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Methods for visualizing cell wall texture

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SUMMARY

Different methods for visualizing cell wall texture are compared: (1) thin-sectioning and staining with potassium permanganate after removal of the cell wall matrix, (2) thin sectioning and on-block staining with uranyl acetate during freeze-substitution, (3) freeze-fracturing of untreated material, and (4) shadow-casting after dry-cleaving of material from which the wall matrix had been removed. Sections mainly give information on the type of texture. The other methods, being surface preparations, yield a clearer picture of the constituent elements, the microfibrils. Thin sections of material fixed in glutaraldehyde and osmium tetroxide and stained on the grid with uranyl acetate and lead citrate proved to be unreliable for determining cell wall texture. The meandering of microfibrils in dry-cleaved and shadow-casted preparations is supposed to be an artefact of this method. It is supposed that the actual width of the crystalline core of the cellulose microfibril is $3{\cdot}6 \pm 1{\cdot}9$ nm, as measured from sections stained with potassium permanganate of material treated with hydrogen peroxide/glacial acetic acid.

Key-words: cellulose microfibril, dry cleaving, *Equisetum fluviatile*, freeze-fracturing, freeze substitution, helicoidal cell wall.

INTRODUCTION

The architectural framework of higher plant cell walls, the cell wall texture, consists of cellulose microfibrils, linear polymers of (1→4)-β-D-glucan.

In electron microscopical preparations of plant cell walls the cell wall texture often remains undetected due to unsuitable preparation. Though often used to study microtubule/microfibril parallelism, staining of sections with uranyl acetate and lead citrate does not always reveal the texture of plant cell walls (Neville & Levy 1984).

Properly stained thin sections can reveal cell wall texture, but do not disclose aspects of wall texture as the density of microfibrils within cell wall lamellae, the angle between microfibrils of adjacent lamellae, nor, in helicoidal walls, the rotation mode of the helicoid.

This paper discusses various methods for visualizing cell wall texture and their limitations. Root hairs of *Equisetum fluviatile*, cells with known helicoidal wall texture (Emons 1986), were used, because the helicoidal wall texture is easily misinterpreted.

MATERIALS AND METHODS

Root hairs of *Equisetum fluviatile* with known helicoidal wall texture (Emons 1986) were used.

Thin sectioning was performed as described by Emons & Wolters-Arts (1983). Roots bearing root hairs were fixed at room temperature in 2% glutaraldehyde, buffered with 0·2 M cacodylate at pH 7·2 for 2 h, washed and treated with hydrogen peroxide/glacial actic acid (H_2O_2/HAc) at 100°C for 30–60 min in order to remove wall matrix material. Roots with hairs were then washed in distilled water and dehydrated in a graded series of aqueous ethanol and flat embedded in Spurr's resin. Sections obtained with a Sorvall Porter Blum ultramicrotome MT 5000 were stained with a 3% aqueous solution of potassium permanganate.

For comparison, the commonly used method for thin-sections was also employed. Roots were fixed as above, post-fixed in 2% osmium tetroxide for 2 h, washed in buffer, dehydrated in ethanol and flat embedded in Spurr's resin. Sections were stained on the grid with saturated aqueous uranyl acetate for 20 mins followed by Reynold's lead citrate for 7 min.

Freeze-substitution and on-block staining was done as described by Emons & Derksen (1986), an adaptation of the method described by Heath & Rethoret (1982). Roots with hairs were placed on pieces of boiled dialysis tubing (as large as a grid) and rapidly frozen by plunging them in to liquid propane, cooled by liquid nitrogen. The specimens on the tubing were transferred to a substitution fluid composed of anhydrous aceton containing 2% osmium tetroxide and 0·1% uranyl acetate precooled at −78°C in a metal vial. The metal vials containing the specimens were kept in a freeze-drying device at a temperature of −80°C (±5°C) for a period of 20 h. The material was brought to room temperature very slowly over a 6-h period in the apparatus. At room temperature the specimens were rinsed with anhydrous aceton several times, infiltrated with Spurr's resin and embedded as a flat layer. Under a light microscope individual hairs, showing no evidence of gross ice crystal damage, were selected. The selected hairs were sectioned with a Sorvall Porter Blum MT 5000 on to formvar coated grids. Additional staining on the grid with uranyl acetate and lead citrate was not needed to obtain good contrast.

