing the microfibril orientation to change under tension in each layer relative to the next. Loss of the capacity for interlayer shear would restrict extension even in the presence of active, growth-promoting enzymes (Carpita and Gibeaut, 1993; McQueen-Mason, 1995) that break cross-links within each layer.

The NMR experiments reported here are not sensitive to the orientation of polymers, nor to the orientation of their motions. Therefore, a change in the mean orientation of the microfibrils can in principle alter the tensile properties of the cell wall without being detectable by NMR. The tensile moduli of composites decrease sharply with fiber angles up to 30° from the stress, but vary little at greater angles (Harris, 1980). The walls of celery collenchyma cells have a helical outer layer, with helicoidal thickenings at the corners that coalesce on maturity to form the thick inner wall (Vian et al., 1993). A wide range of microfibril orientations is therefore present. Polarized FTIR spectrometry was used to assess the mean orientation of polysaccharides within the cell walls (Fig. 5).

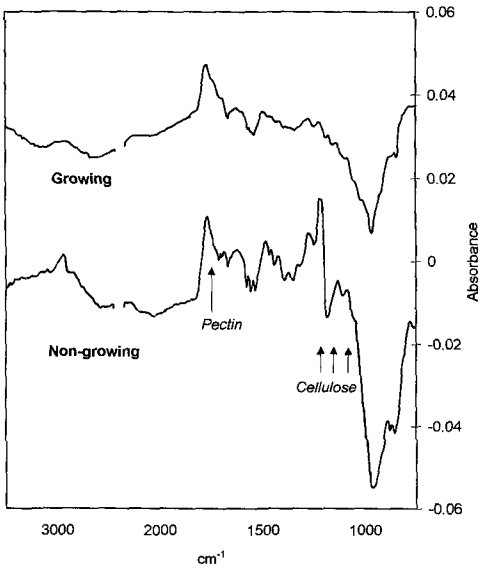

Figure 5. Polarized FTIR difference spectra for isolated collenchyma cell walls from growing and nongrowing celery petioles, indicating the degree of orientation of cellulose and other polymers. The spectra obtained with the plane of polarization parallel to the cell axes were subtracted from the spectra with polarization perpendicular to the cell axes.

In the spectra of the nongrowing cell walls, positive difference bands at 1170, 1121, and 1075 cm^{-1} correspond to those assigned to cellulose (McCann et al., 1992), implying in this case a net orientation of the microfibrils parallel to the collenchyma strands. A difference band at 1750 cm^{-1} may be assigned to pectin. The polarization difference spectra therefore showed that both cellulose and pectins had considerable net orientation in the nongrowing collenchyma cell walls. Difference spectra from the growing cell walls were similar in form, but showed less than one-half the intensity, implying less net orientation. As a control, the spectrum obtained from transverse sections of parenchyma cell walls from the same nongrowing celery petioles (not shown) was almost unaffected by orientation relative to the plane of polarization.

It is concluded that the tensile rigidity of mature collenchyma that is associated with the loss of the capacity for extension growth is the result of both the net longitudinal orientation of its microfibrils and the relative lack of methylated pectins to act as a molecular lubricant between the microfibril layers.

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