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The complex helical texture of the secondary cell wall of *Urtica dioica* root hairs is not controlled by microtubules: a quantitative analysis

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SUMMARY

A detailed quantitative analysis on the orientation of the cortical microtubule arrays and the last layer of cellulose microfibrils deposited in the secondary cell wall has been performed on *Urtica dioica* root hairs.

It was found that cortical microtubules of individual root hairs show a preferential orientation, which ranges in the total root hair population from −20 to +20° with respect to the longitudinal cell axis. Immunofluorescence and thin-section preparations are comparable, as long as the individuality of the root hairs and the modal distribution of the microtubules in the root hairs are considered.

The cellulose microfibrils in the secondary wall are organized in two steep helices. Quantitatively, the majority of the microfibrils are oriented in an S helix, while simultaneously a smaller group is arranged in a Z helix in the same root hair. It is concluded that microtubules do not directly control the orientation of nascent cellulose microfibrils in this complex texture. The organization of the secondary cell wall texture may be a variant of the organization of the primary cell-wall texture.

Key-words: cellulose microfibrils, cell-wall texture, cortical microtubules, root hairs, *Urtica*.

INTRODUCTION

Microtubules are believed to mediate the orientation of the cellulose microfibrils. These two structures often co-align, and there is evidence that changes in the orientation of the microtubules are followed by a change in the orientation of the cellulose microfibrils (reviewed by Seagull 1991). However, contradictory evidence has also been presented (reviewed by Emons *et al.* 1992).

The use of co-alignment as a leading argument for the mediating role of microtubules requires detailed information on the nascent cell-wall texture and the corresponding

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This paper is dedicated to Professor Dr M.M.A. Sassen on the occasion of his retirement.

Abbreviations: GA glutaraldehyde; EGTA ethylene glycol bis(2-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; PFA paraformaldehyde; FITC fluorescein isothiocyanate.

organization of the cytoskeleton, i.e. the cortical microtubules (Wasteneys & Williamson 1987; Emons 1988; Wilms & Derksen 1988; Seagull 1992). Recently, using detailed quantitative analysis, Sassen & Wolters-Arts (1992) and Wolters-Arts & Sassen (1991, 1992) have shown that the texture of primary cell walls can be far more complex than the originally assumed criss-cross or random organization.

A quantitative approach to the problem of demonstrating a relationship between microfibrils and microtubules can easily be used on root hairs as they exclusively exhibit tip growth. The primary and secondary cell walls are deposited simultaneously in the extreme tip and the tubular part of the root hair, respectively. Growing root-hairs are therefore also considered to be excellent material on which to quantitatively study cell-wall deposition in relation to the cortical microtubule organization (Traas *et al.* 1985; Emons 1989).

Urtica dioica root hairs were used in this study as they are believed to have a simple helical wall texture in the secondary wall (Sassen *et al.* 1985), while the cortical microtubular arrays seem to be diverse in orientation (Traas *et al.* 1985). In the previous studies on root hairs, a quantitative analysis of the texture was not performed nor considered necessary as these textures seemed to show a distinct axial, helical or helicoidal organization (Sassen *et al.* 1981).

A detailed quantitative analysis was carried out on the orientations of both microtubules and microfibrils in *Urtica* root hairs in order to detect subtle differences in their organization that might aid in elucidating the presumed interactive relationship of these ultrastructures. Furthermore, the quantitative discrepancies that are present in developing cotton hairs between the microtubular and microfibrillar organization (Seagull 1992) were compared with the organization of these structures in *Urtica* root hairs.

MATERIALS AND METHODS

Plant material

Root hairs of *Urtica dioica* were obtained from roots of seeds germinated in the dark on moist filter paper, and from adventitious roots formed on stem cuttings placed in tap water. Both were incubated at room temperature. Plants were cultured under greenhouse conditions (Sassen *et al.* 1981). It was made sure that only root hairs were used that formed a regular cone at a growing root-tip end. The area of a root hair which was used in our analysis was located between 10 and 1000 μm behind the root-hair tip. Both the cell-wall texture and the microtubular patterns seem to be homogenous in this region of the root hair, which means that tip and transition zone are excluded.

