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Infra-red microspectroscopy of hydrated biological systems: design and construction of a new cell with atmospheric control for the study of plant cell walls

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Summary

A new hydration cell has been constructed that allows wet biological samples, or samples of controlled moisture content, to be analysed *in situ* using infra-red microspectroscopy. The cell has been used to show that there are minor spectral changes associated with the hydration of pectin and tomato pericarp cell walls and slightly more significant changes in onion and carrot epidermal walls. The cell was also used to show that molecular orientations of polymers, previously observed in dry cell walls, were also to be seen in hydrated walls. For cell walls of onion and carrot epidermis, it was shown that the orientations of cell wall polymers are not affected by hydration. Furthermore, the polymer orientations in cell walls of fully elongated onion epidermal cells are different from those of elongating carrot epidermis. By using the hydration cell, it is now possible to investigate both fresh samples and wet systems routinely. The applications of this to the study of biological materials with infra-red microspectroscopy are discussed.

Introduction

Infra-red microscopy or, more correctly, infra-red microspectroscopy is a relatively new tool for investigating biological materials in which infra-red spectra are collected from regions as small as $10 \times 10\,\mu m$. These spectra can be used to determine the composition and structure (e.g. molecular orientation) at discrete points within a sample or many spectra can be collected and used to produce functional group maps, representing the distribution of molecular species within a sample. These techniques have much to offer the microscopist since images can be produced that not only show structural features but also provide the molecular basis for the observed structure. For example, one can determine the spatial arrangement of biopolymers, the mechanism of interaction between them as well as their crystallinity, phase and a wide range of other chemical and physical parameters. As a result, infra-red spectroscopy can provide the microscopist with a great deal more information about the nature of a sample. All this information can be obtained *in situ* without the need for sample destruction and separate analysis and the chemical information can be directly correlated to the visual image. One system where new insight has been obtained using such methods is the plant cell wall.

Several models have been proposed for the molecular structure of higher plant cell walls (McCann & Roberts, 1991; Carpita & Gibeaut, 1993), and for how that structure is modified during cell growth (Carpita & Gibeaut, 1993). However, the diversity of cell wall composition between species, between tissue types and even between domains around a single cell makes it unlikely that a generic model of wall architecture will be appropriate for all systems. It is therefore essential to find methods of cell wall analysis that are applicable at the single cell wall level. Cell wall architecture can be directly visualized in the electron microscope (McCann *et al.*, 1990) and the atomic force microscope (Kirby *et al.*, 1996) to provide basic parameters of wall construction but such methods do not provide direct evidence on the physical and chemical state of molecules or their interactions. However, infra-red microspectroscopy can simultaneously provide both compositional and structural information noninvasively from single plant cell walls (McCann *et al.*, 1992).

Infra-red spectroscopy is a powerful technique for the determination of molecular structure and for quantitative analysis of complex mixtures (George & McIntyre, 1987;

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Wilson & Goodfellow, 1994). It can also provide information on the conformation and, using polarized radiation, orientation of biopolymers including proteins and polysaccharides (Keighley, 1976; Parker, 1983). Infra-red techniques have been used to determine molecular orientation in pea stem cell walls (Morikawa *et al.*, 1978), oat coleoptile cell walls (Morikawa & Senda, 1978) and in pea epidermal cell walls (Hayashi *et al.*, 1980). However, in these examples it was necessary to use relatively large samples and it was not possible to determine the exact relative orientation of the cell walls with respect to the polarization of the infra-red radiation.

More recently, with the addition of a microscope accessory to the Fourier transform infra-red spectrometer, infra-red microspectroscopic techniques have been used to investigate single cell walls. These studies have shown that it is possible to obtain spectral information from regions of a single cell wall as small as $20 \times 20\,\mu m$. Infra-red microspectroscopy was used to follow the compositional changes and molecular interactions during the sequential extraction of onion parenchyma cell walls (McCann *et al.*, 1992), and to observe molecular orientations in cell walls of elongated tobacco cells (McCann *et al.*, 1994) and carrot cells (McCann *et al.*, 1993). In the latter case the authors were able to show that various biopolymers were orientated in cell walls of the stele, epidermis and cortex of a section of aetiolated carrot seedling.

However, the validity of these results has to be in question. The main reason for this doubt arises from the fact that most infra-red spectroscopic studies, and many other microscopic techniques used to probe the cell wall, have used dry material. Water is the major cell wall component, affecting the hydrogen bonding between wall components and the strength of the associations between hydrogen-bonded polymers. It is assumed that dehydrating cell walls will cause a tighter association between hydrogen-bonded polymers. Consequently, cohesion and adhesion forces between cellulose microfibrils as well as matrix polysaccharides would be expected to be greatly affected by changes of hydration. The removal of water from the cell wall may alter biopolymer conformation, resulting in stresses being applied to cell walls that may impose molecular alignment on cell wall components. In order to have confidence in the observations made so far, and for other studies on biological systems, it is vitally important to study hydrated samples and to be able to control carefully the level of hydration of cell walls, plant sections and extracted polymers. However, presenting hydrated samples to an infra-red microscope is technically demanding. The environment within and around such instruments is usually very dry, and the heating effect of a concentrated infra-red beam considerably exacerbates the difficulties in maintaining hydration levels. In this paper we report the design and construction of a cell for use in conjunction with an infra-red microscope that allows the investigation of hydrated cell walls and has further scope for use with biological systems generally. We also report the results obtained with this cell with hydrated pectin, tomato cell walls and molecular orientation in the cell walls of onion epidermis.

