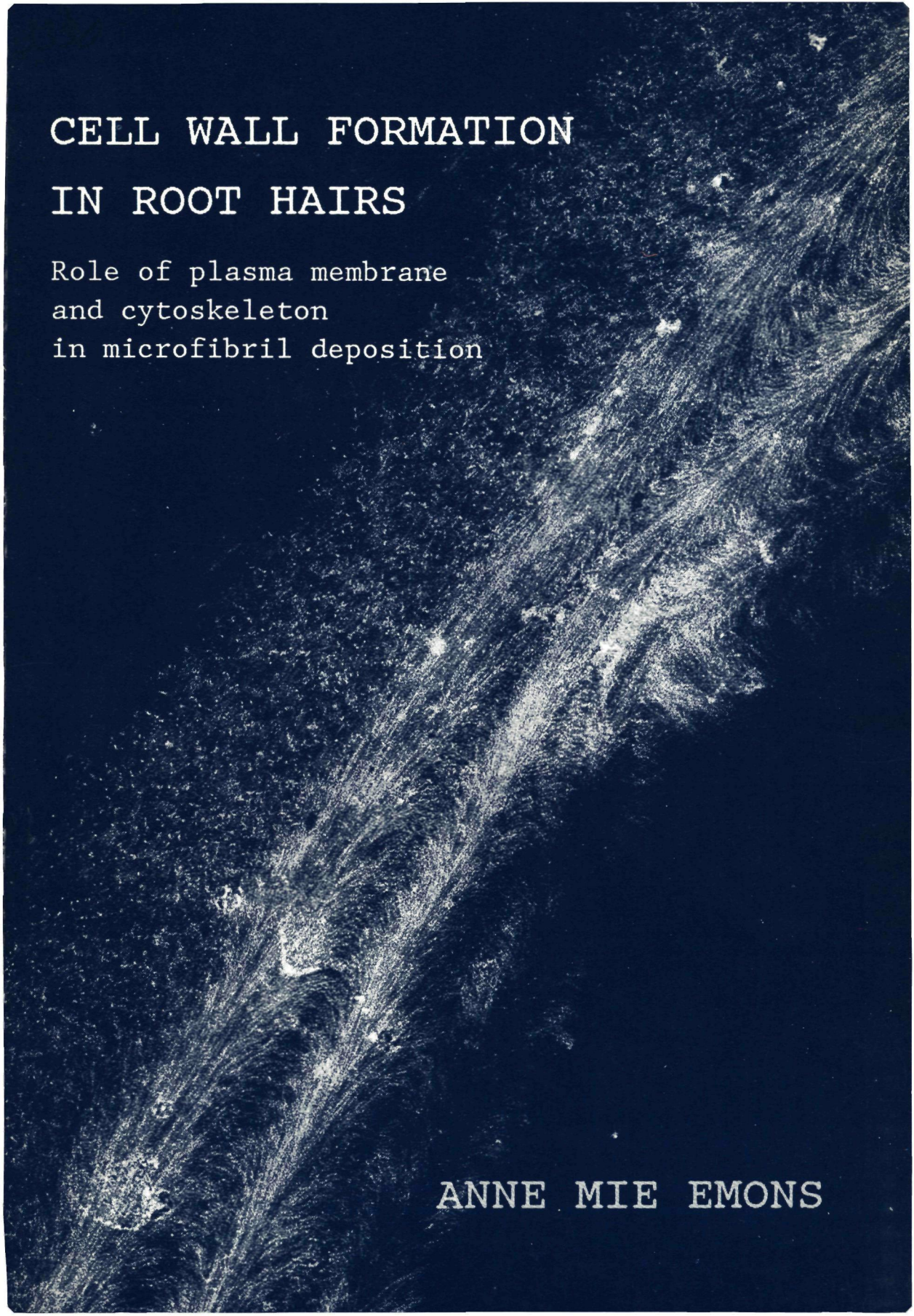
# CELL WALL FORMATION IN ROOT HAIRS

Role of plasma membrane and cytoskeleton in microfibril deposition



ANNE MIE EMONS

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## Role of plasma membrane and cytoskeleton in microfibril deposition

CIP-DATA

Emons, Anna Maria Catharina
Cell wall formation in root hairs:
Role of plasma membrane and cytoskeleton
in microfibril deposition.
Thesis Nijmegen,
with summary in Dutch and references.
ISBN 90-9001385-7
Subject headings: microfibrils, microtubules,
plasma membrane, root hair, plant cell.

# CELL WALL FORMATION IN ROOT HAIRS

## Role of plasma membrane and cytoskeleton in microfibril deposition

PROEFSCHRIFT
ter verkrijging van de graad van
doctor in de wiskunde en natuurwetenschappen
aan de Katholieke Universiteit te Nijmegen,
op gezag van de Rector Magnificus
Prof. Dr. J.H.G.I. Giesbers
volgens besluit van het College van Decanen
in het openbaar te verdedigen
op vrijdag 3 oktober 1986
des namiddags te 1.30 uur precies

door

ANNA MARIA CATHARINA EMONS

geboren te Ottersum

1986
druk: Stichting Studentenpers Nijmegen

PROMOTOR: PROF. DR. M.M.A. SASSEN

Het manuscript kwam tot stand met behulp van het tekstverwerkingsprogramma SuperWriter (1.03) ingevoerd in een Apricot PC en is gezet op een Hewlett Packard Laser Jet+ printer.
Foto's zijn afgedrukt door Dolf Dicke.

# DANKWOORD

Een proefschrift komt tot stand in samenwerking met velen. Daarom wil ik graag iedereen die heeft bijgedragen van harte bedanken.
Ik dank alle medewerkers van Botanie III voor de wetenschappelijke en technische assistentie, de medewerkers van Botanie I voor de prettige sfeer, de medewerkers van de proeftuin voor de verzorging van de planten en de medewerkers van de afdeling illustratie voor de grafische presentatie van de resultaten, Niek van Maaren voor zijn bijdrage aan de hoofdstukken 1 en 2 en Marluus Ruland voor haar bijdrage aan hoofdstuk 11.
In het bijzonder dank ik Dora voor haar steun en stimulans, John voor zijn grote belangstelling en geduld, voor het aanpassen van de benodigde software, voor het printen van de tekst en voor het kritisch corrigeren van alle artikelen, Cynthie, Aldo en Nivja voor hun begrip en de aangename muzikale intermezzo's.

Velp, 3 oktober 1986 Anne Mie Emons

Aan allen die tot mijn vorming bijdroegen

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# GENERAL INTRODUCTION

Plant cells are enclosed in a cell wall. The cell wall defines shape and size of the plant cell and presents a physical barrier to microorganisms and other agents, which may be harmful to the plant (Bateman 1976). Cell walls may contain molecules that control the growth and development of plants (McNeil et al. 1984).

The architectural framework of higher plant cell walls, the cell wall texture, consists of cellulose microfibrils, linear polymers of (1→4)-β-D-glucan, juxtaposed into bundles. In shadowed preparations the crystalline microfibrils vary in width from 10 nm in higher plants to 30 nm in some algae (Preston 1974). Microfibrils are embedded in an amorphous matrix of non-cellulosic substances consisting mainly of pectic polysaccharides, hemicelluloses and proteins (Preston 1974), like steel rods in re-inforced concrete, imparting cohesion, strength and rigidity to the cell wall.

When plant cells increase in volume, as most do shortly after division, their wall components cannot be regarded as inert. Plant cell expansion growth is irreversible and involves a net deposition of wall materials including cellulose.

The statement that cellulose is the most abundant biomolecule on earth has often been made (Preston 1974) and indicates the preponderance of walled organisms. An enormous amount of energy flows through the living world for the sole purpose of making cell walls. Because of the economic value of plant cell walls, as major sources of food, fiber and energy for man, the understanding of plant cell wall formation is of importance for mankind.

The mechanism of cell wall biosynthesis is still not understood.

The cell wall of higher plants is fundamentally involved in the morphology, growth and development of plant cells. Brown (1985) made three assumptions as a background to the spatial control of cellulose assembly: (1) cellulose microfibrils are the reinforcing and constraining structures of

the plant cell wall, (2) the specific orientation of cellulose microfibrils can determine the directionality of differential constraints, and, therefore, cell expansion, (3) the driving force for cell expansion and cell growth is turgor.

Cell walls are classified as primary or secondary. Primary cell walls are deposited by undifferentiated cells, that are still growing, and are dynamic structures. Secondary walls are deposited by full-grown cells and are mechanically more static structures, that determine shape and size of the mature cell. In this respect the root hairs of higher plants are a special case, having tip-growth and depositing a primary wall at the expanding tip, but at the same time depositing a secondary wall in the non-expanding hair tube. The primary wall eventually constitutes a thin sheath on the outer surface of the full-grown root hair. The secondary wall is deposited on the inner surface of the existing primary wall between primary wall and plasma membrane, thus reducing the cell lumen. Primary cell wall and secondary cell wall differ in textures, mechanical properties, and chemical composition (Preston 1974). Cell wall textures are classified as axial, helical, helicoidal and crossed polylamellate, according to the orientation of microfibrils in their constituting lamellae. All these textures have been found in root hairs (Sassen et al. 1985, Lloyd and Wells 1985).

Cytological, autoradiographic and biochemical approaches have provided information on the subcellular events of cell wall synthesis. Endoplasmic reticulum, Golgi apparatus and plasma membrane form a functionally integrated system for the synthesis and transport of cell wall components (Robinson 1977). The polysaccharide deposited into the wall depends on vesicle fusion for the direct transfer of polymers and for the incorporation of enzymes into either the plasma membrane or the cell wall. Cell wall proteins are synthesized on the rough endoplasmic reticulum. Hemicellulosic and pectic polysaccharides are formed in the Golgi apparatus and reach the plasma membrane via vesicles (Sievers and Schnepf 1981). The vesicles fuse with the plasma membrane releasing their contents to the cell wall (cf. for pollen

tubes: Sassen 1964). Cellulose microfibrils are assembled on the plasma membrane.

The cytoplasmic side of the plasma membrane contains coated pits. In root hairs they have been shown in *Raphanus sativus* as early as 1966 (Bonnett and Newcomb). In animal cells coated pits function in receptor mediated endocytosis and membrane turnover (Pearse and Bretscher 1981, Bretscher and Pearse 1984). Their function in plant cells is controversial. Coated pits are also seen near dictyosomes and it has been suggested that they arise at the Golgi complex and deliver cell wall substances at the plasma membrane (Franke and Herth 1974, van der Valk and Fowke 1981, Robertson and Lyttleton 1982). However, Joachim and Robinson (1984) and Tanchak et al. (1984) have shown that cationic ferritin is taken up by plant protoplasts by the coated pit-coated vesicle pathway.

Roelofsen (1958) has suggested that an enzyme at the tip of the growing microfibril might be responsible for the polymerization of glucose and its crystallization into the microfibril. He predicted that it might be possible by means of electron microscopy to visualize the cellulose synthesizing complex. Helicoidal walls, however, have been argued to be formed by self-assembly (Neville 1985, 1986) in the periplasmic space (Roland and Vian 1979). Therefore the question arises whether a terminal synthesizing complex is present in the plasma membrane of cells which deposit a helicoidal wall. Putative synthesizing complexes in higher plants exist of a terminal globule on the extraplasmic-face of the plasma membrane and a particle rosette on the protoplasmic-face of the plasma membrane (Brown 1985). The synthesizing complexes are free to move in the plane of the fluid membrane while microfibrils are being generated. The occurrence of these complexes in the plasma membrane is seen as proof that microfibrils lengthen by tip-growth (Willison 1982), which they might not do if cell walls were self-assembling like liquid crystals. But what determines the direction of the mobile synthesizing complexes that leave microfibrils in their wake?