Electron microscopy

In order to visualize the microtubules, root hairs were fixed in 2% glutaraldehyde (GA) in a 0·2 M cacodylate buffer (pH = 6·8) for 2 h at room temperature. After a successive fixation in 2% OsO_4, the preparations were rinsed in buffer, dehydrated in ethanol and finally embedded in Spurr's resin (Spurr 1969). Thin sections (grazing, oblique) were made on a Sorvall Porter Blum ultramicrotome and stained with uranyl acetate/lead citrate. The frequency distribution of microtubular orientation was determined from micrographs of 79 different root hairs. Only cortical microtubules from the back wall were measured (cf. Emons & Wolters-Arts 1983). While several different techniques were used to study the wall texture, each technique is based on wall material extracted with H_2O_2/HAc (1:1, v/v).

The extraction process took 0·5–1·0 h at 80–100°C (Emons & Wolters-Arts 1983; Sassen *et al.* 1985). The material was then excessively washed in deionized water.

After extraction, the root hairs were embedded in Spurr's resin. Sections of 0·2 μm thickness were mounted on grids. The resin was removed with sodium methoxide in methanol–benzene for 2 min and washed successively in methanol–benzene and methanol (Mayor *et al.* 1961). The wall material in these preparations turns over on the grid and allows observation of the microfibrils after regular shadowcasting (Pluymaekers 1982).

In another set of experiments, whole roots were adhesively attached to glass slides and frozen in liquid nitrogen. The root hairs were then detached from the root using a razor blade, extracted as described above, re-frozen and finally carefully ground in glass tubes. Cell-wall fragments were mounted on carbon-enforced, Formvar-coated grids and shadowcasted at 45° with platinum.

Extracted root hairs were also dry-cleaved. After dehydration in ethanol, preparations were mounted on poly-L-lysine coated grids, critically point dried, cleaved and shadowcasted with platinum and reinforced with carbon (Sassen *et al.* 1986).

The microfibril orientation was analysed from micrographs taken at two final magnifications of 60 000 × and 90 000 × from both liquid nitrogen-crushed and dry-cleaved preparations. A pointed trellis was used to randomly select microfibrils (Erickson 1980). The directions of the microfibrils coinciding with the point markers on the trellis were determined using a Kontron Videoplan with a standard programme (Sassen & Wolters-Arts 1986). The results were plotted in frequency distributions using discrete angular intervals.

Immunofluorescence

Root hairs were treated as described by Traas *et al.* (1985). A 20 mM potassium phosphate buffer (pH = 6·8) containing 5–10 mM EGTA and 2–5 mM $MgSO_4$ was used. Root fragments with root hairs still attached were fixed in 4% paraformaldehyde (PFA) in this buffer for 3 to 4 h. Permeabilization of the cell wall was accomplished by a 10- to 15-min treatment in 5% cellulase (Onozuka R-10, Serva, Heidelberg, Germany). The primary antibody was a rat monoclonal anti-tubulin (MAS 077, Sera labs); the second antibody, a fluorescein-isothyocyanate (FITC) labelled goat anti-rat (Nordic BV, Tilburg, The Netherlands).