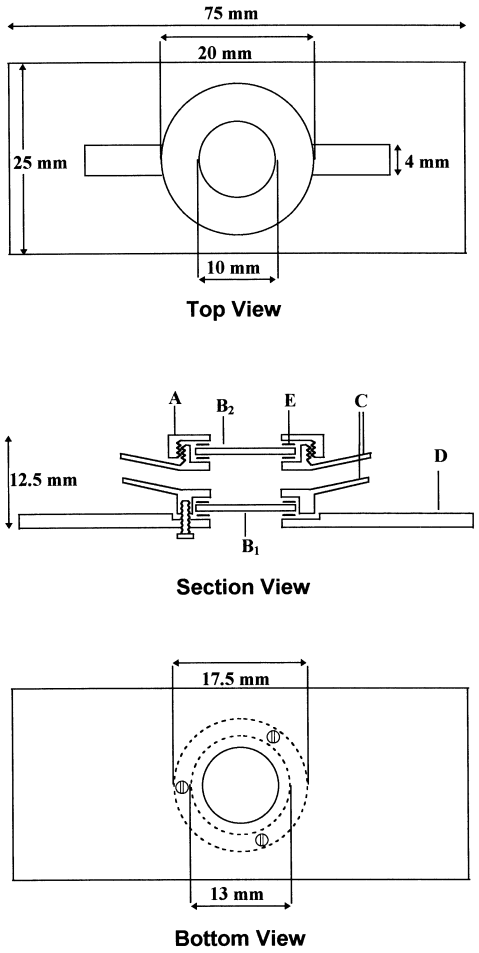

Fig. 1. The schematic of hydration cell (not to scale). The cell consists of two 13-mm-diameter × 1-mm-thick polished barium fluoride discs (B1, B2). The lower disc is secured to holder (D) by cell body (C) with O-ring seals (E). The top disc is held in place by a screw-on top (A) with O-ring seals. The cell is connected to controlled atmosphere via 4-mm-diameter pipes (full description in text).

Materials and methods

Cell design and construction

The cell was constructed for use with the standard X–Y stage and 15× Cassegrainian objective of a Spectra-Tech IR-Plan infra-red microscope. This necessitated that the dimensions of the cell holder be no larger than 75 mm × 25 mm with a maximum height of 12·5 mm. The

cell is illustrated in Fig. 1. The holder (D) was made of stainless steel with a hole of 10 mm diameter drilled through it. The holder was recessed around this hole to a depth of about 0·5 mm to a diameter equal to the outer diameter of the brass cell body (C) to assist cell assembly. A 13-mm-diameter barium fluoride disc (B1) (Crystran Ltd, Poole, Dorset) was mounted in the recessed base of the cell body. The recess was ≈ 1·5 mm to allow for a window and two O-ring seals (E). The window was held in place by the cell body which was mounted to the holder by three small countersunk screws attached from the bottom of the holder. The small O-ring seals were used between the barium fluoride disc and the cell body to ensure a good seal and prevent damage to the disc. The disc thickness was 1 mm. The cell body held the lower disc in place when it was attached to the holder by three small screws, and was recessed to receive a second, upper, barium fluoride disc. The upper disc (B2) was held in place with a screw-on cap made of brass (A) and sealed with O-rings. When the cell was assembled the internal gap between the two windows was 7 mm. The body of the cell was fitted with 4-mm-diameter tubes allowing the cell to be connected via plastic tubes to a chamber containing water or saturated salt solutions. The use of saturated salt solutions (Greenspan, 1977) allowed the relative humidity within the cell to be carefully controlled for cell wall hydration or to prevent samples from drying out when exposed to the infra-red beam. In practice, a peristaltic pump was used to equilibrate the atmosphere in the cell with that over the saturated salt solutions. Alternatively, where the need was only to prevent sample drying, small water-saturated sponges could be inserted into the tubes (C) before the ends were sealed.

Material and spectral acquisition

Commercial citrus pectin (type 192) was obtained from Citrus Colloids Ltd. Mature-green tomatoes (cv. Ragu) were kind gifts from Dr M. J. Gidley, Unilever plc. Cell wall material (CWM) of tomatoes was prepared from pericarp tissue by grinding in liquid nitrogen as previously described (McCann *et al.*, 1990). The pectin sample and the CWM samples were applied to the lower disc (Fig. 1, B1) of the hydration cell from aqueous suspensions in order to produce a thin and even layer of sample on the disc. Onions (cv. Jumbo) were obtained locally. The epidermal monolayer from between two layers of a fresh onion bulb was prepared by separating the layers, removing the epidermal layer and carefully washing and scraping it of parenchyma cells adhered to one side of the epidermis. A strip (≈ 10 mm long and 2–3 mm wide) of the epidermis was placed flat on the lower barium fluoride disc of the hydration cell directly after washing and scraping. The carrot epidermal layer was obtained from the aetiolated stem of a carrot seedling grown on agar in a sealed Petri dish in the dark for 7 days. A segment of the stem was squashed flat on a barium fluoride disc and rinsed thoroughly with distilled water.