The alignment of the fibrils within the wall has an important bearing on the physical properties of cells. The orientation of microfibrils is a matter of discussion (Robinson 1977). In many cells microtubules have been found in parallel to the nascent microfibrils and they have therefore been hypothesized to orientate the microfibrils during their deposition (Heath 1974). Heath has suggested that cellulose synthesizing complexes are located in the plasma membrane and that they are associated with a component which crosses the inner side of the plasma membrane and interacts with adjacent mictotubules, whereby the microfibril synthesizing complex is moved through the membrane. This is possible because the membrane has a fluid mosaic structure (Singer and Nicholson 1972). Recent reviews on this subject include: Robinson and Quader 1982, Gunning and Hardham 1982, Lloyd 1984. In cases where microfibril-microtubule paralellism has not been found (Chafe and Wardrob 1970) this has been regarded as a stage in reorientation prior to the deposition of a new lamella, rather than evidence that microtubules are not involved. Also Sawhney and Srivastava (1975) found lack of paralellism between microtubules and microfibrils, but regarded this as evidence for a shift in the orientation of new microfibrils in normal thickening of polylammellate walls.

A role for the microtubules in microfibril orientation is supported by experiments with anti-microtubular agents. The destruction of microtubules by these agents disturbs microfibril deposition in many plant species (Robinson and Quader 1982).

Many different hypotheses have been proposed on the role of the cytoskeleton in microfibril orientation (Heath and Seagull 1982) but none of these hypotheses received sufficient proof. Microfibril deposition during treatment, which depolymerizes microtubules, may be random (Schnepf et al. 1975), in swirls (Hepler and Fosket 1971), smeared (Brower and Hepler 1976), unidirectional (Quader et al. 1978), helicoidal (Takeda and Shibaoka 1981, Quader et al. 1978), or undisturbed (Itoh 1976, Schnepf and Deichgräber 1979). In some cells microfibrils are deposited in an orderly pattern while microtubules are absent (Hahne and Hoffmann 1985).

Schnepf (1978) found more or less parallel microtubules associated with dispersed microfibrils in the endostomium of the moss; microtubules run in the direction of the longitudinal axis of the cell. Microtubules have several other functions in plant and animal cells: maintenance of cell shape, internal organization of the cytoplasm, movement of cytoplasmic components, cytokinesis, endo- and exocytosis (Dustin 1984).

This thesis reports a study on cell wall texture and microfibril deposition in root hairs and the role cytoplasmic elements play in this process. The main object used is the root hair of *Equisetum hyemale*, which has a helicoidal wall (Sassen et al. 1981). In a helicoidal wall, fibril lamellae are subsequently deposited in successively rotated orientations. Therefore, the helicoidal wall texture is an excellent system to test the hypothesis that microtubules orientate microfibrils. Because root hair growth can be easily followed and the cells are accessable for chemical- and physical fixation root hairs are suitable for studies of wall texture and mode of synthesis of microfibrils of higher plants.

Root hairs develop as outgrowths of epidermal cells of the roots of many plant species. Because they increase the root surface area and hence the volume of soil contacted they are likely to be involved in the uptake of nutrients (Itoh and Barber 1983, Robinson and Rorison 1983), and water (Jones et al. 1983). In addition, root hairs may also have a role in the anchorage of plants (Stolzy and Barley 1968), the maintenance of contact between roots and soil (Russell 1977) and in root exudation (Baht and Baldwin 1976). Furthermore, they are important for the formation of symbiosis between legumes and *Rhizobium* (Turgeon and Bauer 1985).

In chapter 1 the hypothesis that root hairs of aquatic plants have helicoidal wall texture, while root hairs of terrestrial plants have axial or helical wall texture (Sassen et al. 1981, Sassen et al. 1985) is tested.

Chapter 2 reports a study on wall textures of root hairs of all european species of *Equisetum*.

Chapters 3 and 4 present a detailed description of helicoidal wall texture and of microtubule alignment in the cortical cytoplasm of young and full-grown root hairs of *Equisetum hyemale*.

In chapter 5 data on microtubule alignment obtained with thin-sectioning of chemically fixed material are compared with data obtained by means of the freeze-substitution method. Data obtained by the dry-cleaving method for cell walls (Sassen et al. 1985) are used to give a detailed description of the inner side of the cell wall.

The alignment of microfibrils, microtubules and microfilaments of the trichoblastic part of the root hair cell is described in chapter 6. Microfibrils are visualized by thin-sectioning of cells from which matrix material has been removed, by means of the freeze-substitution and dry-cleaving methods. Microtubules are visualized by immunofluorescence and freeze-substitution. Microfilaments are stained with rhodamine-labelled phalloidin.

The influence of colchicine -which depolymerizes microtubules- on helicoidal wall deposition is studied in chapter 7.

In chapter 8, by means of freeze-fracturing, the extraplasmic (EF) and protoplasmic (PF) fracture faces of the plasma membrane of root hairs of *Equisetum hyemale* are shown to contain the putative microfibril synthesizing complexes.

The relation of the particle rosettes of the PF-face of the plasma membrane to arrays of hexagonally ordered patterns found in the plasma membrane of these root hairs and in other plant cells is worked out in chapter 9.

Chapter 10 deals with the occurrence, distribution, dimensions and ultrastructure of coated pits and coated vesicles on the cytoplasmatic side of the plasma membrane. Coated pits and coated vesicles are visualized by means of the dry-cleaving method. Based on these data the function of these structures in plant cells is hypothesized.

Chapter 11 describes the ultrastructural organization of the cytoplasm of root hairs of *Equisetum hyemale* and *Limnobium stoloniferum* after freeze-substitution. Especially wall vesicles and microfilament bundles are described. Cytoplasmic streaming in developmental stages of root hairs of *Equi-*

setum hyemale *is shown. A relation is shown between elements of the cytoskeleton and cytoplasmic streaming.*

Finally, on the basis of the data described in this thesis, in chapter 12 a mathematical model for helicoidal wall deposition is proposed.

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# CHAPTER 1

## Helicoidal Cell Wall Texture in Root Hairs

# HELICOIDAL CELL WALL TEXTURE IN ROOT HAIRS

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**PLanta, in press.**

## ABSTRACT

It is shown that root hairs of most aquatic plants have a helicoidal cell wall texture. Cell walls of root hairs of the aquatic/marshland plant Ranunculus lingua, however, have axial microfibril alignment. The occurrence of helicoidal wall texture is not limited to root hairs of aquatic plants: the terrestrial plant Zebrina purpusii has a helicoidal root hair wall texture. With the exception of the grasses, the occurrence of root hairs with helicoidal cell wall pertains to species with predetermined root hair forming cells, trichoblasts. The rotation mode of the helicoid is species specific. The average angle between fibrils of adjacent lamellae varies from 23° to 40°.

In Hydrocharis morsus ranae cortical microtubules have a net-axial orientation and thus do not parallel nascent microfibrils.

Helicoidal cell wall deposition is discussed.

KEY WORDS: cellulose microfibril, helicoidal cell wall, microfibril orientation, plant cell, root hair

## INTRODUCTION

In helioidal cell walls each lamella is built up of parallelly oriented microfibrils. In subsequent lamellae there is a progressive change in microfibril orientation (Neville 1985, 1986). Sassen and coworkers (1985) have suggested that the helicoidal texture is characteristic of root hairs of aquatic plants. So far only two aquatic plants have been studied with respect to root hair wall texture: *Limnobium stoloniferum* (Pluymaekers 1982) and *Ceratopteris thalictroides* (Sassen et al. 1981, Meekes 1985). The helicoidal cell wall texture has been found in young root hairs of the

terrestrial plant *Equisetum hyemale* (Sassen et al. 1981, Emons 1982). However, root hairs of *Equisetum hyemale* acquire an additional helical microfibril layer (Emons and Wolters-Arts 1983) similar to the texture in root hairs of some terrestrial plants (Sassen et al. 1981). In water without connection to soil, stem fragments of *Equisetum hyemale* grow into new plants complete with strobili (Wagner and Hammitt 1970). *Equisetum hyemale* appears to be very well equiped for life in water.

Root hairs of *Equisetum fluviatile*, a species the natural habitat of which is water, have a helicoidal wall. They do not acquire a helical wall layer when full-grown (Emons 1986).

To test the hypothesis that root hairs of aquatic plants have a helicoidal root hair cell wall, we studied 13 species of aquatic plants: 6 with the electron microscope and 7 additional species with the polarizing microscope, a method which gives strong indications whether or not a cell wall is helicoidal. These aquatic plants naturally grow rooted in mud in shallow water, but also produce roots in water when not in contact with the ground.

In addition, root hairs of 4 terrestrial plants were studied: *Zebrina purpusii*, the cell wall of which appears isotropic in polarized light (Sassen et al. 1981), *Lepidium sativum* with a positive birefringent wall (Sassen et al. 1981), *Pteris cretica* and *Asplenium viviparum* which, like the aquatic *Ceratopteris thalictroides*, are ferns.

## MATERIAL AND METHODS

The species with water as their natural habitat were grown in aquarium tanks. The tanks contained a layer of pond mud and were kept at a temperature of 18°C-25°C, in a regime of 16 h light and 8 h dark. With the electron microscope root hairs of the following species were examined: Hydrocharis morsus-ranae (Hydrocharitaceae), Stratiotes aloides (Hydrocharitaceae), Phragmites australis (Gramineae), Ranunculus lingua (Ranunculaceae), Ruppia maritima (Potamogetonaceae), Butomus umbellatus (Butomaceae). With the polarizing microscope root hairs of the 6 species mentioned as well as Aponogeton distachyos (Aponogetonaceae), Azolla caroliniana (Azollaceae), Potamogeton crispus (Potamogetonaceae), Potamogeton pectinatus (Potamogetonaceae), Zannichellia peltata (Zannichelliaceae), Cyperus asper (Cyperaceae) and Sagittaria sagittifolia (Alismataceae) were studied.

Of the terrestrial species root hairs of Pteris cretica (Polypodiaceae) and Asplenium viviparum (Polypodiaceae) were taken from plants grown in soil, and studied with the polarizing microscope. Root hairs of Zebrina purpusii (Commelinaceae) from cuttings grown in tap water and root hairs of Lepidium sativum L. (Cruciferae) from seedlings grown on petri dishes in moist air were studied with both the polarizing microscope and the electron microscope.

### Polarizing microscopy

Having no preferential crystal alignment, helicoidal cell walls are isotropic between crossed polarizers (cf. Preston 1974). Birefringence of untreated hairs as well as of hairs treated with hydrogen peroxide/glacial acetic acid (1:1,v/v) ($H_2O_2$/HAc) was determined with the polarizing microscope (Leitz HM Pol).
Single wall preparations were prepared as described for electron microscopy.