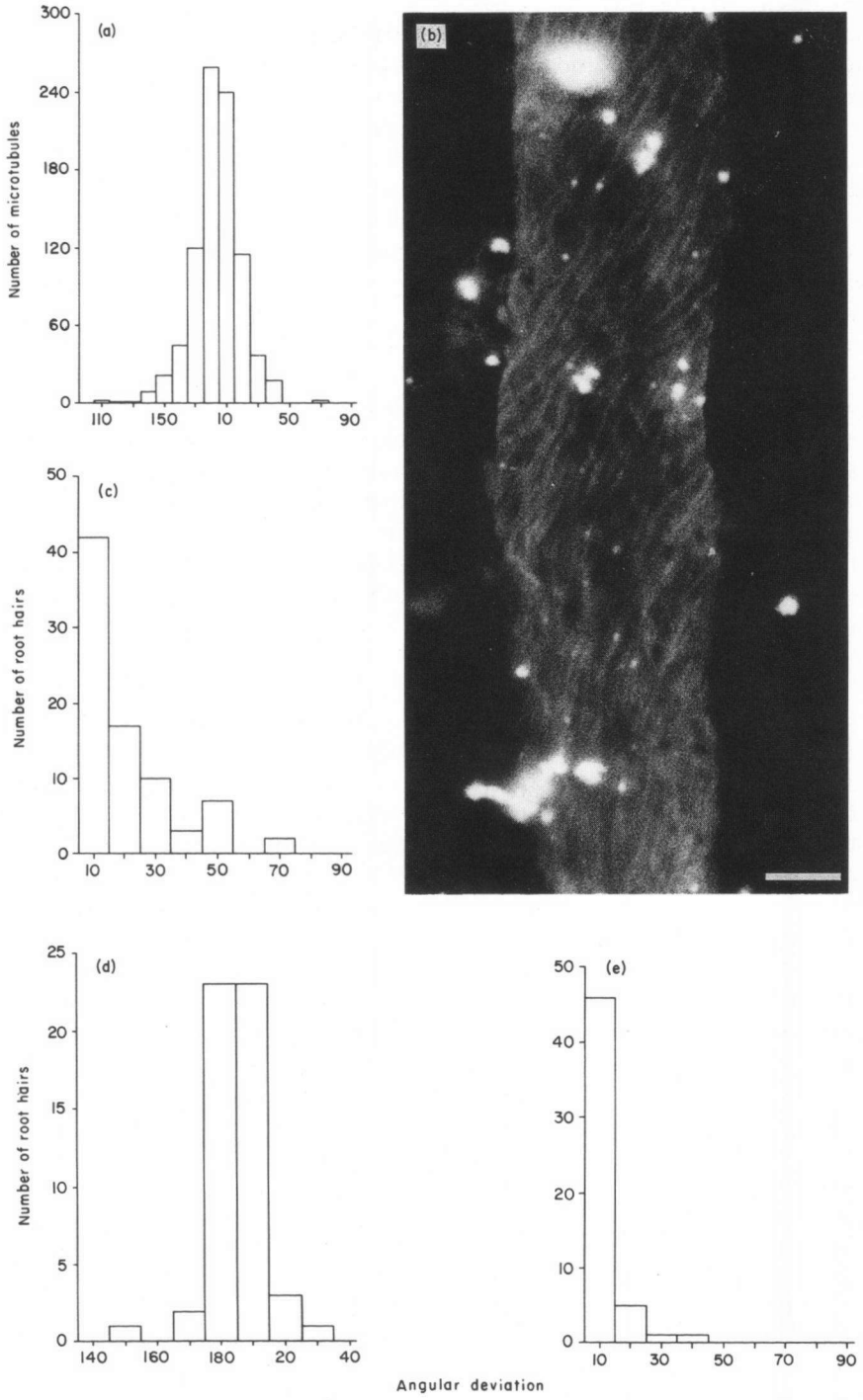
A total of 81 root hairs, originating from 5 cuttings, were examined using a Leitz Orthoplan microscope Orthomat combination with the appropriate filters and photographed on 400 ASA professional film to establish the main orientation of the cortical microtubules in individual root hairs.

RESULTS

Microtubules

Emons & Wolters-Arts (1983) and Emons (1982) have obtained and shown large numbers of micrographs of microtubules in thin sections of root hairs. Our micrographs showed the same type and quality of preparations. A quantitative evaluation of microtubular orientations is provided in the frequency profile of Fig. 1a. This histogram shows an axial orientation of the microtubules with a considerable deviation from the longitudinal cell axis.