Freeze-fracturing was done as described by Emons (1985). Without any prior treatment with cryo-protectant or fixative the root hairs, still attached to the root, were placed in a specimen holder consisting of two thin copper plates, and ultra rapidly frozen in liquid propane in a cryo-jet (QFD Balzers). The material was fractured by opening the specimen holder in a freeze etching apparatus (Balzers). After a short etching of 1 min, to sublimate ice, the material was platinum/carbon replicated at −108°C. The replicas were floated on a cleaning solvent of 40% chromic acid to remove contaminating biological material, washed in distilled water, and allowed to dry onto Formvar-coated grids.

The dry-cleaving technique for cell walls (Sassen *et al.* 1985) was used as a shadow-casting technique. After fixation and extraction of wall matrix materials with H_2O_2/HAc at 100°C for 30–60 min, the roots were washed in distilled water, the root hairs were placed on poly-L-lysine coated grids, critical-point-dried, broken open by lightly tipping the material on the grid against adhesive tape, and finally shadowed with platinum and carbon. Microfibrils of the inner cell wall layer are thus revealed.

Preparations were examined with a Philips EM 201 electron microscope at 80 kV.

RESULTS

The arced patterning, revealing the helicoidal nature of the root hair wall, is very clearly seen after extraction of the matrix material by treatment with H_2O_2/HAc for 30 min at 100°C and staining on the grid with $KMnO_4$ (Fig. 1a). Microfibrils visualized by this

procedure measured 3·6 ± 1·9 nm in width. Microfibril width was measured with a calibrated 10 × ocular from micrographs with a magnification of 192 500 × (Fig. 1b).

Figure 2 shows the cell wall of a root hair, from which matrix material had not been extracted, stained with aqueous uranyl acetate and lead citrate. It is clear that the procedure used is inadequate to reveal arced patterning, even an axial pattern can be observed.

On-block uranyl acetate-stained freeze-substituted material did, however, reveal the helicoidal nature of the cell wall (Fig. 3). By this procedure the measured microfibril width varied greatly within the same cell type, from 6 nm to 20 nm.

Figure 4 shows a freeze-fractured cell wall preparation of a root hair. Microfibrils in this preparation lie straight. Also microfibril imprints of the last-deposited lamella, visible through the EF-face of the plasma membrane, show straight microfibrils (Fig. 5). Microfibril width in freeze-fractured preparations was 8·5 nm ± 1·5 nm including shadow deposit. Microfibril width was measured on microfibrils lying in the shadowing direction.

Dry-cleaving is an improved version of the traditional shadow-casting technique. Figure 6a and b show dry-cleaved shadow-casted inner cell wall preparations. Dry-cleaved preparations of helicoidal cell walls show the last-deposited microfibril lamellae, with microfibrial orientation regularly shifted in each lamella. Prepared by means of dry-cleaving, microfibrils within a lamella show a meandering pattern. However, with respect to overall microfibril orientation the wall texture seen by the dry-cleaving technique after removing matrix material with H_2O_2/HAc (Fig. 6) is similar to the wall texture seen by freeze-fracturing (Figs 4 and 5). Microfibril width in dry-cleaved preparations was 8 nm ± 1 nm including shadow deposit. Microfibril width in freeze-fractured and dry-cleaved preparations depends on the amount of platinum used for shadowing.

DISCUSSION

Thin sections stained with potassium permanganate, taken from material from which the wall matrix is dissolved, reveal the helicoidal texture of a plant cell wall. Helicoidal walls show parabolas in thin sections (Fig. 1a). A helicoidal wall texture consists of one microfibril-thick lamellae. The orientation of microfibrils in each lamella rotates regularly, like the steps of a spiral staircase (Neville 1986, for root hairs: Emons & van Maaren 1987). The impression of arcs seen in thin sections is produced by short strands of microfibril from subsequent lamellae. The width of helicoidal arcs depends on obliqueness of sections. Sections perpendicular to the component layers do not show an arced pattern unless sections are tilted in the electron microscope.

Staining with uranyl acetate and lead citrate of fixed root hairs did not reveal the microfibrils of the cell wall (Fig. 2). Data based on sections stained with uranyl acetate and lead citrate are not reliable. Nevertheless, similar preparations have often been used to show that microfibrils lie parallel to cortical microtubules. Uranyl acetate and lead citrate have no chemical affinity for cellulose. What is actually stained by this procedure is not known. Cox & Juniper (1972) have reported that uranyl acetate and lead citrate do stain cellulose microfibrils, but that this staining is physical in nature and removed by washing of the material on the grids. Indeed, on-block staining, as performed in the freeze-substitution procedure, in which the material is washed in acetone, did reveal the microfibrils (Fig. 3). Occasionally, uranyl acetate and lead citrate staining on the grid of chemically fixed material did reveal the actual wall texture. This might be caused by inadequate washing.