After assembling the barium fluoride disc with the sample and the other parts of the hydration cell, the hydration cell was mounted on the stage of the infra-red microscope (Spectra-Tech, IR-Plan). The microscope was connected to a Bio-Rad FTS60 Fourier transform infra-red (FTIR) spectrometer. In all experiments, a background single beam spectrum was collected of a clean, sample-free area of the barium fluoride disc just before or after a single-beam infra-red spectrum of the sample was obtained, in order to convert the single beam spectrum to an absorption spectrum. The same spectral acquisition parameters and aperture size were used for both the sample spectrum and the background spectrum. An infra-red polarizer (KRS5 substrate, Graseby Specac, Orpington, U.K.) could be inserted into the beam for obtaining spectra with polarized light and the incident light could be arranged to be either parallel or perpendicular to a fixed axis of the sample plane.

Spectra were recorded at a resolution of 8 cm^{-1} with 64 interferograms being co-added before Fourier transformation, except for the spectra taken from freshly prepared CWM of tomato, where a resolution of 4 cm^{-1} and 256 scans were used. All the experiments described here were repeated three times and the spectra acquired under the same experimental conditions were found to be highly reproducible.

Hydration of citrus pectin and freeze-dried CWM

An aqueous suspension of citrus pectin (type 192) or a freeze-dried sample of tomato CWM was applied to the disc of the hydration cell and was then dried in an oven at 37 °C and stored in a desiccator over phosphorus pentoxide before the cell top and upper disc were attached. After infra-red spectra of a dry sample in the closed hydration cell were obtained, the sample was then exposed to an atmosphere of 94% relative humidity (RH) above saturated potassium sulphate solution at 22 °C. During exposure, equilibration was assisted by a peristaltic pump pumping the air through the cell with a flow rate of 15 mL min^{-1}. Spectra were collected after the equilibration. In the next step, to hydrate the sample fully, the plastic tubes connecting the cell to the atmosphere above the saturated potassium sulphate solution were removed, water-saturated sponges were inserted into the pipes and the outer ends of the side tubes of the hydration cell were sealed. Again, after equilibration, spectra of the fully hydrated sample were collected. Finally, the sponges were removed and the cell was connected to a drying atmosphere over phosphorus pentoxide. Spectra were collected when the pectin or the CWM were completely dry.

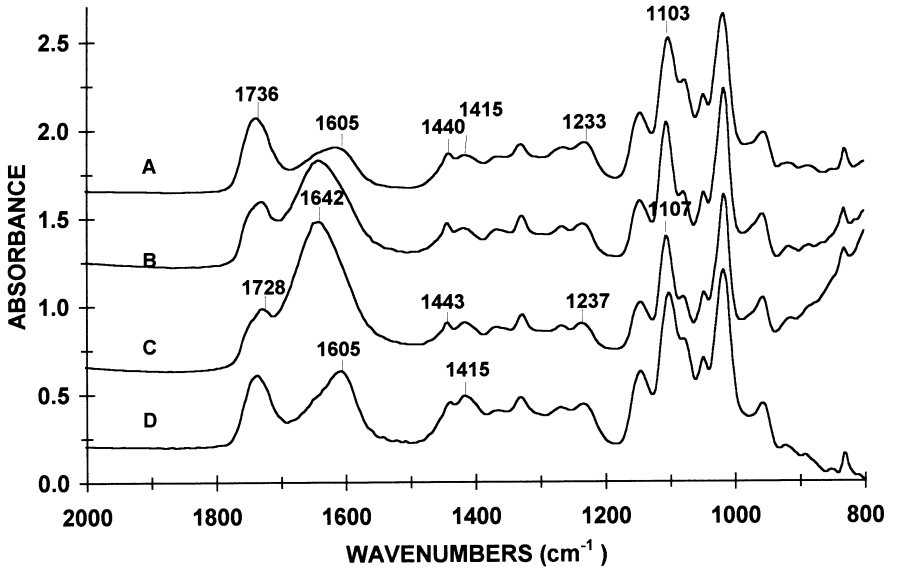

Fig. 2. Spectra of dry, hydrated and re-dried pectin in hydration cell. Top to bottom: spectrum of initial dry pectin (A); pectin exposed to 94% RH (B); fully hydrated pectin exposed to water-saturated sponge (C); and pectin re-dried after hydration (D).

Dehydration of freshly prepared CWM

An experiment was carried out to investigate the drying process using freshly prepared CWM from pericarp of mature-green tomatoes. An aqueous suspension of CWM was applied to the lower disc of the hydration cell. Infra-red absorption spectra were obtained from the wet CWM sample first. Spectra were then taken from the sample in equilibrium at 94% RH and finally from the sample dried completely as described above. All spectra were collected with constant aperture size and from the same area of CWM for direct comparison.