The microtubules in immunofluorescence preparations (Fig. 1b) show a predominantly helical orientation. As the *in toto* immunofluorescence preparations show microtubules in


(a)
Number of microtubules
300
240
180
120
60
0
110
150
10
50
90
(b)
(c)
Number of root hairs
50
40
30
20
10
0
10
30
50
70
90
(d)
Number of root hairs
25
20
15
10
5
0
140
160
180
20
40
(e)
50
40
30
20
10
0
10
30
50
70
90
Angular deviation

either an S or a Z helix (approx. 1:1) only the absolute deviation from the cell's longitudinal axis was determined (Fig. 1c).

The distribution of the orientation of the microtubules is quite diverse over the population of root hairs. However, summing the absolute modes (Fig. 1d) of the orientation of the microtubules in the various root hairs a profile (Fig. 1e) arises that is equivalent to the one found in immunofluorescence preparations. The class distribution of modes of the root-hair population thus shows a preferential orientation of microtubules in individual root hairs (Fig. 1d).

Microfibrils

The cell wall of *Urtica* is distinctly double layered, as can be seen in transverse sections after removal of the resin (Fig. 2a). The difference in the organization of the microfibrils in each layer is apparent at higher magnification (Fig. 2b).

The type of organization, or texture, is much clearer and only determinable in liquid nitrogen-crushed (Fig. 2c) or dry-cleaved preparations (Fig. 2d). The outer primary wall texture is described as being dispersed, as we did not perform an analysis on its organization. The inner, secondary wall layer clearly shows microfibrils organized in a helical fashion, yet aberrations on this clear-cut helical configuration can be observed (Fig. 2d).

The quantitative analysis of the cell-wall texture was performed on the innermost, last layer of microfibrils deposited on the secondary wall. Various parameters were tested that might influence the measurement of microfibril orientation. Firstly, the influence of different micrograph magnifications on determining microfibril orientation was objectively established from liquid nitrogen-crushed cell-wall fragments. If we compare Fig. 3a (low magnification) with Fig. 3b (high magnification) no drastic profile differences occur. The profile in Fig. 3a is slightly cruder than that in Fig. 3b, this is caused by a smaller suitable sample size and the fact that fewer microfibril orientations can be determined in micrographs of lower magnification. The majority of the microfibrillar orientations are restricted to the 165–175° angular interval. Secondly, we determined the distribution of microfibrils in dry-cleaved preparations in high-magnification micrographs (Fig. 3c). This profile is almost identical to the one shown in Fig. 3b with the exception that a second peak at 10–15° is more pronounced. Further interpretations on the profile of microfibrillar distributions were performed on the combined data from (high magnification) liquid nitrogen-crushed and dry-cleaved preparations (Fig. 3d). This overall profile is applicable to every examined cell-wall fragment at any location in the root hair between 10 and 1000 μm from the root tip.

Two areas could be designated in the overall profile. The first one covers 50–80% of all microfibrils, which qualitatively is recognizable as the S-helically organized texture

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Fig. 1. Distribution of cortical microtubule orientation in root hairs of *Urtica dioica* in reference to the cell's longitudinal axis (180°/0°). Angular deviation is given in 10° increments. Classes on the *x*-axis are labelled by the corresponding class middles. N = number of root hairs used; n = number of microtubules measured. (a) Microtubule orientation of the pooled results from thin-section preparations of all root hairs. Mean = 178·7°, SD = 15·3, $N = 79$, $n = 871$. (b) Immunofluorescent preparation showing the helical arrangement of cortical microtubules. Bar: 5 μm. In Fig. 1c–1e each column in a histogram represents the number of root hairs with the particular modal orientation of their cortical microtubules. (c) Absolute distribution of modal microtubule orientation in root hairs from immunofluorescence preparations. $N = 81$. (d) Distribution of modal microtubule orientation in root hairs from thin-section preparations using fragments which show at least ten microtubules per fragment. $N = 41$, $n = 703$. (e) Absolute distribution of modal microtubule orientation in root hairs from thin-section preparations using fragments which show at least ten microtubules per fragment. Opposite mode classes are superpositioned. $N = 41$, $n = 703$.