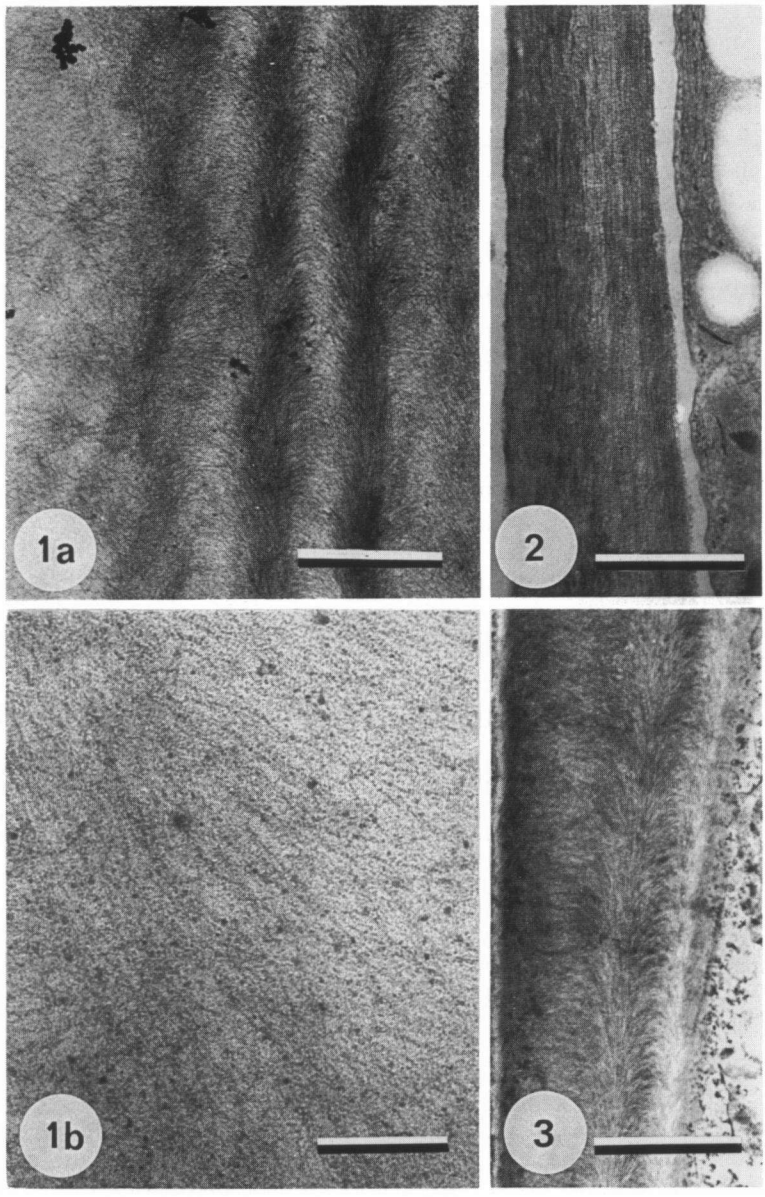

Fig. 1. Thin sections of the cell wall of root hair of *Equisetum fluviatile* treated with hydrogen peroxide/glacial acetic acid and stained with permanganate, a procedure revealing the helicoidal nature of the cell wall. (a) Bar: 500 nm, (b) Bar: 100 nm.

Fig. 2. Thin section of the cell wall of root hair of *Equisetum fluviatile* fixed in glutaraldehyde and osmium tetroxide and stained with uranyl acetate and lead citrate, a procedure inadequate to reveal the arcs of the helicoidal wall. Bar: 500 nm.

Fig. 3. Thin section of the cell wall of root hair of *Equisetum fluviatile* prepared by freeze-substitution and on-block staining with uranyl acetate, showing the helicoidal arcs. Bar: 500 nm.