Polarized FTIR microspectroscopy of epidermal cell walls

Spectra of onion epidermal cell walls were obtained from single cells using an aperture of about 50 μm (along the elongation axis) × the width of the cell. Spectra of carrot epidermal cells were obtained from areas containing a few cells using aperture sizes of about 50 μm (along the elongation axis) and 30 μm wide. The spectra of dried cells were obtained after freshly prepared samples were applied to the barium fluoride plates wet and allowed to dry gently in an oven at 40 °C. For recording spectra of hydrated onion and carrot epidermal samples, the freshly prepared sample was applied wet and water-saturated sponges were inserted into the side tubes of the cell to prevent excessive sample drying. By introducing polarized light to the sample, a pair of spectra were taken with the incident light polarized parallel and perpendicular to the long axis of the cells. Each pair of single beam spectra for the two polarization directions were taken with the sample position unchanged. The background spectrum used for each spectrum was taken with the same polarization as the corresponding sample spectrum. To obtain the difference spectrum between each pair of spectra for the two polarization directions, a scaling factor of unity was used for the subtraction since the same sample area was used for taking both spectra.

Polarized spectra of both dry and wet cell walls were collected in the same way and fresh samples were used in each case.

Results

Hydration of citrus pectin

Hydration of citrus pectin 192, a pectin with 37–42% degree of esterification, was carried out at first in order to test the performance of the hydration cell and to determine the effect of water on the conformation of the polymer, and the consequent spectral changes induced by water. Pectin is one of the major components in many dicotyledonous plant cell walls and the most hydrophillic one. Hydration of freeze-dried mature-green tomato CWM was then carried out in the same way for the purposes of comparison.

The results of the spectroscopic study of the hydration of pectin are shown in Fig. 2. The four spectra in the figure are intensity normalized and offset for clarity. From top to bottom the four spectra were recorded at the four stages: A, dried; B, hydrated at 94% RH; C, fully hydrated and D, re-dried.

The assignment of the bands in infra-red spectra of pectins are still under discussion because infra-red spectra of pectins depend on the source of pectin and the methods used for obtaining the pectins. However, some assignments (Filippov, 1992) are summarized here. Most of the bands in the carbohydrate fingerprint region 900–1200 cm^{-1} are the vibrations associated with the skeletal rings of the sugar

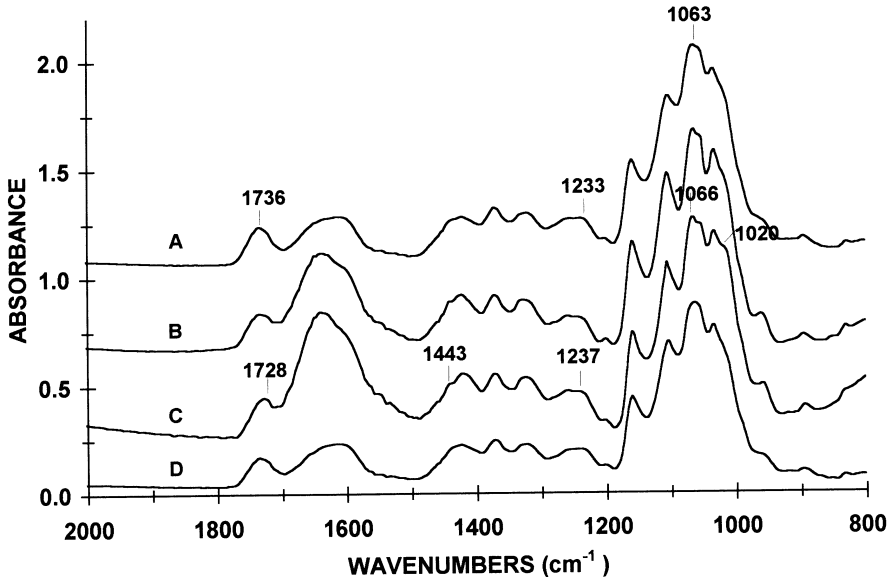

Fig. 3. Spectra of dry, hydrated and re-dried pericarp CWM of freeze-dried mature-green tomatoes in hydration cell. Top to bottom: spectrum of initial dry tomato cell walls (A); tomato cell walls exposed to 94% RH (B); fully hydrated tomato cell walls exposed to water-saturated sponge (C); and tomato cell walls re-dried after hydration (D).

monomers of pectin. The absorption bands around 1210–1330 cm^{-1} originate from hydroxyl groups of the polysaccharide backbones and the absorptions at 1330–1500 cm^{-1} originate mainly from CCH and OCH vibrations. The absorptions at 1605 and 1415 cm^{-1} are the asymmetric and symmetric stretching of the carboxylic acid ionic form and the band at 1736 cm^{-1} arises from the C=O stretch of the ester groups.

When the sample was exposed to the humidified vapour, water absorption bands appeared, as expected, resulting in increases of the band intensities around 1642 cm^{-1} and below $\approx$1000 cm^{-1} (Fig. 2B,C). The small variations in background around 1500 cm^{-1} originated from atmospheric water vapour. Close examination of the difference between spectra of dried and hydrated pectins revealed small shifts in peak position and changes in the relative peak intensities as a result of hydration.

The shift of the ester band in wave-number from 1736 to 1728 cm^{-1} indicated the formation of hydrogen bonds which affect the C = O stretching. The slight shift in the peak from 1103 to 1107 cm^{-1} after hydration indicates possible conformational changes of the main chain of the polymer, since the absorption at 1103 cm^{-1} is from the contributions of the ring vibration and the angle deformation the C–C–C, C–O–C and C–C–O of the pyranose ring structures of pectins. Furthermore, an absorption band at 1233 cm^{-1} shifted to 1237 cm^{-1} and a small peak at 1440 cm^{-1} shifted to 1443 cm^{-1} when the sample is hydrated.