### Electron microscopy

Thin sectioning and shadow-casting were carried out as described by Emons and Wolters-Arts (1983). The dry-cleaving technique for cell walls (Sassen et al. 1985) was used for Zebrina purpusii. This method does not differ essentially from shadow-casting, but cells are critically-point-dried prior to cleaving and larger surfaces of inner wall may be obtained.

Specimens were examined with a Philips EM 201 electron microscope.

## RESULTS

### *Birefringence*

Table 1 shows birefringence of young growing root hairs of all species studied in this respect including the species of the present study.

Of the 16 species of aquatic plants examined, only root hairs of *Ranunculus lingua* are positively birefringent, root hairs of the other species are optically isotropic (Table I).

Isotropy in the polarizing microscope is a strong indication for helicoidal cell wall texture. But a random texture is also isotropic and a helical texture with alternating 45° helices as well as a single helix with 45° pitch will also be nearly isotropic in whole mounts (Frey-Wyssling 1976). The possibility of a single helix could, however, be cancelled out by examining single wall preparations.

Young root hairs of the terrestrial plants (Table 1) are positively birefringent with the exception of all Equisetaceae, *Zebrina purpusii*, *Tradescantia albiflora*, *Impatiens*

*walleriana* and *Coleus blumei*, which are isotropic in polarized light.

*Cell wall texture in aquatic plants*

Thin transverse as well as longitudinal sections of *Hydrocharis morsus ranae* root hairs show two arcs of the helicoidal wall (Fig. 1). The outer layer consists of randomly oriented microfibrils.

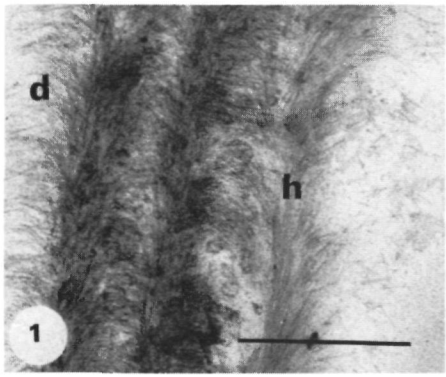
Surface preparations show the inner wall microfibrils aligned in parallel within a lamella. Underneath the inner lamella the second innermost lamella is seen and often it is possible to discern a third lamella. An angle of approx. 35° can be observed between the orientations of microfibrils in successive lamellae (Fig. 3). Looking from the cytoplasm and going from former to later deposited lamellae the rotation mode of the helicoid is clockwise. In *Stratiotes aloides* up to 4 helicoidal arcs are formed in the full-grown hair (Fig. 2). The angle between microfibril orientations in adjacent lamellae is approximately 30°. The rotation mode is clockwise.

A helicoidal layer consisting of only one half arc is formed in Butomus umbellatus, three arcs in Phragmites and up to twelve arcs in Ruppia maritima (data not shown).

The root hair wall of *Ranunculus lingua* was prepared by shadow-casting (Fig. 4), and proved to be exceptional for an aquatic plant. Microfibrils align more or less in the direction of the long axis of the hair, which agrees with the observation that the wall is positive birefringent in the polarizing microscope. This wall texture is comparable to the root hair wall texture of most terrestrial plants.

*Cell wall texture in terrestrial plants*

The inner layer of the root hair of *Lepidium sativum* (Fig. 5) has axial microfibril alignment. It has to be noted that in cell wall preparations of root hairs with axial microfibril alignment some transverse single microfibrils do always occur.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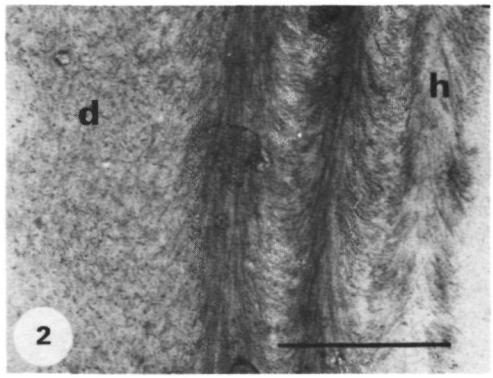
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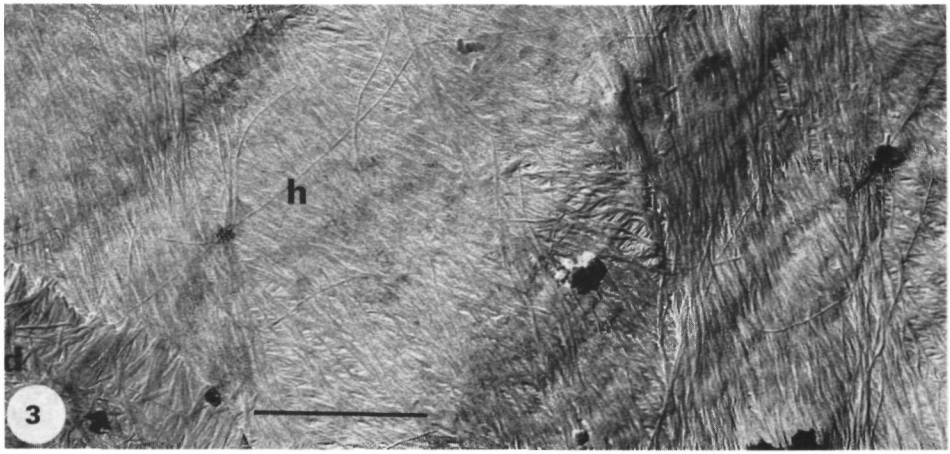
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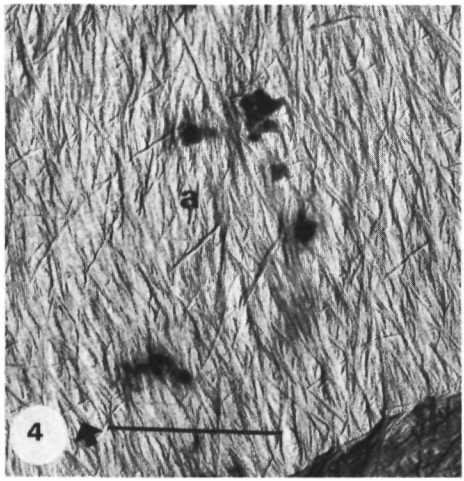
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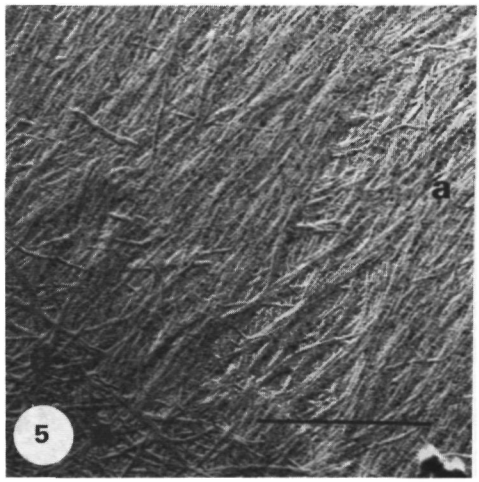

a
5

Fig. 1.
Thin section of cell wall of root hair of Hydrocharis, showing two arcs of the helicoidal cell wall. x45 500. Bar: 500 nm.

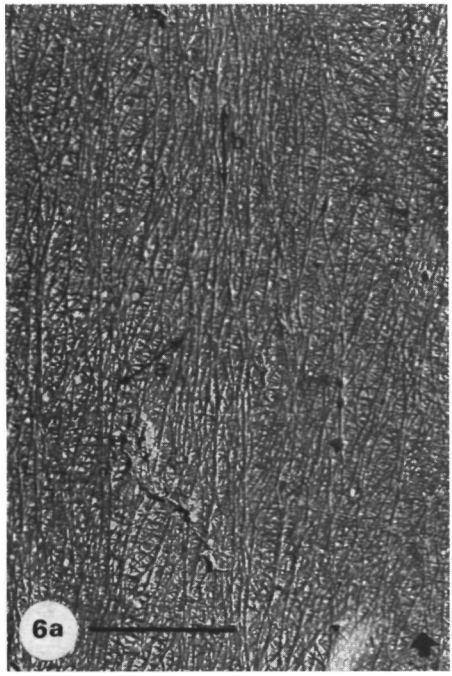
Fig. 2.
Thin section of cell wall of root hair of Stratiotes aloides, showing three helicoidal arcs. x45 500. Bar: 500 nm.

Fig. 3.
Surface view of cell wall inner side of Hydrocharis root hair, showing two consecutive fibril lamellae with an angle of approx. 35° between microfibril orientations in adjacent lamellae and a clockwise helicoid rotation. Rotation mode is determined looking from the cytoplasm and going from former to later deposited fibril lamellae. Pointer: long axis of hair. x45 500. Bar: 500 nm.

Fig. 4.
Surface view of inner cell wall of root hair of Ranunculus lingua, prepared by shadow-casting, showing microfibrils more or less in the direction of the long axis of the hair. Pointer: long axis of hair. x45 500. Bar: 500 nm.

Fig. 5.
Surface view of inner cell wall of root hair of Lepidium sativum, prepared by shadow-casting, showing microfibrils predominantly in the longitudinal direction. Pointer: long axis of hair. x45 500. Bar: 500 nm.

Fig. 6 a-d.
Surface views of inner cell wall preparations of root hair of Zebrina purpusii, obtained by dry-cleaving, taken at various distances from the hair tip, showing that microfibrils of the innermost wall lamella occur at different orientations with respect to the long axis of the hair. The angle between adjacent microfibril lamellae is approximately 40°. The rotation mode is counterclockwise. Pointer: long axis of hair. x19 200. Bar: 1000nm


a
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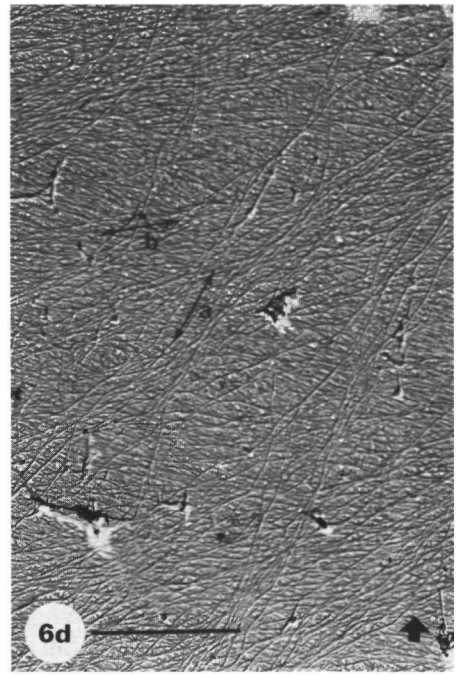
6a


a
b
6b


a
b
6c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The inner cell wall of the *Zebrina purpusii* root hair is shown in Figs. 6a-d. The micrographs are taken at various distances from the hair tip and show that microfibrils of the innermost wall lamella occur at various orientations, a typical feature for helicoidal cell walls of tip-growing cells. The angle between successive lamellae is approx. 40°; the rotation mode is counterclockwise, different from the rotation mode in *Hydrocharis* and *Stratiotes*.

## DISCUSSION

Shadow-casting and dry-cleaving yield surface views of the inner cell wall and thus give 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. Because microfibrils in freeze-fractured material are straight (Emons 1985) wavy patterns of microfibrils observed in dry-cleaved preparations are likely to be induced by the method. However, in dry-cleaved preparations net microfibril orientation is not altered.