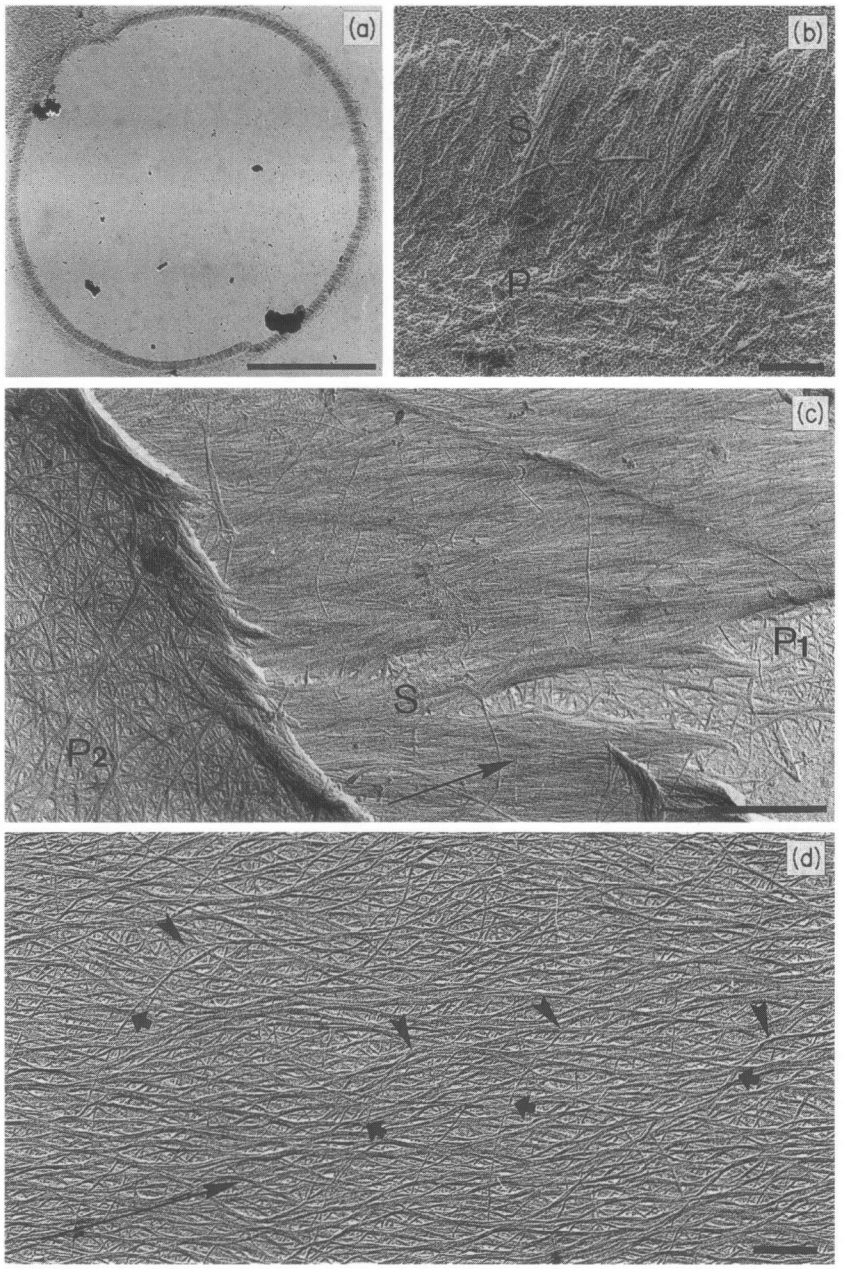

Fig. 2. Cell-wall texture after hydrogen peroxide/glacial acetic acid extraction of root hairs. Large arrows indicate the cell's longitudinal axis. (a) Transverse section (0·2 μm thick) of a root hair showing total circumference of a root hair. The cell wall has overturned after the removal of Spurr's resin. Bar: 5 μm. (b) Detail from (a) Notice the distinct difference in organization of microfibrils between the primary wall (P) and the secondary wall (S). Bar: 0·125 μm. (c) Surface view of the inner cell-wall texture after crushing in liquid nitrogen. Notice the difference in microfibril organization between the primary (P) wall, seen from the inside (P_1) and outside (P_2) of the root hair, and the ordered microfibrils in the secondary wall layer (S). Bar: 0·45 μm. (d) Surface view of the inner cell-wall texture after dry cleaving showing undulating cellulose microfibrils. Small arrows indicate single or small bundles of microfibrils in opposite direction to the majority of microfibrils. Notice that these microfibrils sometimes seem to derive from microfibrils oriented in an S helix (arrowheads). Bar: 0·5 μm.