Most of the changes due to the hydration were reversible when the sample was re-dried. However, it is interesting to note that the spectrum of pectin re-dried after hydration shows differences in intensity of the bands from the carbonyl groups in the region 1800–1400 cm^{-1} compared with the original spectrum of dry pectin. There is an increase in the relative intensities of the bands at 1605 and 1415 cm^{-1} and a slight decrease in the intensity of the ester band at 1736 cm^{-1}. These effects were, in fact, exacerbated in samples subjected to repetitive hydration and drying. It showed that partial de-esterification has taken place during hydration since this phenomenon disappeared when the pectin sample was incubated in 80% alcohol in a 70–80 °C water bath for 30 min prior to the hydration work to inactivate hydrolytic enzymes.

Hydration of freeze-dried and fresh tomato CWM

Figure 3 shows the spectroscopic results obtained for hydration and drying of freeze-dried mature-green tomato CWM. As in Fig. 2, the four spectra in Fig. 3 are intensity normalized and offset. From top to bottom the four spectra were recorded at the four stages: A, dried; B, hydrated at 94% RH; C, fully hydrated; and D, re-dried. Similar changes are observed to those seen in the hydration of pectin; Fig. 3 shows the ester band shifted from 1736 to 1728 cm^{-1} by the hydration. The shift of the band at 1233–1237 cm^{-1} and the appearance of the small peak at 1443 cm^{-1} can be seen weakly in the spectra of the hydrated sample compared to the dry sample.

Sharpening of many bands around 1050–950 cm^{-1}, a phenomenon not observed during the hydration of pectin, was seen during the cell wall hydration, probably as a result of the crystallized cellulose in the cell wall being affected by water. A relatively broad band at 1063 cm^{-1}, which seemed to be composed of at least two unresolved bands, became sharper and shifted to higher wave-number when the sample was hydrated, and a shoulder appeared simultaneously at a lower wave-number. There was also an increase in intensity of the shoulder band at 1020 cm^{-1}

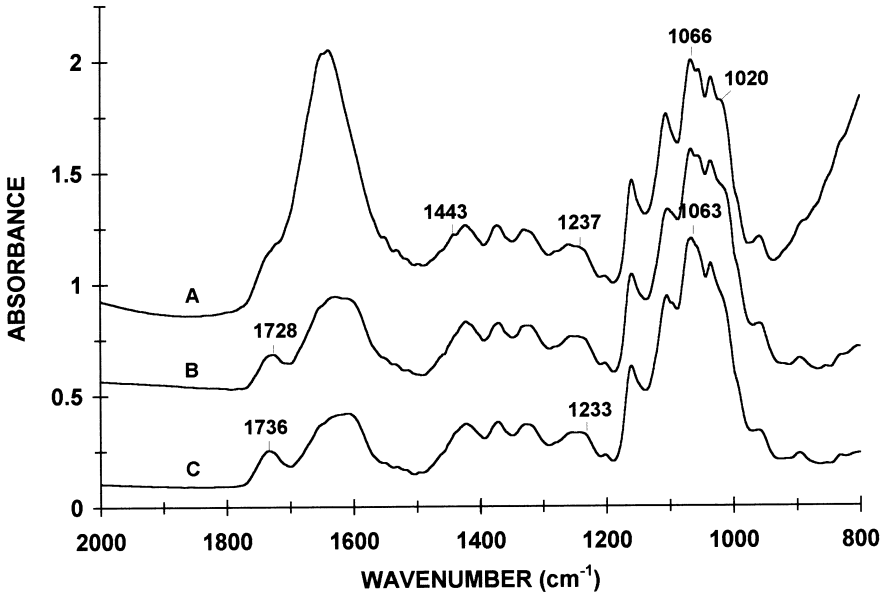

Fig. 4. Spectra of wet and dry cell walls, freshly prepared from pericarp of mature-green tomatoes, in hydration cell. Top to bottom: spectrum of freshly made wet CWM (A); tomato CWM exposed to 94% RH (B); and dried tomato CWM (D).

in the spectrum of the hydrated sample. Most of the spectral changes caused by hydration were subtle, except for the increase of the water absorption bands. Any changes observed were difficult to assign to specific macromolecules owing to the complexity of the spectra, resulting from the overlap of absorptions from the main cell wall polysaccharides. Although all of the changes observed in the spectra of hydrated tomato CWM were minor, these changes could be observed in other cell wall systems such as onion parenchyma cells and in the polarized spectra of the walls of epidermal cells of onion and carrot seedling discussed later in this paper.

The hydration effects observed on the freeze-dried CWM could be seen more clearly in the freshly made CWM where higher resolution was used for acquiring spectra (Fig. 4). In Fig. 4, three spectra were recorded at different drying stages. From top to bottom, these stages are: A, wet; B, 94% RH; and C, dry. The band sharpening and peak shifts were observed as in Fig. 3. In spectrum A, the ester band overlapped with the strong water absorption band at 1642 cm^{-1} since the hydration level is very high. Freeze-drying of the cell wall material seems to have no significant effect on its infra-red spectrum. It is important to note, however, that in these samples the effects of hydration are not pronounced, possibly showing that there are very few major conformational changes during hydration in these particular systems.