### *The helicoidal cell wall texture in root hairs*

In biological systems the chemistry of helicoidal structures varies. The basic requirements seem to be long crystalline rods and a suitable matrix (Neville and Levy 1984). In plant cells helicoids are mostly built of cellulose microfibrils in a polysaccharide matrix. In the helicoidal root hairs studied, surface views show that fibrils within a lamella are not contiguous. Fibril density varies between preparations, and is dependent on the stage of lamella completion. The angle between microfibrils of adjacent lamellae in root hairs ranges from an average angle of 23° in *Limnobium stoloniferum* (Pluymaekers 1982) to 40° in *Equisetum hyemale* (Emons, Wolters-Arts 1983). Roland et al. (1983) have hypothesized that the rotation mode is the same for all helicoidal plant cell walls. However, in root hairs the rotation mode, looking from the cytoplasm and according to the sequence of deposition may be clockwise as in *Hydrocharis*

(Fig. 3) and *Stratiotes*, as well as in *Limnobium* (Pluymaekers 1982) and *Ceratopteris* (Meekes 1985) or counter clockwise as in *Zebrina* (Figs. 6 a-d) and *Equisetum* (Emons and Wolters-Arts 1983), but is constant within one species. This observation constitutes constraints for possible models of helicoid formation. Neville (1985) speculated that all cell wall helicoids may have left-handed rotation as a consequence of the hypothesis that "Hemicellusoses self-assemble to form a cholesteric liquid crystal, which is in turn responsible for interacting with, and orienting cellulose microfibrils into a helicoid".

*Occurrence of helicoids in cell walls of root hairs*

The helicoidal texture has been argued to be the most effective wall texture for root hairs of aquatic plants (Sassen et al. 1985). Indeed, all but one of the studied hairs of aquatic plants have a helicoidal wall texture. The exception is *Ranunculus lingua*, which is rooted in the submerged substratum. However, other aquatic species, for instance *Stratiotes*, may also root in the mud. In no species a difference in structure was found between root hairs of free floating roots and roots growing in mud. Absence of influence of the substrate has been shown for *Equisetum hyemale* (Emons, 1986).

Root hair initiating cells, trichomes, may be of two types, type I or type II (Leavitt 1904, Cormack 1962). In type I any rhizodermal cell may form a hair after the cell has extended considerably. Trichomes of type II originate early in the development of the rhizodermis. Only predetermined cells, trichoblasts, form root hairs. An asymmetric division leads to the formation of a trichoblast and has been shown for *Hydrocharis* to be predicted by the presence of a pre-prophase band of microtubules (Gunning et al 1978).

Most species with a helicoidal cell wall investigated in the present study fall within type II (Table I), also the terrestrial Commelinaceae and Equisetaceae. *Ranunculus lingua*, which proved to be exceptional with respect to wall texture, has trichome type I root hairs. The grasses, however, do not show coincidence between trichome type and wall texture (Table 1).

TABLE 1

Cell wall texture of young growing root hairs of different plant species, studied with polarizing microscopy (Birefringence) and electron microscopy (Texture); distribution of trichome type I and II cells according to Leavitt (1904). Trichome type I cells are not predetermined in the nascent epidermis; trichome type II cells are predetermined to differentiate in root hair forming trichoblasts. (I) and (II) means that trichome type is hypothesized from related species.

| Plant species | Trichome Type | Birefringence | Wall Texture | Environment |
|---|---|---|---|---|
| EQUISETINAE | | | | |
| Equisetum arvense Equisetaceae | II | none[a)] | | terrestrial |
| Equisetum fluviatile Equisetaceae | II | none | helicoidal[a)] | aquatic |
| Equisetum palustre Equisetaceae | (II) | none | helicoidal | marshland |
| Equisetum pratense Equisetaceae | (II) | none[a)] | | terrestrial |
| Equisetum sylvaticum Equisetaceae | (II) | none[a)] | | terrestrial |
| Equisetum telmateia Equisetaceae | (II) | none[a)] | | terrestrial |
| Equisetum hyemale Equisetaceae | II | none | helicoidal[b)] | terrestrial |
| Equisetum ramosissimum Equisetaceae | II | none[a)] | | terrestrial |
| Equisetum scirpoides Equisetaceae | II | none[a)] | | terrestrial |
| Equisetum variegatum Equisetaceae | II | none | helicoidal[a)] | terrestrial |
| FILICES | | | | |
| *Pteris cretica Polypodiaceae | I | pos. | | terrestrial |
| *Asplenium viviparum Polypodiaceae | (I) | pos. | | terrestrial |
| Ceratopteris thalictroides Parkeriaceae | | none | helicoidal[c)] | aquatic |
| *Azolla caroliniana Azollaceae | II | none | | aquatic |

(continued on next page)

(TABLE 1 continued)

| | | | | |
|---|---|---|---|---|
| DICOTYLEDONAE | | | | |
| Urtica dioica<br>Urticaceae | (I) | pos. | helical[d)] | terrestrial |
| Urtica cannabina<br>Urticaceae | (I) | pos.[d)] | | terrestrial |
| Parietaria officinalis<br>Urticaceae | (I) | pos.[d)] | | terrestrial |
| *Ranunculus lingua<br>Ranunculaceae | (I) | pos. | | aquatic/<br>marshland |
| *Lepidium sativum<br>Cruciferae | (I) | pos. | axial | terrestrial |
| Sinapis alba<br>Cruciferae | (I) | pos. | axial[e)] | terrestrial |
| Raphanus sativus<br>Cruciferae | (I) | pos. | axial[f)] | terrestrial |
| +Impatiens walleriana<br>Balseminaceae | (I) | none[d)] | | terrestrial |
| +Coleus blumei<br>Labiatae | (I) | none[d)] | | terrestrial |
| MONOCOTYLEDONAE | | | | |
| *Sagittaria sagittifolia<br>Alismataceae | (II) | none | | aquatic |
| *Butomus umbellatus<br>Butomaceae | | none | helicoidal | aquatic |
| *Stratiotes aloides<br>Hydrocharitaceae | II | none | helicoidal | aquatic |
| *Hydrocharis morsus ranae<br>Hydrocharitaceae | II | none | helicoidal | aquatic |
| Limnobium stoloniferum<br>Hydrocharitaceae | (II) | none | helicoidal[g)] | aquatic |
| *Potamogeton crispus<br>Potamogetonaceae | (II) | none | | aquatic |
| *Potamogeton pectinatus<br>Potamogetonaceae | (II) | none | | aquatic |
| *Ruppia maritima<br>Potamogetonaceae | (II) | none | helicoidal | aquatic |
| *Zannichellia peltata<br>Zannichelliaceae | II | none | | aquatic |
| *Aponogeton distachyos<br>Aponogetaceae | II | none | | aquatic |

(continued on next page)

(TABLE 1 continued)

| | | | | |
|---|---|---|---|---|
| °Allium cepa<br>Liliaceae | | | helicoidal[h)] | terrestrial |
| Tradescantia albiflora<br>Commelinaceae | (II) | none[d)] | | terrestrial |
| *Zebrina purpusii<br>Commelinaceae | (II) | none | helicoidal | terrestrial |
| *Cyperus asper<br>Cyperaceae | | none | | aquatic |
| Triticum monococcum<br>Gramineae | | pos | axial[i)] | terrestrial |
| *Phragmites australis<br>Gramineae | II | none | helicoidal | aquatic |
| Zea mays<br>Gramineae | | pos | axial[j)] | terrestrial |

* these species investigated in this study

+ these species need further investigation

° Allium is a special case. In many Allium species root hairs do not originate in the epidermis, but in the hypodermal layer, or exoderm. Leavitt (1904) compares the short piliferous cells of the exoderm with trichome type II.

a) Emons 1986
b) Emons 1982, Emons and Wolters-Arts 1983
c) Meekes 1985
d) Sassen et al. 1981
e) Belford and Preston 1961
f) Newcomb and Bonnett 1965
g) Pluymaekers 1982
h) Sassen et al. 1985
i) Pluymaekers 1979
j) Frey-Wyssling and Muhlethaler 1949

Microtubules have been hypothesized to control microfibril orientation (Robinson and Quader 1982). Hence, the nascent microfibrils would be expected to parallel the microtubules. However, this is not observed in root hairs with helicoidal cell walls: *Hydrocharis* (Traas, personal communication), *Equisetum hyemale* (Emons 1982, Emons and Wolters-Arts 1983), *Ceratopteris thalictroides* (Meekes 1985), *Limnobium stoloniferum* (Traas et al. 1985).

Because coincidence in orientation between microtubules and the extracellular crystalline rods has never been reported in animal cells, self-assembly has been considered as an alternative model for the control of helicoid formation (Neville and Levy 1984, Neville 1985, 1986). The self-assembly process is thought to occur in the periplasm (Roland 1979) and is easily disturbed (Reis et al. 1985). In self-assembly the face involved in helicoidal control is the inner surface of the previously deposited wall lamella.

The control of microfibril orientation is far from being understood. The hypothesis that microtubules control microfibril orientation shifts the problem to explaining the orientation of microtubules. Coincidence in microtubule- and microfibril alignment has been found especially in expanding cells (see review: Robinson and Quader 1982). This coincidence has also been found with freeze-substitution in the expanding part, the tip, of young growing root hairs (Emons and Derksen 1986). The growing cotton hair is different from the root hair in that the cotton hair expands not only at the tip but over its total length (Willison 1982), while the root hair expands only at the tip (Sievers and Schnepf 1981). Indeed, in the cotton hair, microtubules and microfibrils are in parallel (review: Ryser 1985).

Microtubule and microfibril parallelism has further been found in non-expanding cells where local wall thickening occurs, as in differentiating x,lem vessels (Falconer and Seagull 1985, review: Robinson and Quader 1982), but not in full-grown cells with wall deposition over the entire surface of the cell.

Microtubules and microfibrils both function in morphogenesis and are parallel in expanding cells (Hardham 1982, Busby and Gunning 1984), but there is insufficient proof that microtubules orientate the nascent microfibrils. Also Hahne and Hoffmann (1985) concluded that microtubules exert no influence on the orientation of microfibrils.

The study of helicoidal cell wall deposition may prove to be particularly important to discover the orientating mechanism of microfibrils in plant cells.

ACKNOWLEDGEMENT

We thank Prof. Dr. M.M.A. Sassen for his support and Dr. J. Derksen for critically reading the manuscript.

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# CHAPTER 2

## Cell Wall Texture in Root Hairs of the Genus Equisetum

# CELL WALL TEXTURE IN ROOT HAIRS OF THE GENUS EQUISETUM

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Can. J. Bot. 64 (1986), in press

## ABSTRACT

Two groups can be distinguished within the ten species of Equisetum, listed in the Flora Europaea, based on cell wall texture of root hairs. This distinction coincides with the division of the genus Equisetum into two subgenera: Equisetum (horsetails) and Hippochaete (scouring rushes). All species of the subgenus Equisetum have a helicoidal cell wall texture in young growing root hairs as well as in full-grown hairs. All species of the subgenus Hippochaete deposit an additional inner cell wall layer against this helicoidal layer when elongation has stopped. The microfibrils in this additional layer do not form a helicoidal texture, but are helically arranged, forming a Z-helix.