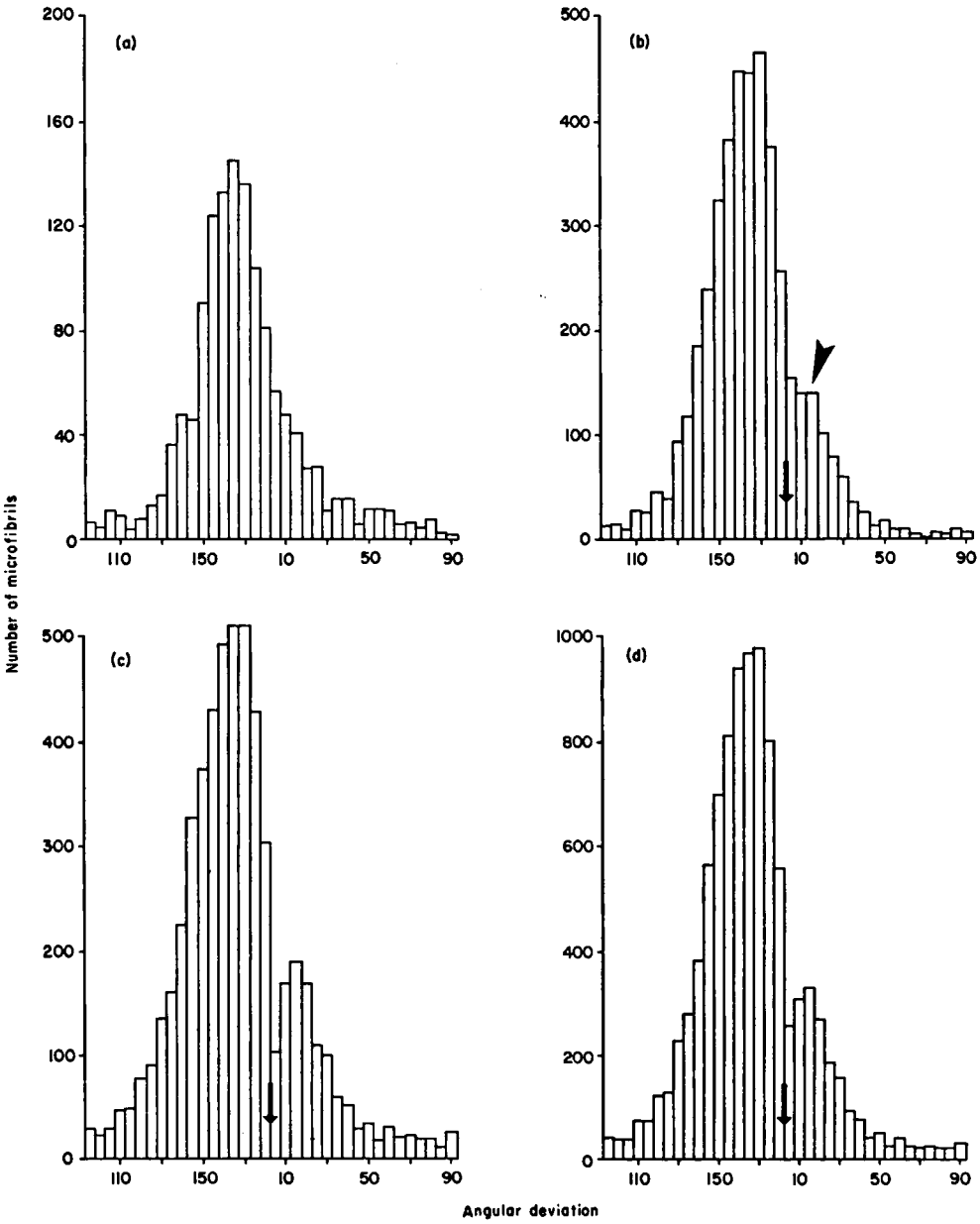

Fig. 3. Distribution of microfibril orientation in root hairs of *Urtica dioica* determined from the last deposited secondary cell-wall layer. Arrows indicate the cell's longitudinal axis. Angular deviation is given in 5° increments. Classes on the *x*-axis are labelled by the upper boundary of each class. N = number of root hair fragments used; n = number of microfibrils measured. (a) Microfibril orientation in nitrogen-crushed samples using low (60 000 ×) magnification micrographs. $N = 11$, $n = 1341$. (b) As in Fig. 1a using high magnification (90 000 ×) micrographs. Notice the onset of the small peak at the right sight located shoulder of the main microfibril direction (arrow head). $N = 30$, $n = 5493$. (c) Microfibril orientation in dry-cleaved samples (micrograph magnification 90 000 ×). $N = 16$, $n = 4354$. (d) Combined data of nitrogen-crushed and dry-cleaved samples (micrograph magnification 90 000 ×). Mean = 164·5°, SD = 28·7, $N = 41$, $n = 9847$. The small peak is significantly present ($P < 0{\cdot}001$, Sign Test).

depicted earlier. A second area encloses up to 20% of all the microfibrils, yet now arranged in a Z-helix. Assuming a symmetric Gaussian distribution the smaller peak is highly significant ($P<0{\cdot}001$, Sign Test). The profile of cellulose microfibril distributions ranges from 0 to 180°. Deviations from a definite orientation are partially caused by the undulating nature of the bundled microfibrils due to the extraction method applied (Emons 1988); especially in fast-frozen, deep-etched material (McCann *et al.* 1990; Tamura & Senda 1992) undulating microfibrils are hardly observed.