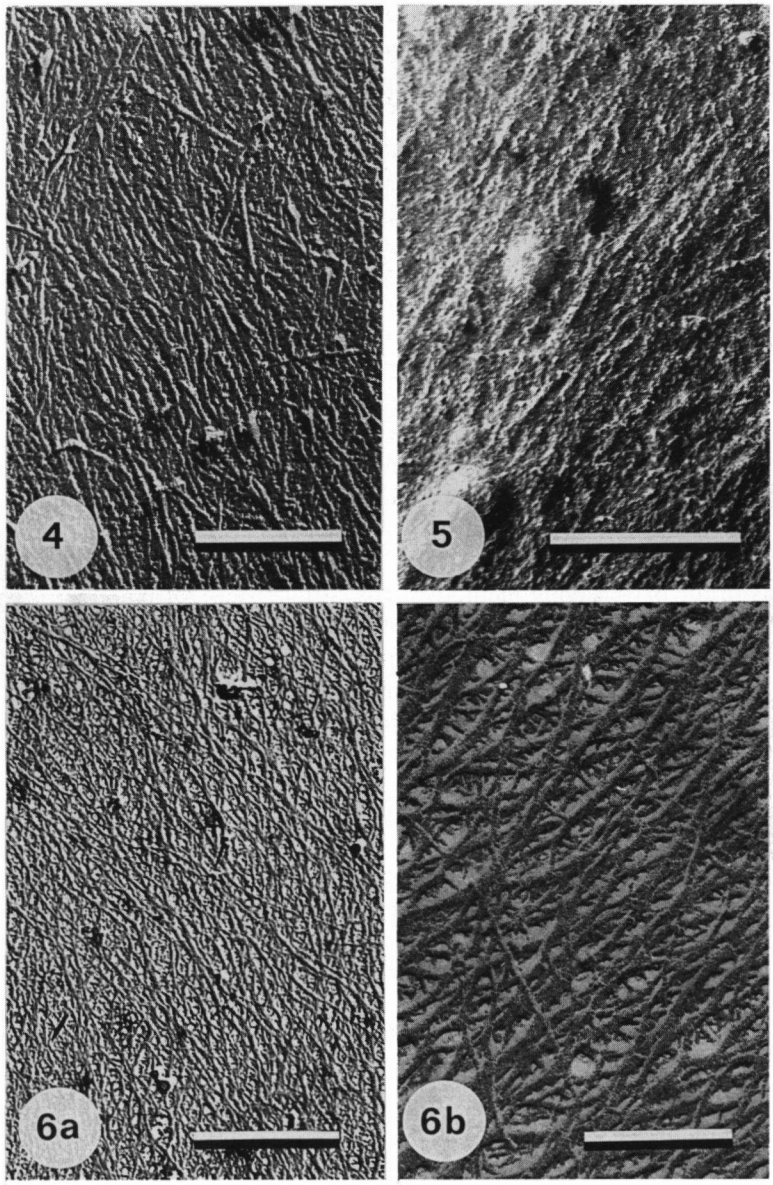

Fig. 4. Freeze-fracture preparation of the cell wall of untreated root hair of *Equisetum fluviatile*, showing straight microfibrils. Bar: 500 nm.

Fig. 5. EF-fracture face of the plasma membrane of root hair of *Equisetum fluviatile*, showing straight imprints of the last deposited wall microfibrils in the plasma membrane. Bar: 500 nm.

Fig. 6. Surface view of the inner cell wall of root hair of *Equisetum fluviatile* prepared by the dry-cleaving technique after removing matrix material with hydrogen peroxide/glacial acetic acid, showing microfibrils meandering in the plane of the membrane. (a) Bar: 500 nm, (b) Bar: 500 nm.

Furthermore, artefacts of initial chemical fixation are ruled out using freeze-substitution. Therefore, freeze-substitution of on-block stained material will be an important tool to investigate whether or not microtubules and microfibrils lie in parallel (review: Lloyd 1984; cf. Emons 1982, Emons & Wolters-Arts 1983, Lloyd & Wells 1985, Traas *et al.* 1985), especially, if, with further improvement of the technique, microtubules and microfibrils are visualized at the same time in the same preparation (Sassen & Wolters-Arts 1986).

Another method for microfibril visualization is the method employed by Roland and co-workers (Roland *et al.* 1977). It consists of enzymatic extraction of wall matrix material and a subsequent positive staining for polysaccharides with PATAG (periodic acid thiocarbohydrazide-silver proteinate) (Thiery 1976). With respect to wall texture, results are similar to those obtained with extraction with H_2O_2/HAc and staining with $KMnO_4$, but because the extraction of matrix material is enzymatic, this method is especially suitable for *in situ* cytochemical studies of cell walls.