The presence of a helical layer in full-grown hairs is not a prerequisite for growth in soil but an exclusively helicoidal root hair wall texture might be favourable for life in water. The wall texture is not influenced by the consistency of the substratum.

La "Flora Europaea" distingue dix espèces du genre Equisetum. La présente étude montre que la texture des parois cellulaires des poils radicaux permet de distinguer deux groupes parmi les espèces. Cette distinction coïncide avec la division du genre Equisetum en deux sous-genres: Equisetum et Hippochaete. Toutes les espèces du sous-genre Equisetum ont une texture hélicoïdale, aussi bien dans les poils jeunes en croissance que dans les poils pleinement développés. Toutes les espèces du sous-genre Hippochaete déposent une couche supplémentaire à l'intérieur d'une couche hélicoïdale de la paroi cellulaire une fois que la croissance s'est arrêtée. Les microfibrilles de la couche supplémentaire ne sont pas déposées hélicoïdalement mais s'arrangent dans une hélix-Z.

La présence d'une couche hélicale dans des poils pleinement développés n'est pas une qualité requise pour la croissance en terre, mais une texture de parois entièrement hélicoïdale pourrait être favorable à la situation aquatique. La texture cellulaire n'est pas influencée par la consistence du substratum.

The Equisetinae, belonging to the Pteridophyta, appeared and speciated in the Palaeozoic (ARNOLD 1947). Today, only one family, the Equisetaceae, which appeared in the Carboniferous, persists. It is represented by the single genus *Equisetum*.

BRAUN (1844) divided the genus into two groups: *Equiseta speiropora*, the horsetails, with irregularly dispersed stomata over the whole surface of the grooves of the stem and *Equiseta stichopora*, the scouring rushes or winter-Equiseta, with irregularly dispersed stomata in two distinct lines or bands, one on each side of the grooves.

The stomata of the horsetails appear as pores, flush with the epidermis; the stomata of the scouring rushes are sunken, a difference considered by MILDE (1865) to be important enough to separate the group into two genera, *Eu-Equisetum* and *Hippochaete*. MANTON (1950) demonstrated a marked difference in chromosome size in comparing *Eu-Equisetum* with *Hippochaete*, *Eu-equisetum* having smaller chromosomes; she, however, considered them to be subgenera. HAUKE (1963,1978) also, in his monographs on *Equisetum*, rejected a division into two genera since the similarities in general morphology, anatomy and reproductive system outweigh the differences in stomatal structure and chromosome size. He argues, however, that treating them as sections would underestimate the differences that do exist. He therefore considered the family Equisetaceae to be monogeneric, the single genus *Equisetum* consisting of two subgenera, *Equisetum* and *Hippochaete*.

In an extensive study on the cell wall texture of the root hair of *Equisetum hyemale* (EMONS and WOLTERS-ARTS 1983) the secondary wall layer, i.e. the texture of microfibrils deposited in the not growing hair tube against the randomly textured primary wall layer of the young tip-growing hair, was shown to be of the helicoidal type. The term helicoidal was first applied to plant cell walls by Bouligand (1972). The helicoidal cell wall texture is made up of a stack of parallel-fibred lamellae, one fibril thick; microfibril orientation in subsequent lamellae is progressively rotated

(NEVILLE and LEVY 1984, EMONS and WOLTERS-ARTS 1983). The helicoidal wall texture has been found primarily in root hairs of aquatic plants (SASSEN et al. 1981), but in the full-grown hair of *Equisetum hyemale* a helical wall texture is deposited against the helicoidal wall layer, causing positive birefringence in the polarizing microscope (EMONS and WOLTERS-ARTS 1983). The helical layer is not found in aquatic plants, but terrestrial plants show only an axial or helical microfibril orientation in the secondary wall layer of their root hairs (SASSEN et al. 1981). Although *E. hyemale* is a terrestrial plant, it has become adapted for dispersal from stem fragments carried by water (WAGNER and HAMMITT 1970).

The aim of the present study was to investigate a). whether the cell wall of young root hairs of *Equisetum hyemale* is also helicoidal (the aquatic trait) in soil and in agar media of different consistencies, b). whether the other terrestrial species of *Equisetum* acquire the additional helical wall layer (the terrestrial feature) and c). whether *Equisetum fluviatile*, an aquatic, and *Equisetum palustre*, a marshland plant, lack the additional helical wall layer (the terrestrial feature). In the first instance, data were obtained with the polarizing microscope.

Two problems were addressed using the transmission electron microscope: 1). Do the isotropic hairs have a helicoidal wall texture, as described for *E. hyemale*? This question is relevant, because a hair with random microfibril alignment and with alternating 45° helices will also be isotropic. As examples *E. fluviatile*, *E. palustre* and *E. variegatum* were taken. 2). Do the full-grown isotropic hairs indeed lack a helical fibrillar alignment. The helical layer may be too thin to detect with the polarizing microscope. As examples *E. fluviatile* and *E. palustre* were studied.

All of the European species of *Equisetum*, covered in the Flora Europaea (TUTIN et al. 1964), were studied.

## MATERIAL AND METHODS

### Growth conditions

Ten different species of Equisetum were grown in the botanical garden of the University of Nijmegen: E. arvense L., E. fluviatile L. (= E. limosum L., E. heleocharis Ehrh.), E. hyemale L., E. palustre L., E. pratense Ehrh., E. ramosissimum Desf., (= E. campanulatum Poiret), E. scirpoides Michx, E. sylvaticum L., E. variegatum Schleicher ex Weber & Mohr, and E. telmateia Ehrh. (= E. maximum auct., E. majus Gars.). Root hairs were collected from plants living in situ: water in the case of E. fluviatile, marsh in the case of E. palustre and soil in all other cases. In addition, stem cuttings containing several nodes were grown on an aqueous soil extract (for preparation, cf. Meekes 1985) under greenhouse conditions: 18° C to 25° C, 1000 lux, with a light/dark regime of 16/8 h. In this soil extract, stem cuttings of E. fluviatile, E. hyemale, E. palustre, E. ramosissimum, E.scirpoides and E. variegatum developed roots with root hairs. However, E. arvense, E. pratense, E. sylvaticum and E. telmateia did not develop roots on stem cuttings in an aqueous growth medium. Root hairs of Equisetum hyemale, grown in different agar concentrations (0.5%, 1%, 1.5%, 2%) in a similar soil extract, were also studied.

### Light microscopy

Birefringence of root hairs of different ages was determined by means of a polarizing microscope (Leitz HM Pol) using the procedure of Preston (1974). Both fresh root hairs and hairs treated with hydrogen peroxide/glacial acetic acid ($H_2O_2$/HAc 1:1, v/v) were used. This treatment removes the cell cytoplasm and the cell wall matrix, leaving the cellulose microfibrils intact. For fibril angle measurement, single wall preparations obtained by cutting root hairs obliquely with a razor blade, were treated with the dichroitic stain chlorozinc-iodine to enhance birefringence (cf., EMONS and WOLTERS-ARTS 1983).

### Electron microscopy

Two procedures were used to visualize cell wall microfibrils . Whole roots with hairs, fixed in 2% glutaraldehyde in phosphate buffer pH 7 for l hour, were treated with $H_2O_2$/HAc for 1 hour at 100° in order to remove all matrix substances, which obscure the microfibrils. The material was washed in buffer, dehydrated in aqueous ethanol and flat embedded in Spurr's resin. Thin sections were stained with a 3% aqueous solution of potassium permanganate.

To visualize the innermost layer of the cell wall, i.e. the last deposited microfibril lamella situated at the plasma membrane, fixed hairs treated with $H_2O_2$/HAc were mounted on poly-L-lysine coated grids, critical point dried, cleaved, and shadowed with platinum and carbon (for procedure cf. Sassen et al. 1985).

Specimens were examined in a Philips EM 201 electron microscope at 60 KV.

Herbarium vouchers of the material studied are deposited in the Rijksherbarium of the University of Leiden.

*Polarizing microscopy*

Isotropy in the polarizing microscope means that none of the orientations of crystalline microfibrils of the cell wall predominates. Positive birefringence is caused by a predominance of the axial alignment of the wall crystalline microfibrils.

The mature hair is incrusted with a tawny brown substance, which appears at the time that hairs become positive birefringent. To be sure that birefringence is not caused by this substance both fresh hairs and hairs treated with $H_2O_2$/HAc, which removes all material except the microfibrils, were used. But no difference in birefringence was found between treated and untreated hairs.

Table 1 summarizes the data for the various *Equisetum* species. In all species, the young growing hairs were isotropic in polarized light. The full-grown hairs in all of the species of the subgenus *Equisetum* were isotropic while those of all species of the subgenus *Hippochaete* showed positive birefringence, a phenomenon which started at the base of the hair. Hairs grown in soil and hairs grown in an aqueous medium exhibited similar optical behaviour in polarized light.

The influence of the consistency of the medium on root hairs of *E. hyemale* is shown in Table 1. Hairs grown in different media exhibited the same optical behaviour: young, growing hairs were isotropic and full-grown hairs showed positive birefringence.

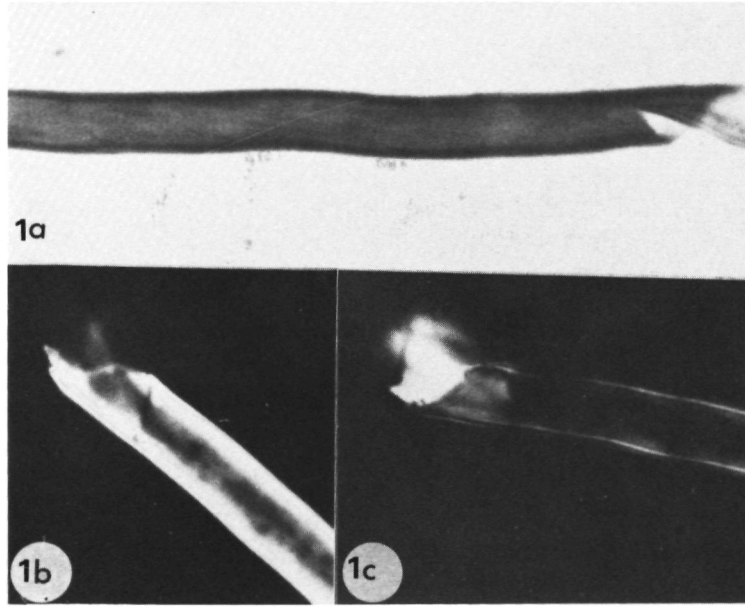
In full-grown hairs of the species of the subgenus *Hippochaete* an angle of mean microfibril direction with the long axis of the hair could be measured in single wall preparations (Table 2). This angle varied between 9° and 16° in *E. ramosissimum* and was 25° to 42° in *E. variegatum*, indicating that the overall fibril direction in full-grown hairs can be described as a helix with a pitch angle of more than 45°, causing positive birefringence in the polarizing microscope. In all *Hippochaete* a Z-helix was found.

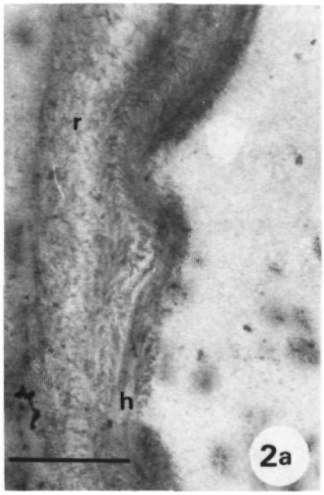
Fig. 1.