Molecular orientation in onion epidermis cell walls

After the preparation and drying down, the epidermal cells of onion and carrot seedling appeared to be undistorted and regularly arranged along the elongating axis. The width of the onion cells was about 30–60 μm and the length of the cells was 2–5 times greater than their width. The carrot epidermal cells were typically about 5–15 μm wide and 20–50 μm long.

A pair of polarized spectra of the dry sample of onion epidermal cell walls are shown in Fig. 5(a). There are clearly differences in some band intensities between the perpendicular and parallel polarization directions, indicating that there are molecular alignments within the walls. In order to determine which molecules are aligned, the difference spectrum (Fig. 5b) was obtained by subtracting the spectrum taken with the perpendicular polarization from that with parallel polarization ($\parallel - \perp$) with respect to the long axis of the cell. In the region 900–1200 cm^{-1} in the difference spectrum, all bands were more intense with parallel polarization. The pattern of the difference spectrum in Fig. 5(b) is mostly consistent with the spectrum of cellulose in this region (Liang & Marchessault, 1959). In highly crystalline cellulose samples, infra-red signals in this region were observed to be more intense when the polarized light is parallel to the cellulose backbone (Liang & Marchessault, 1959), so that most of the bands in the difference spectrum can be attributed to cellulose microfibrils. Thus the signals representing cellulose backbone vibrations of the polymer chain were more intense with the parallel polarization, which suggests that cellulose is arranged predominantly parallel to the long axis of the epidermal cells. The signals at 1740 and 1605 cm^{-1} represent the vibrations from pectin ester and the carboxylate groups, respectively. They are negative in the difference spectrum indicating that the signals of the carbonyl groups from the pectin are stronger in the perpendicular polarization. The polarization effects of

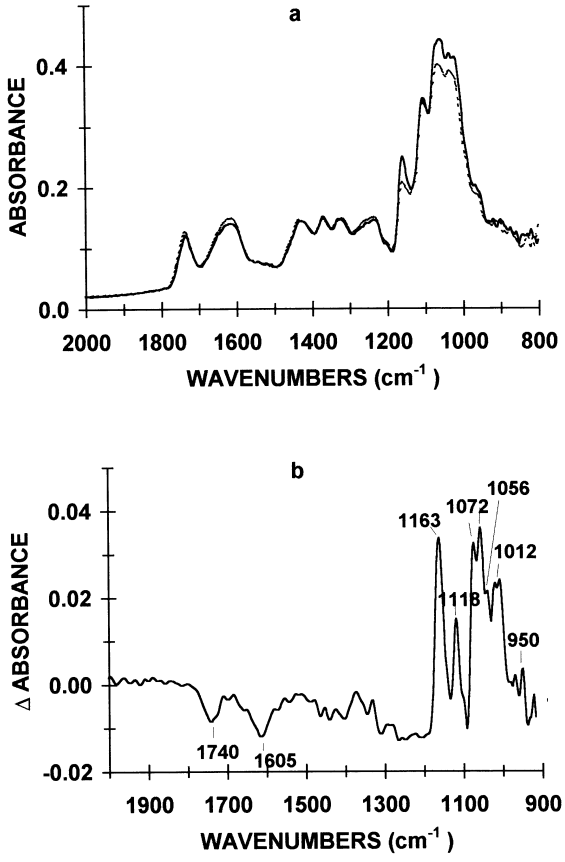

Fig. 5. (a) Spectra of dry onion epidermal cell walls taken with parallel (solid line) and perpendicular polarization (dotted line) with respect to the long axis of the cell, and (b) the difference spectrum between parallel and perpendicular polarization directions.

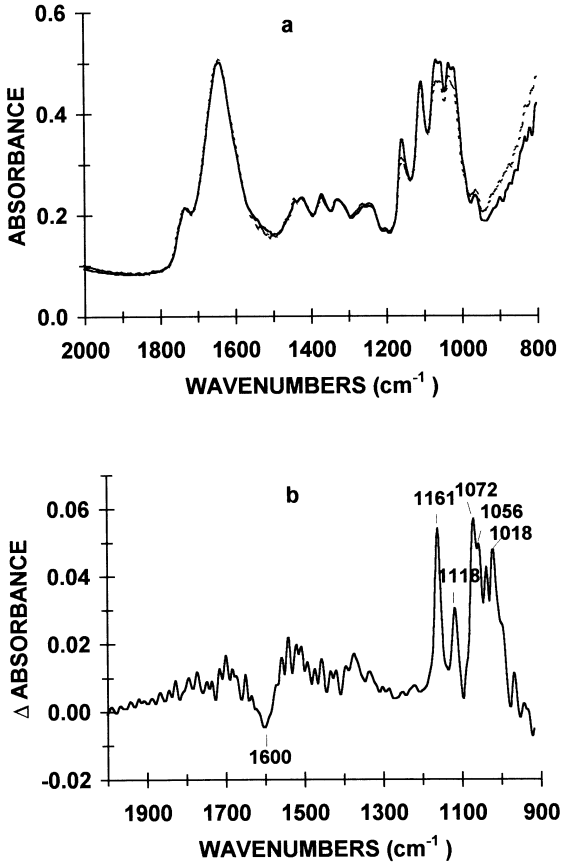

Fig. 6. (a) Spectra of hydrated onion epidermal cell walls taken with parallel (solid line) and perpendicular polarization (dotted line) with respect to the long axis of the cell, and (b) the difference spectrum between parallel and perpendicular polarization directions.

orientated pectin in the lower frequency range (900–1200 cm^{-1}) are likely to be masked by the signals from the cellulose.