Freeze-fracturing (Fig. 4) visualizes microfibrils by a short etching, in fact a mere sublimation of ice. It also reveals microfibril imprints in the plasma membrane (Fig. 5). Because neither chemical fixation nor drying are applied, microfibril orientation is least disturbed by this method. Microfibrils in freeze-fractured material lie straight. Freeze-fracturing is not always appropriate for studying cell wall texture because the cleavage plane cannot be controlled.

Shadowing with platinum/carbon after the wall matrix is dissolved and cells are cleaved, as in the dry-cleaving method, yields surface views of the inner cell wall and, better than freeze-fracturing, gives data on fibril density within a lamella, fibril angle between lamellae, fibril angle with long axis of the cell, and rotation mode of the helicoid. Dry-cleaving does not differ essentially from traditional shadow-casting, but produces much larger inner cell wall preparations and is, therefore, to be preferred. As with the traditional methods, however, wavy patterns of microfibrils are found (Sassen *et al.* 1985).

Boyd & Foster (1975) have hypothesized that this meandering of microfibrils seen in shadow-casted specimens is evidence for bonding of microfibrils within lamellae and between adjacent lamellae. Willison & Abeysekera (1985), however, explained the wavy pattern of microfibrils in shadow-casted specimens as the result of flattening a spiralized structure, on the basis of their observations that single cellulose microfibrils commonly exhibit helical twisting. They suggest that microfibril spirals arise at the time of microfibril synthesis by properties intrinsic to the cellulose molecules. Despiralization of microfibrils then occurs during treatment with H_2O_2/HAc, and microfibrils become wavy during the drying process.

In freeze-fractured preparations microfibrils of root hairs of *Equisetum* measured 8·5 nm ($\pm$1·5 nm) including shadow deposit. In dry-cleaved preparations microfibrils of this plant material measured 8 nm ($\pm$1 nm) including shadow deposit. A width of 6–10 nm is a regular measure for shadowed microfibrils of higher plant cell walls (Preston 1974). The shadow deposit has to be subtracted to obtain the actual width. In thin sections of material from which the cell wall matrix had been dissolved, the microfibrial diameter was much smaller ($3{\cdot}6 \pm 1{\cdot}9$ nm). The diameter of microfibrils prepared with freeze-substitution varied from 6 nm to 20 nm. This variation may suggest that a hydrophylic sheath of less-crystalline glucans, which surrounds the crystalline core, is positively stained.

By means of high-resolution platinum/carbon replication of tobacco epidermal leaf cells, Ruben & Bokelman (1987) have shown $3{\cdot}68 \pm 0{\cdot}3$ nm wide microfibrils, which

consist of three $1{\cdot}78 \pm 0{\cdot}22$ nm wide submicrofibrils twisted in left-handed fashion around their axes. This microfibrial width agrees with the width measured using H_2O_2/HAc treatment and staining with $KMnO_4$. Therefore, the last method seems to enable measurement of actual microfibril width. The physico-chemical basis of cellulose microfibril staining with $KMnO_4$ is not yet understood. It might be physical absorption (for discussion see Desphande 1976). Washing with water does not remove the stain.

Ultrastructural details of microfibrils, such as those shown by Ruben & Bokelman (1987), put constraints on possible models of cellulose microfibril biosynthesis at the plasma membrane. There is general agreement that in most algae and in all higher plant cells cellulose synthesis occurs at the plasma membrane (Delmer 1987). It has been hypothesized that particle rosettes, clusters of six particles visible by means of freeze-fracturing on the PF-face of the plasma membrane, play a role in microfibril synthesis (reviews: Brown 1985, Herth 1985, Delmer 1987, Emons 1988 in press). If, during D-glucan polymerization, i.e. the formation of microfibrils, rosettes rotate around their central axes in the plane of the membrane, their products will be twisted into a helix.

In conclusion, staining of thin sections after chemical or enzymatic removal of wall matrix molecules reliably discloses cell wall texture. If stained on-bloc without subsequent washing with water, as in freeze-substitution, uranyl acetate staining also reveals the type of wall texture. If the type of texture is known, shadow-casting, especially after dry-cleaving, provides additional data on the texture.

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REFERENCES

Boyd, J.D. & Foster, R.C. (1975): Microfibrils in primary and secondary wall growth develop trellis configurations. *Can. J. Bot.* **53:** 2687–2701.

Brown, R.M., Jr. (1985): Cellulose microfibril assembly and orientation: recent developments. *J. Cell Sci.* **77** (Suppl. 2): 13–32.