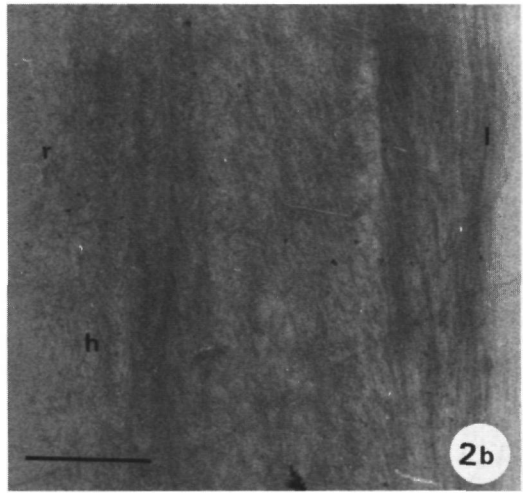
Root hair of Equisetum hyemale in the polarizing microscope: a: hair seen without analyzer to show the shape of the flared end; b and c: hair between crossed polarizers: b: hair positioned 45° to the directions of polarizer and analyzer: the whole hair is brightest in this direction, which is perpendicular to the extinction direction; c: hair rotated such that the single wall layer (flared end) is brightest. The angle between the orientations b and c corresponds with the mean angle microfibrils in the root hair make with the long axis of the hair (cf. EMONS and WOLTERS ARTS 1983). x1800.

Fig. 2,3,4.

Longitudinal thin sections of the root hair wall of E. hyemale (2a,b), E. fluviatile (3a,b) and E. palustre (4a,b); a: young hairs, b: full-grown hairs. r: outer wall layer with randomly oriented microfibrils. This layer is the same in all species, is deposited at the tip of the growing hair and lines the tube of the whole hair; h: helicoidal layer, consisting of parallel-fibred lamellae in rotating orientations; L: helical layer in which microfibrils make an angle of less than 45° with the long axis of the hair. This layer is present in E. hyemale but not in E. fluviatile and E. palustre. Scale bar: 500 nm.




r
h
2a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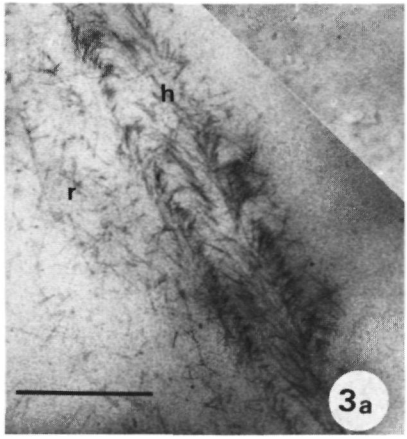
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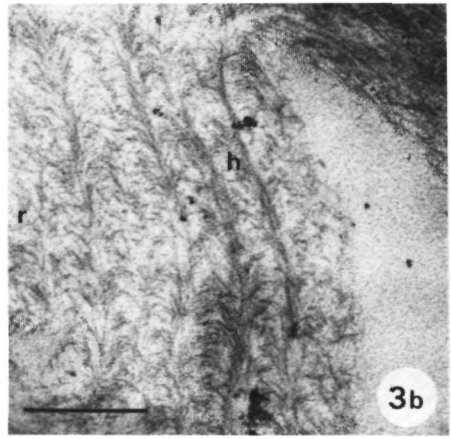
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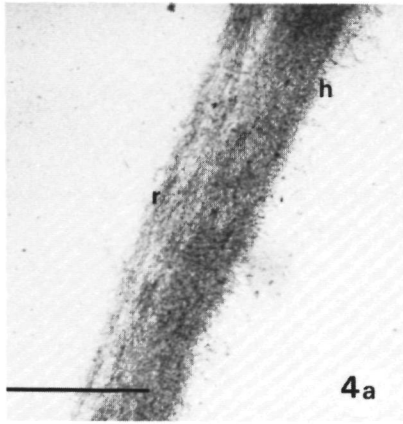
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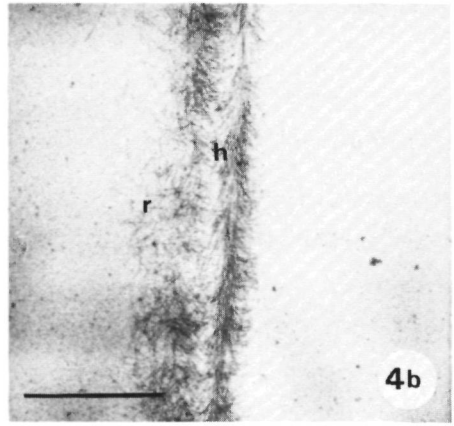

h
r
4b

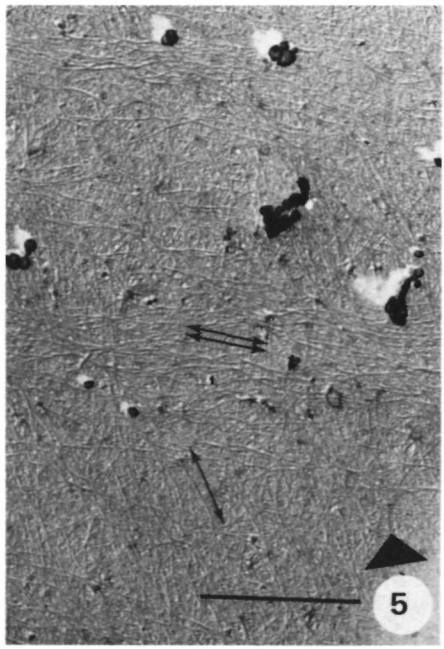
Fig. 5,6.

Shadow-cast preparations of young growing root hairs of E. hyemale (5) and E. variegatum (6), showing the inner wall layer, the layer against the plasma membrane, in surface view: the lamella under deposition (double arrow) and the previous lamella (single arrow) in a different orientation can be observed. Fibrils within a completed lamella are not contiguous. Pointer designates long axis of root hair. Scale bars: 1000 nm.

Fig. 7a,b

Fig. 7a is a schematic representation of the cell wall texture in an Equisetum root hair. Randomly oriented microfibrils, deposited at the hair tip, form the outside of the whole hair. The helicoidal texture consists of superimposed lamellae, each of which is built up of parallelly oriented microfibrils. In subsequent lamellae there is a progressive rotation of the microfibril orientation. In full-grown hairs of the subgenus Hippochaete the innermost layer consists of microfibrils oriented in a Z-helix.

Fig.7b shows how the helicoidal wall is seen in section and in surface view.



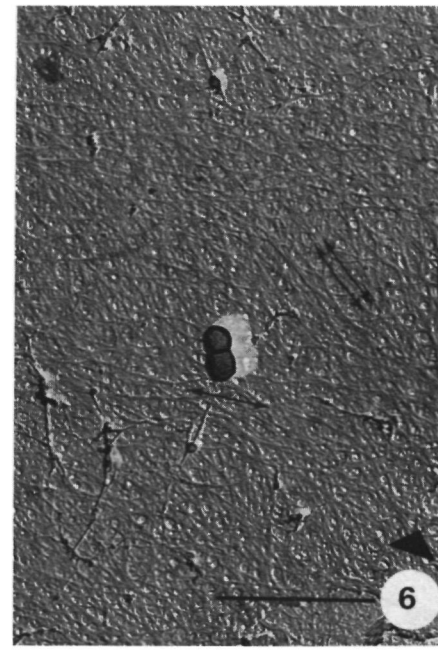



Fig. 1 shows a fractured hair with flared end of *E. hyemale* between crossed polarizers: the angle between the positions of the hair in 1b, the position in which the whole hair is brightest, and 1c, position in which the single wall is brightest, corresponds to the mean angle microfibrils make with the long axis of the hair.

Single wall preparations of the subgenus *Equisetum* showed no birefringence, ruling out the possibility of wall texture with a 45° helix, which would also be nearly isotropic in whole mounts.

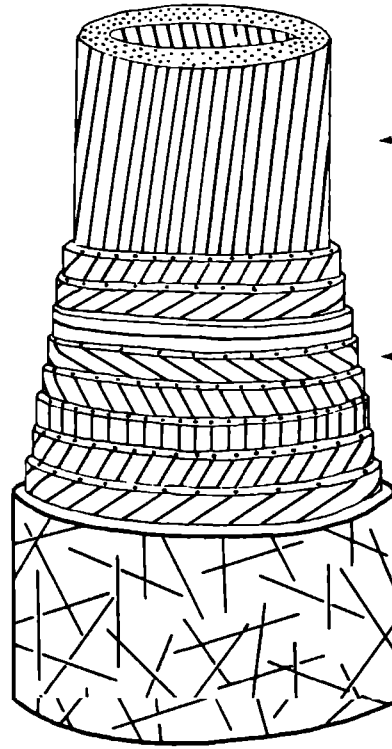
*Electron microscopy*

Data on wall texture, obtained with the polarizing microscope, were verified with the electron microscope. It is not necessary to confirm helical wall texture with the electron microscope in positive birefringent hairs, since, in single wall preparations, the mean microfibril angle can be measured.

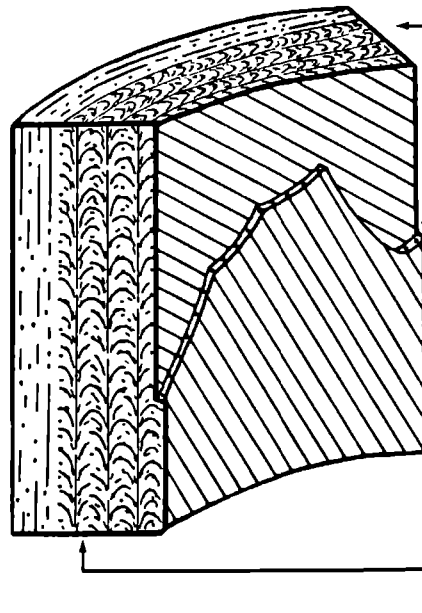
Figs. 2a, 3a and 4a show the wall texture in thin longitudinal sections of young growing hairs of *E. hyemale*, *E. fluviatile* and *E. palustre* respectively. Fig. 2a shows one and a half arcs of the helicoidal wall in *Equisetum hyemale*, Fig. 3a shows two and a half arcs in *E. fluviatile* and in young *E. palustre* (Fig. 4a) the random wall and an initial alignment of the helicoidal layer are observed.

A schematic representation of the various wall orientations is given in Figs. 7a and 7b. The ambiguity in the terminology of right and left-handed helices in the botanical bibliography on chiral problems is avoided by calling the helix shown in Fig. 7a a Z-helix and a helix of opposite sign an S-helix (Frey-Wyssling 1976).

The helicoidal wall seen from the cytoplasmic side of the hair shows successive fibril lamellae oriented in progressively further rotated planes. Fig. 5 shows this for a young *E. hyemale* root hair. Fig. 6 shows the helicoidal pattern in surface view from the cytoplasmic side in a root hair of *E. variegatum*: the lamella under deposition can be seen in a transverse orientation to the long axis of the hair, overlying a lamella with oblique orientation. From Figs. 5 and 6


←helical
z-helix
←helicoidal
←random

7a


←transverse
section
←surface view
longitudinal
section

7b

it is clear that microfibrils within a lamella are not contiguous; there is much space between adjacent microfibrils.