Relaxation and/or shrinkage of the cell wall after extraction might lead to the asymmetric bending of the microfibrils, which could give rise to favoured tangent angles. Another contribution to the increase in the distribution of microfibrils is the unavoided measurement of microfibrils from layers that are located more to the outside of the wall. These features will, however, only contribute to a general increase in the quantitative variation of the detected profile. An alteration of the profile is not considered to have occurred in this investigation.

DISCUSSION

From the *in toto* immunofluorescence preparations it is evident that cortical microtubular arrays vary from an axial to a helical orientation between root hairs. They show an absolute modal angle of 0–20° with respect to the cell's longitudinal axis. As both right- and left-handed helices occur in equal numbers, the *in vivo* discrete distribution of modes ranges from approximately −20° to +20° (see also Traas *et al.* 1985).

Variations in the pitch of microtubule helices in single root hairs may result from expansion of the root-hair tube in addition to tip growth, which has been proposed for *Urtica* and *Allium* by Traas *et al.* (1985). Lloyd & Wells (1985) accept these pitch variations to be consistent with the dynamic helical model (Lloyd 1984). We do not expect a drastic change in microtubule orientation merely from using a specific immunofluorescence technique, but due to the relative instability and low fluorescence of a single microtubule, the model distribution of bundled microtubules might be slightly over-emphasized.