The spectra of hydrated onion epidermal cell walls with parallel and perpendicular polarization are shown in Fig. 6(a). Comparing the spectra in Fig. 6(a) with those from the dry sample in Fig. 5(a) of the corresponding polarizations, there are some notable relative intensity differences in the spectra of hydrated walls compared with the dry samples. These changes are basically the same as those between dry and hydrated tomato cell walls discussed in Figs. 3 and 4, but are more pronounced than for tomato cell walls or for pectin alone (Fig. 2).

By inspection of the difference in band intensities of the two polarized spectra shown in Fig. 6(a), it is clear that some molecules are orientated in the wet sample. Figure 6(b) shows the difference spectrum obtained by subtracting the spectrum taken with the perpendicular polarization from that with parallel polarization. Although the difference spectrum of the wet sample is greatly affected by water vapour in the region 1400–1800 cm^{-1}, by comparison of Fig. 6(b) with Fig. 5(b) it can be seen that the two difference spectra are basically very similar and that cellulose was aligned in the same way in both dry and hydrated samples. The band at 1605 cm^{-1} for the pectin carboxylate groups still shows the same polarization behaviour in both dry and wet samples, but the polarization of the pectin ester band at 1740 cm^{-1} is not as obvious because of the strong water absorption in this region. Nevertheless, there is a strong indication that the pectin was aligned. It becomes apparent that the orientation of cell wall polymers inside the walls was not greatly affected by the hydration or drying conditions.

Molecular orientation in carrot epidermis cell walls

Polarized infra-red spectra of a dry sample from the epidermal cell wall of a 25-μm-thick section of carrot seedling have been investigated previously (McCann *et al.*, 1993). It was found that the signals of the carbonyl groups from the pectin (1745 cm^{-1} for pectin ester bands,

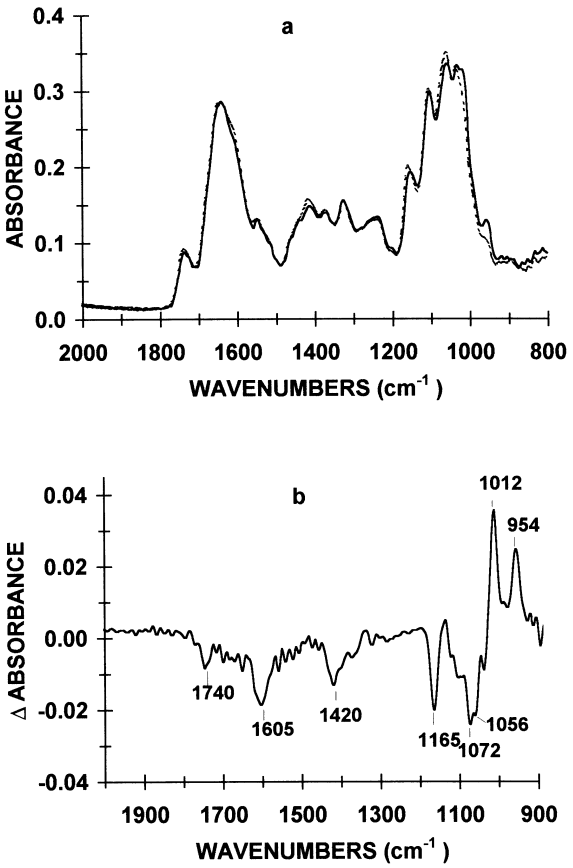

Fig. 7. (a) Spectra of dry carrot epidermal cell walls taken with parallel (solid line) and perpendicular polarization (dotted line) with respect to the long axis of the cell, and (b) the difference spectrum between parallel and perpendicular polarization directions.

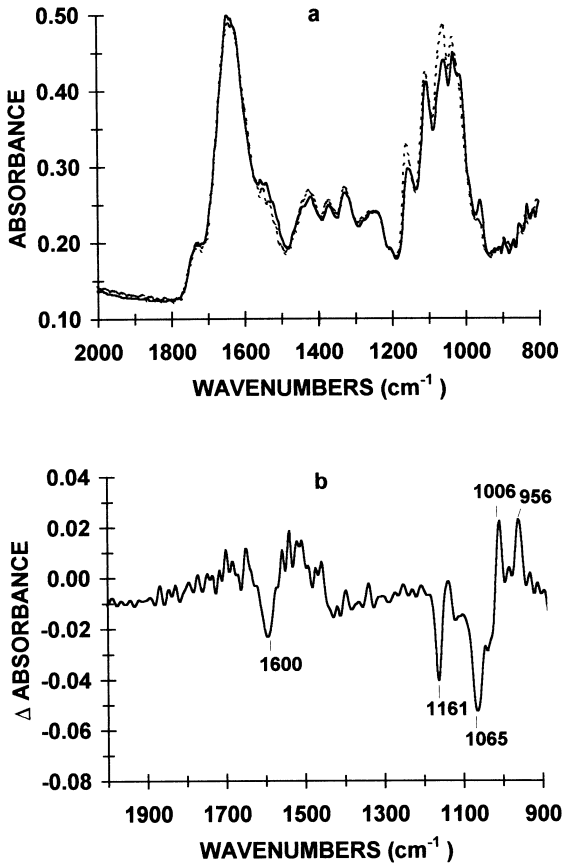

Fig. 8. (a) Spectra of hydrated carrot epidermal cell walls taken with parallel (solid line) and perpendicular polarization (dotted line) with respect to the long axis of the cell, and (b) the difference spectrum between parallel and perpendicular polarization directions.