Fig. 2b shows this helical layer in a thin longitudinal section. Though lamellae with different microfibril orientations do alternate, in all of the lamellae the microfibrils form a Z-helix of more than 45° pitch angle around the hair. Neither lamellae with transversely oriented microfibrils nor S-helices were found at this stage of microfibril deposition. It is the helically arranged layer which causes the positive birefringence in the polarizing microscope. The layer becomes very thick in these persistent hairs, making the lumen of the cell very narrow. Full-grown hairs of *E. palustre* and *E. fluviatile*, subgenus *Equisetum*, were analyzed with the electron microscope up to a known age of 5 weeks and never exhibited this helical wall texture (Figs. 3b and 4b). Also the persistent hairs of *E. fluviatile* and *E. palustre* grown in situ and much older than 5 weeks were isotropic in polarized light, whereas the *E. hyemale* root hairs showed positive birefringence at the base of the hair when they were 3 to 9 days old. In hairs at an age of 5 weeks 6 arcs of the helicoidal wall were formed in *E. fluviatile* and not more than 2 arcs in *E. palustre*.

## DISCUSSION

A helicoidal wall texture consists of successive lamellae with parallel fibrils in each of them. In each subsequent lamella the fibril orientation is rotated, in a regular manner, with respect to the orientation in the previous lamella. In thin slightly oblique sections, this wall texture appears as arcs. One arc corresponds to a 180° rotation of the helicoid. For discussion of this wall texture compare SASSEN et al. 1981, PLUYMAEKERS 1982, EMONS 1982, EMONS and WOLTERS-ARTS 1983, NEVILLE 1984, BONFANTE FASOLO and VIAN 1984, REIS et al. 1985.

Boiling of hairs in $H_2O_2$/HAc does not alter microfibril orientation in plant cells: Equisetum hyemale root hairs prepared by freeze-fracturing show a similar helicoidal cell wall texture (EMONS 1985).

Table 1
Birefringence of root hairs of Equisetum species, grown in different media

| subgenus | species | medium | birefringence young hairs | biregringence full-grown hairs |
|---|---|---|---|---|
| Equisetum | E. arvense | soil | none | none |
| | E. fluviatile | water | none | none |
| | E. palustre | marsh | none | none |
| | | water | none | none |
| | E. pratense | soil | none | none |
| | E. sylvaticum | soil | none | none |
| | E. telmateia | soil | none | none |
| Hippochaete | E. hyemale | soil | none | positive |
| | | water | none | positive |
| | | agar, 0.5% | none | positive |
| | | agar, 1% | none | positive |
| | | agar, 1.5% | none | positive |
| | | agar, 2% | none | positive |
| | E. ramosissimum | soil | none | positive |
| | | water | none | positive |
| | E. scirpoides | soil | none | positive |
| | | water | none | positive |
| | E. variegatum | soil | none | positive |
| | | water | none | positive |

Table 2.
Mean microfibril angle with the long axis of full-grown hairs measured on single wall preparations in the polarizing microscope

| species | microfibril-angle | number of hairs |
|---|---|---|
| E. hyemale | 15°-41° | 116 |
| E. ramosissimum | 9°-16° | 14 |
| E. scirpoides | 21°-42° | 11 |
| E. variegatum | 25°-42° | 20 |

*Cell wall texture and environment*

The helicoidal wall texture has been found in root hairs of aquatic plants, while hairs of terrestrial plants have an axial or helical microfibril pattern (SASSEN et al. 1981, SASSEN et al. 1985). The *E. hyemale* root hair was found as an exception, being a terrestrial plant and having a helicoidal wall texture in the root hairs, though only when young and growing (SASSEN et al. 1981, EMONS and WOLTERS-ARTS 1983). This helicoidal wall texture is now shown to be present not only in young hairs grown in water, but also in hairs grown in soil and hairs grown in different agar concentrations. This trait, therefore, is not influenced by the consistency of the substrate, but is species specific.

*E. fluviatile*, an aquatic *Equisetum*, and *E. palustre*, a marshland *Equisetum* lack a helical fibril pattern when full-grown. *E.sylvaticum*, *E. pratense*, *E. telmateia* and *E. arvense*, normally growing in soil, however, also do not acquire this additional wall texture, indicating that a terrestrial plant may have only a helicoidal wall texture in the root hair cell wall and that the presence of a helical (or axial) layer is not a prerequisite for growth in soil as the natural medium.

*Cell wall texture as a systematic trait*

All of the species without the additional helical cell wall layer in full-grown root hairs belong to the subgenus *Equisetum*, and none of the species with this layer is included in this subgenus. It is clear that none of the studied species of the subgenus *Equisetum* shows positive birefringence, while the species in the subgenus *Hippochaete* all have positive birefringent thick walled root hairs when full-grown. The angle of this helix with the long axis of the hair (90°-helix pitch angle), measured on single wall preparations, varied from 9° to 16° for *E. ramosissimum* and from 25° to 42° for *E. variegatum*. The significance of this difference is not clear. How microfibril helices in root

hairs are oriented is not known. In *Equisetum hyemale* the cortical microtubules, which are hypothesized to orientate the nascent microfibrils (HEATH and SEAGULL 1982) are not parallel to the last-deposited microfibrils (EMONS 1982). Also in *E. fluviatile*, microtubules remain axially aligned, while microfibrils are deposited in sussessively differing orientations (EMONS, unpublished). Neville and Levy (1984) have suggested self-assembly for helicoidal cell walls.

In conclusion, the additional helical inner cell wall layer in full-grown hairs is not a special trait of the terrestrial species but of the subgenus *Hippochaete*. This corroborates the division of the genus *Equisetum* into two subgenera.

## ACKNOWLEDGEMENT

I wish to thank Niek van Maaren for making the thin sections of E. fluviatile and E. palustre, H.P.G. Helsper for the determination of the different species, Dr. P. Baas (Rijksherbarium, University of Leiden) for critically reading the manuscript, and am greatly indebted to Prof. Dr. M.M.A. Sassen.

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# CHAPTER 3

## Microtubules do not Control Microfibril Orientation in a Helicoidal Cell Wall

Protoplasma 113, 85—87 (1982)

PROTOPLASMA


# Microtubules Do Not Control Microfibril Orientation in a Helicoidal Cell Wall

*Brief Report*

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Received March 22 1982
Accepted April 8 1982

## Summary

The secondary cell wall layer of the young root hair of *Equisetum hyemale* (L) has a helicoidal texture The cortical microtubules in these hairs maintain an axial alignment while microfibrils are being deposited with a different orientation in each subsequent layer The role of cortical microtubules in microfibril orientation is disputed

*Keywords* Microtubules Microfibril orientation, Helicoidal cell wall Root hair *Equisetum hyemale*

## 1. Introduction

In many plant cell walls the cellulose microfibrils are laid down in a highly organized pattern Studies on microfibril alignment center around the hypothesis that microfibril orientation is determined by the direction of microtubules subjacent to the cell wall (NEWCOMB 1980) Ever since its postulation (LEDBETTER and PORTER 1963) this hypothesis has found much experimental support, especially from cell walls with parallelly oriented microfibrils (ROBINSON 1977), and has been generally accepted Little is known, however, about microtubule behaviour during deposition of a helicoidal cell wall A helicoidal wall is composed of successive parallel fibred lamellae in each of which fibril orientation is rotated with respect to that of the previous lamella This texture is transient in a growing wall, or consolidated in a typical secondary wall (ROLAND 1981) The present observations on the secondary wall behind the extending tip of root hairs of *Equisetum hyemale* (L), common scouring rush, contravenes the hypothesis that cortical microtubules direct the microfibrils of the cell wall as a general rule

## 2. Material and Methods

Stem cuttings of *Equisetum hyemale*, containing several nodes, were grown on an aqueous soil extract under greenhouse conditions To visualize the microfibrils in the cell wall untreated root hairs, still attached to the root, placed between copper plates, were frozen with liquid propane in a Cryo-jet device of Balzers Samples were fractured at − 100 C in a Balzers double replica device, etched for 2 minutes at − 100 C, at 10 $^{-5}$ torr and replicated with Platinum/Carbon in a Balzers freeze etch unit Replicas were cleaned with 70% sulfuric acid In addition whole roots with hairs were treated, for extraction of cell wall matrix with $H_2O_2$ HOAc (hydrogen peroxide/glacial acetic acid) (1 1, v/v) for 1 hour at 100 °C, dehydrated and flat embedded in Spurrs medium Sections of root hairs were stained with 3% $KMnO_4$ for 30 minutes

To visualize the microtubules whole roots with hairs were primarily fixed at room temperature for 2 hours in the culture medium to which glutaraldehyde buffered with 0 2 M cacodylate pH 7 2 (final concentration 2% ) was added postfixed for 2 hours with 2% osmium tetroxide dehydrated, and flat embedded in Spurrs medium Sections of root hairs were stained with saturated aqueous uranylacetate for 20 minutes and with leadcitrate for 7 minutes Sections were obtained with a Sorvall Porter Blum ultramicrotome MT 2 or MT 5000 Specimens were examined in a Philips EM 201 or EM 300 electronmicroscope at 60 kV

## 3. Results and Discussion

In polarized light the young root hair of *Equisetum hyemale* is isotropic in plane view The cell wall consists of an outer layer with a dispersed texture to which an

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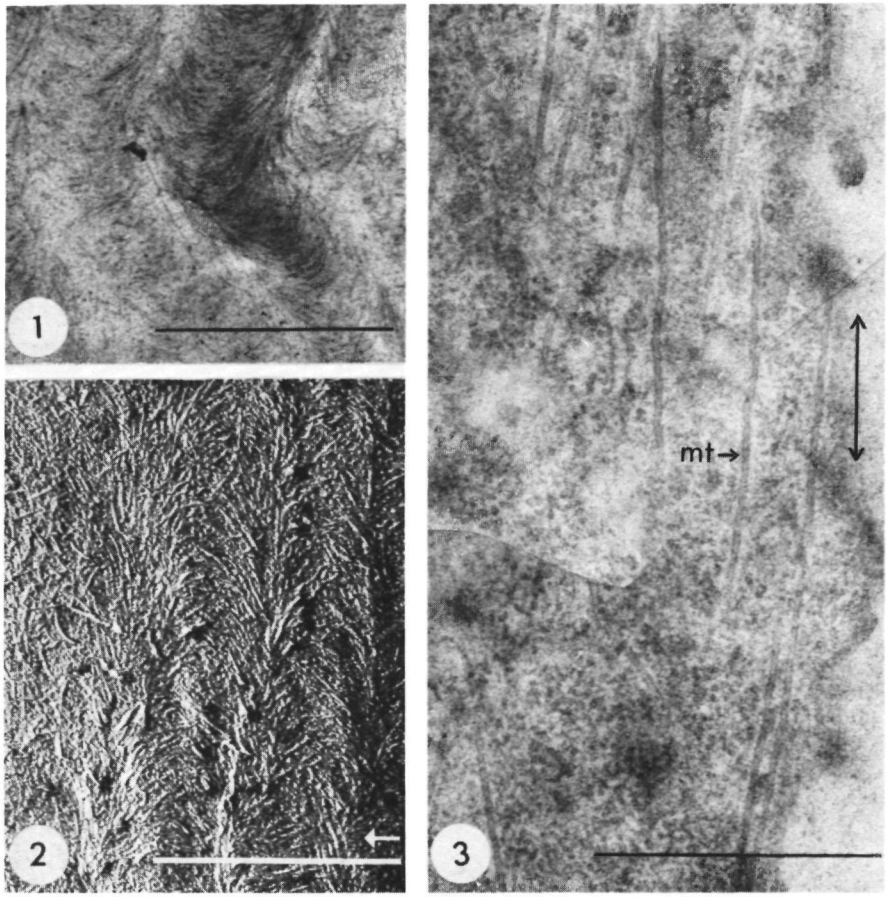