Cox, G. & Juniper, B. (1972): Electron microscopy of cellulose in entire tissue. *J. Microsc.* **97:** 343–355.

Delmer, D. (1987): Cellulose biosynthesis. *Ann. Rev. Plant Physiol.* **38:** 259–290.

Desphande, B.P. (1976): Observations on the fine structure of plant cell walls I. Use of permanganate staining. *Ann. Bot.* **40:** 433–437.

Emons, A.M.C. (1982): Microtubules do not control microfibril orientation in a helicoidal cell wall. *Protoplasma.* **113:** 85–87.

— (1985): Plasma-membrane rosettes in root hairs of *Equisetum hyemale*. *Planta* **163:** 350–359.

— (1986): Cell wall texture in the genus *Equisetum*. *Can. J. Bot.* **64:** 2201–2206.

— (1988): The role of rosettes and terminal globules in cellulose synthesis. In: P. Weimer and C.H. Haigler (ed.): Vol. 1 of: *Cellulose: Science and Technology* R. Atalla (ed.): Marcel Dekker, Inc., New York (in press).

— & Derksen, J.W.M. (1986): Microfibrils, microtubules and microfilaments of the trichoblast of *Equisetum hyemale*. *Acta Bot. Neerl.* **35:** 311–320.

— & van Maaren, N. (1987): Helicoidal cell wall texture in root hairs. *Planta* **70:** 145–151.

— & Wolters-Arts, A.M.C. (1983): Cortical microtubules and microfibril deposition in the cell wall of root hairs of *Equisetum hyemale*. *Protoplasma.* **117:** 68–81.

Heath, I.B. & Rethoret, K. (1982): Mitosis in the fungus *Zygorynchus moelleri:* Evidence for stage specific enhancement of microtubule preservation by freeze-substitution. *Eur. J. Cell Biol.* **28:** 180–189.

Herth, W. (1985): Plant cell wall formation. In: A.W. Robards (ed.): *Botanical Microscopy 1985*. Oxford University Press, Oxford. pp. 285–310.

Lloyd, C.W. (1984): Toward a dynamic helical model for the influence of microtubules on wall patterns in plants. *Int. Rev. Cyt.* **86:** 1–51.

— & Wells, B. (1985): Microtubules are at the tips of root hairs and form helical patterns

corresponding to inner wall fibrils. *J. Cell Sci.* **75:** 225–238.

Neville, A.C. (1986): The physics of helicoids, multidirectional "plywood" structures in biological systems. *Phys. Bull.* **37:** 74–76.

— & Levy, S. (1984): Helicoidal orientation of cellulose microfibrils in *Nitella opaca* internode cells: ultrastructure and computed theoretical effects of strain reorientation during wall growth. *Planta* **162:** 370–384.

Preston, R.D. (1974): *The Physical Biology of Plant Cell Walls.* Chapman & Hall, London.

Roland, J.C., Vian, B. & Reis, D. (1977): Further observations on cell wall morphogenesis and polysaccharide arrangement during plant growth. *Protoplasma* **91:** 125–141.

Ruben, G.C. & Bokelman, G.H. (1987): Triple-stranded, left-hand-twisted cellulose microfibril. *Carb. Res.* **160:** 434–443.

Sassen, M.M.A. & Wolters-Arts, A.M.C. (1986): Cell wall texture and cortical microtubules in growing staminal heirs of *Tradescantia virginiana. Acta Bot. Neerl.* **35:** 351–360.

—, — & Traas, J.A. (1985): Deposition of cellulose microfibrils in cell walls of root hairs. *Eur. J. Cell Biol.* **37:** 21–26.

Thiery, J.P. (1976): Mise en evidence des polysaccharides sur coupes fines en microscopie electronique. *J. Microsc.* **6:** 987–1017.

Traas, J.A., Braat, P., Emons, A.M.C., Meekes, H.T.H.M. & Derksen, J.W.M. (1985): Microtubules in root hairs. *J. Cell Sci.* **76:** 303–320.

Wardrob, A.B., Wolters-Arts, A.M.C. & Sassen, M.M.A. (1979): Changes in microfibril orientation in the walls of elongating plant cells. *Acta Bot. Neerl.* **28:** 313–333.

Willison, J.H.M. & Abeysekera, R.M. (1985): On the form and arrangement of cell wall microfibrils. In: A.W. Robards (ed.): *Botanical Microscopy 1985.* Oxford University Press, Oxford, pp. 181–203.