1600 cm^{-1} and 1414 cm^{-1} for pectin carboxylate groups) were stronger in the perpendicular polarization as observed here for the onion epidermal walls, but those in the carbohydrate fingerprint region (1200–900 cm^{-1}) were also stronger in the perpendicular polarization, unlike onion epidermal walls. To check if this difference of polymeric alignment between the onion and carrot epidermal cell walls is genuine, and also to check whether the polarization behaviour of cell wall polymers is affected by hydration, experiments were performed on both wet and dry samples of carrot epidermis in the same way as for the onion samples.

The polarized spectral pair for the dry and wet carrot epidermal cell walls are shown in Fig. 7(a) and Fig. 8(a), respectively, and the corresponding difference spectra for both spectral pairs obtained by subtraction are shown in Fig. 7(b) and Fig. 8(b). As found previously by McCann *et al.* (1993) bands from pectin ester groups (1740 cm^{-1}), from pectin carboxylate groups (1605 and 1420 cm^{-1}) and from cellulose (1165, 1072 and 1056 cm^{-1}) were all more intense with the light polarized perpendicular to the long axis of the cells. Two bands at 1012 and 954 cm^{-1} were stronger with parallel polarization.

Although pectin is aligned judging by the polarization of the carbonyl groups of the pectin, it is difficult to say unambiguously what is the relative orientation of the pectin with respect to cellulose. The pectin signals in the carbohydrate fingerprint region were strongly affected by all the other polysaccharides, especially cellulose. Pectin carbonyl groups may well have a different orientation to that of the pyranose rings. The two bands at 1012 and 954 cm^{-1} which were more intense with parallel polarization in Figs. 7(b) and 8(b) are likely to originate from vibrations from pectin rings and glycosidic bridges between the rings. In the difference spectra of onion epidermal cell walls, the bands at 1012 and 954 cm^{-1} also had the opposite sign to the signals of pectin carbonyl groups at 1740, 1605 and 1415 cm^{-1} (see Fig. 5b). From our observations it is possible that pectin backbones are predominantly aligned in a direction parallel to the long axis of both carrot and onion epidermal cells.

Discussion

The results clearly show that the hydration cell provides a convenient way to measure infra-red microspectra of plant cell walls and biopolymer extracts. In use, no real problems were found. The cell appeared able to maintain samples in the hydrated state and allowed the hydration level to be changed relatively easily. We did not notice any degradation in spectroscopic performance compared with using dry cell walls on a single barium fluoride plate. Although there was a slight increase in the intensity of water vapour bands in the single beam spectra, this did not affect the quality of the final ratioed spectra as the atmosphere within the cell remains constant. We did not experience any problems with condensation forming on the windows. The main conclusion is therefore that it is now possible to measure infra-red spectra of very small areas of biological materials in their natural state. Although we made only discrete measurements using this cell in this paper the cell can easily be used with mapping stages to allow infra-red functional group mapping, making possible the generation of microscopical images providing information on the distribution of individual molecular species as well as their chemical and physical state. The technique could become a very powerful addition to the range of methods available to the microscopist and represents a significant step forward in the analysis of biological materials.

Studies of the hydration and drying of pectin show that the hydration cell functions well in both hydration and drying modes using either the connection to controlled humidity atmospheres or used with water-soaked inserts. However, the results also show that hydration can induce spectral changes. In pectin and tomato cell walls these effects are not very pronounced and are probably due to molecular conformational changes and hydrogen bonding changes. In the onion and carrot epidermal walls there is a more marked difference between the dry and wet cell wall and this may indicate a more significant conformational change on hydration. Clearly, the magnitude of hydration effects will vary from system to system. The polarization studies on carrot and onion epidermal cell wall are reassuring in that they not only show that molecular orientation can be measured in hydrated systems but also that hydration apparently does not affect the orientation of molecules. This confirms that molecular orientation is intrinsic and does not result from induced stress on drying and that previous work on cell walls showing unusual molecular orientations of biomolecules is likely to be valid. However, we conclude that accurate, rigorous models of cell wall architecture and the roles of individual molecules in these structures can only be constructed using data collected on hydrated samples, particularly if a full structural analysis including molecular conformation and crystallinity is required. This technique is likely to be important since earlier studies of plant–water relations have dealt mainly with associated changes of turgor pressure and their effects on growth during physiological conditions or effects on hydraulic conductivity, or the effect of water deficiency on the cell wall and how the cell wall adjusts to it. Very little is known about the effects of hydration on polymer conformations and interactions in the walls.

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