The variations in and between root-hair samples of thin-sectioned material are brought about by the small size of the studied fragments and the limited number of determinable microtubules (see also Seagull 1986). Furthermore, bends or wavelets in individuals or bundled microtubules add to the over-emphasis of actual variations. Serial sectioning, especially of freeze-substituted material, could provide more accurate answers to whether the variations in microtubule orientation are a result of actual orientation or are related to the applied method, but this is not an issue in this study. Nevertheless, our results show that there is no discrepancy in average microtubule orientation between the pooled results of thin sections (Fig. 1a) and those of immunofluorescent preparations (Fig. 1d). However, both the individuality of root hairs and the modes of microtubule orientation should be taken into account.

From recent work by our group it has become apparent that simple protractor measurements are not adequate to establish subtle variations in textures. In the present article, we have demonstrated that even a difference of 50% in micrograph magnification shows important details, i.e. the minor peak at 10–15° (Fig. 3e). It also appeared necessary to use relatively large and serried cell-wall surfaces, as shown in the dry-cleaved preparations, to unambiguously detect specific details of the texture.

The quantitative presence of the peak at 10–15° (Fig. 3) is qualitatively discernible in micrographs as individual or small bundles of microfibrils (Fig. 2c). Furthermore, they are interwoven or lie on the bundles of microfibrils that form the major peak at 165–175°. In some instances these individual microfibrils even seem to descend from the main direction of microfibrils. In summary, the secondary cell wall of *Urtica* root hairs shows the presence of two, oppositely arranged helices of microfibrils. The major S helix is never changed into a dominant Z helix. The earlier observation describing a helical texture in *Urtica* root hairs can thus be extended (Sassen *et al.* 1981; 1985).

The implications of the presence of a left- and right-handed helix of microfibrils are yet unclear. If the alleged dispersed orientation of microfibrils in the primary wall of *Urtica* has a similar organization as in other primary cell walls (Sassen & Wolters-Arts 1992; Wolters-Arts & Sassen 1991, 1992) then the temporal and spatial control, or the organization, of the secondary cell-wall texture might be a variant of the primer initial wall control. From *Urtica* root hairs it is known that between the tip and the tubular area there is a transition zone present that shows microfibrils arranged axially and progressively helically. This part of the root hair, although difficult to access, might show how the transition between primary and secondary cell wall texture takes place. Future research on *Urtica* root hairs should focus on this region.

Detailed quantitative analyses are prerequisites to describing the organization of both the wall texture and the cortical microtubule arrays. A quantitative analysis of developing cotton hairs was carried out by Seagull (1992). The means and the variances of the orientations of the microtubules and the microfibrils were compared using *t*-tests to study the relationship between these structures. The differences in the means and the variances were tested per variable among the different fibre ages, and between variables at each fibre age. The microtubules and the microfibrils showed similar, but not equal changes in the mean orientations. The variances showed significant differences between the variables throughout the age of the fibres.

We have refrained from such an analysis as it can only be applied to populations for which the essential properties of the tested variables are known. We do not know, for example, whether the variables in *Urtica* are interdependent. Moreover, cellulose microfibrils are dispersed over at least two populations. The latter observation alone is sufficient reason to reject a *t*-test analysis. Discrepancies in microtubule and microfibril orientations in developing cotton hairs were thought permissible by assuming that only a part of the microtubule population is involved in orienting microfibrils and that mathematical significance does not necessarily imply a biological one.

The discrepancies between the orientations of microtubules with either an S or Z helix configuration and microfibrils with a consistent major S helix in *Urtica* root hairs are such that we cannot support the idea that microtubules directly determine the orientations of the nascent cellulose microfibrils, and thus could bring about the complex texture observed. However, the participation of microtubules in the establishment of the cell wall as a whole cannot be rejected either, as microtubules function in targeting exocytosis (Haigler & Koonce 1992; Hogetsu 1991) and determining the site of endocytosis (Kengen & Derksen 1991, 1992).

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