Fig. 1–3. Transmission Electron micrographs of young root hairs of *Equisetum hyemale*
Fig. 1. Longitudinal thin section of the secondary cell wall showing the characteristic parabolic pattern of the helicoidal wall. Marker bar 1 μm
Fig. 2. Freeze fracture image of the cell wall. Arrow indicates direction of shadowing. Marker bar 1 μm
Fig. 3. Longitudinal thin section through the cortical cytoplasm of the root hair showing microtubules more or less axially aligned. Arrow indicates long axis of root hair. *mt* = microtubule. Marker bar 1 μm

inner non-extending layer has been deposited (SASSEN *et al.* 1981). In sections of hairs treated with $H_2O_2$/HOAc and stained with $KMnO_4$ this inner layer shows parabolic patterns (Fig. 1); a similar pattern is shown after freeze-fracturing of untreated hairs (Fig. 2). All observations clearly demonstrate that the inner secondary cell wall layer of the young root hair of *Equisetum hyemale* has a helicoidal texture. A similar cell wall texture has been observed in root hairs of *Limnobium stoloniferum* (PLUYMAEKERS 1982, in press). Fixation of root hairs with glutaraldehyde/osmium-tetroxide and staining with uranylacetate/leadcitrate did not reveal the cell wall texture conclusively, although electron-dense lines were observed in these preparations. Observations obtained with this method have been incorrectly interpreted as indication for axially aligned cellulose microfibrils in the cell wall of *Equisetum hyemale* root hairs (HARRIS 1979).

Microtubules were visualized by this last method. Cross sections of young root hairs show transverse profiles of microtubules only. In tangential sections through the cortical cytoplasm the microtubules are oriented more or less parallel to the long axis of the hair (Fig. 3). The microtubules remain axially aligned

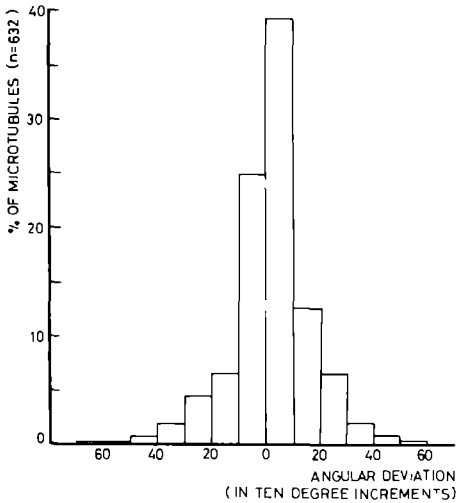


Fig 4 Angular deviation of microtubules from the long axis of young root hairs of *Equisetum hyemale* 0 – long axis of the hair 632 microtubules from 57 sections from 16 root hairs

during wall deposition, although they subtend microfibrils with a different orientation in each subsequent layer In this study 632 microtubules from 57 sections from 16 root hairs were examined, they show a pattern of angular deviation from the long axis of the hair (Fig 4) which is similar to the microtubule deviation pattern in root hairs of radish (*Raphanus sativus*) (SEAGULL 1980) In radish, however, the secondary wall microfibrils are axially aligned, thus reflecting the orientation of the subjacent microtubules

Microtubules in young root hairs of *Equisetum hyemale*, not participating in the orienting of microfibrils, might function in maintaining polarity of the cytoplasm during apical growth A similar role has been postulated for microtubules in caulonema tip cells of the moss *Funaria hygrometrica*, which also elongate exclusively by tip growth (SCHMIEDEL and SCHNEPF 1980)

As microfibrils are not directed by microtubules, there must be another cause for their ordered deposition A self-assembly process for the morphogenesis of helicoidal structures in biological systems has been suggested (NEVILLE 1976), analogous to the spontaneous helicoidal assembly of cholesteric liquid crystals The self-assembly process could take place on the fluid mosaic structure of the plasma membrane where the cellulose microfibrils probably are synthesized (NEWCOMB 1980) Alternatively, deposition of microfibrils might be directed by a plasma membrane associated apparatus (MONTEZINOS and BROWN 1978). In either case, from the results of this study it seems unlikely that the force directing the cellulose synthetase complex is generated by cortical microtubules as postulated in HEATH's theory (1974).

I gratefully acknowledge the support of Professor Dr M M A SASSEN and the technical assistance of M WOLTERS-ARTS

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# CHAPTER 4

## Cortical Microtubules and Microfibril Deposition in the Cell Wall of Root Hairs of Equisetum Hyemale

Protoplasma 117 68–81 (1983)

PROTOPLASMA


# Cortical Microtubules and Microfibril Deposition in the Cell Wall of Root Hairs of *Equisetum hyemale*

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Received October 20 1982
Accepted in revised form November 13 1982

## Summary

The cell wall of root hairs of *Equisetum hyemale* is shown to be composed of three different cell wall textures The growing cell wall at the tip of the hair is composed of a dispersed texture of microfibrils which continues along the outside of the whole hair With increasing distance from the tip an increasing number of helicoidally arranged lamellae is deposited These findings correspond with the observed isotropism of young hairs in polarized light

Hairs of approximately 4 days old become positive birefringent indicating that longitudinally oriented layers prevail over layers with a transverse direction This phenomenon starts at the base of the hair Full grown hairs are positive birefringent up to the tip and concordantly show a thick additional inner cell wall layer which forms a helical pattern the length of the hair with a mean microfibril angle of 25 with the cell axis

Cortical microtubules subjacent to the dispersed the helicoidal and the helical wall texture are axially aligned and thus not in co alignment with the last deposited microfibrils

Coated and smooth vesicles are present in the cortical cytoplasm of both growing and full grown hairs Electron dense profiles (20 nm in diameter) surrounded by a halo (of 50 nm) were observed on the wall plasmalemma interface in full grown hairs only A relation of these structures with microfibril deposition could not be demonstrated They might represent channels transporting material to the wall which in full grown hairs is heavily impregnated with a tawny brown substance

The general hypothesis that cortical microtubule orientation directs microfibril deposition is disputed

*Key words* Cell wall texture Cortical microtubules Microfibril deposition *Equisetum hyemale* Root hair

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## 1. Introduction

Crystalline cellulosic microfibrils provide the architectural framework of the cell wall of most green plants and, together with the non-cellulosic matrix materials and cytoskeletal components, they determine cell shape (review HEPLER and PALEVITZ 1974) Much attention is currently devoted to the nature of the mechanisms that are supposed to orient these microfibrils during their deposition on the inner surface of the plant cell wall The site of microfibril formation is conceived to be on the surface or outside of the plasma membrane (*e g*, HERTH 1980) and polymerization and crystallization to be consecutive processes (HAIGLER *et al* 1980 and HERTH 1980) Microtubules have been demonstrated in the cortical cytoplasm of many types of plant cells during primary and secondary wall deposition In most instances they have been observed in parallel alignment with the cellulose microfibrils of the underlying wall and hence, have been generally hypothesized to play an orienting role in microfibril deposition (HEATH 1974, reviews by HEPLER and PALEVITZ 1974, HEPLER 1981, GUNNING and HARDHAM 1982) Microtubule disrupting agents, for instance colchicine lead to an aberrant microfibrillar pattern (review ROBINSON 1977) Although this corroborates the above hypothesis, it is no final proof for a cause and effect relationship Moreover exceptions to the parallelism between microtubules and microfibrils have been reported (review HEPLER and PALEVITZ 1974)

The root hair of *Equisetum hyemale* (L), common scouring rush, being easily accessible for fixation and *in vivo* observation, has been chosen for investigating microfibril deposition and the relationship between microtubule and microfibril orientation Until recently the cell wall of root hairs was conceived to be composed of a typical primary wall with randomly oriented microfibrils and in the lateral walls an additional inner layer of parallel, mainly axially oriented microfibrils (review SIEVERS and SCHNEPF 1981) However, the aquatic plants *Ceratopteris thalictroides* (SASSEN *et al* 1981) and *Limnobium stoloniferum* (PEETERSMAEKERS 1982) have an inner cell wall in their root hairs with a helicoidal texture, consisting of successive parallel-fibred lamellae in each of which fibril orientation is rotated with respect to the previous lamella, whereas in the inner cell wall layer of *Urtica dioica* microfibrils align according to a helix (SASSEN *et al* 1981) In a previous paper (EMONS 1982) it was reported that the inner layer of young root hairs of *Equisetum hyemale* has a helicoidal texture, while cortical microtubules in the subtending cytoplasm are axially aligned Hence, it was concluded that microtubules cannot exert directional control of microfibril alignment in the case of this helicoidal texture

The present paper deals with microfibril deposition at the growing tip of root hairs of *Equisetum hyemale* and with microfibril deposition and microtubule orientation in full-grown hairs The control of the organization of microfibrils in the cell wall is discussed

## 2. Material and Methods

### 2 1 Plant Material

Stem cuttings of *Equisetum hyemale* (L ) common scouring rush containing several nodes were grown on an aqueous soil extract under greenhouse conditions After six days roots emerged at the nodes and produced extensive root hair populations To decide upon root hair age root length was measured four times a week

### 2 2 Light Microscopy

To track root hair growth lengths of root hairs along the root were measured under a dissecting microscope Birefringence of root hairs of different age was determined with the polarizing microscope (Leitz HM Pol) Both fresh root hairs and hairs treated with hydrogen peroxide glacial acetic acid ($H_2O_2$ HOAc 1 1 v v) were used For fibril angle measurement single wall preparations were stained with the dichroitic cellulose staining chlorozinc iodine to enhance birefringence (*cf* LENEY 1981)

### 2 3 Electron Microscopy

In order to visualize microfibrils roots with hairs were frozen between glass slides in liquid nitrogen and after removing all root parts the frozen hairs were scraped from the slides with a razor blade This procedure causes the root hairs to break thus revealing the inner layer of the cell wall

For extraction of cell wall matrix the root hair fragments were treated with H O HOAc (1 1 v v) for 1 hour at 100 C Cleaned wall preparations placed on formvar coated grids were shadowed with platinum

In addition whole roots with hairs were treated with $H_2O_2$ HOAc for 1 hour at 100 C dehydrated in a graded series of aqueous ethanol and flat embedded in Spurr s medium For determination of distances of the sections from the root hair tip hairs were marked with *Petunia* pollen grains (for procedure of flat embedding and marking *cf* PEETERSMAEKERS 1982) sections were stained with a 3% aqueous solution of potassium permanganate ($KMnO_4$)

To visualize microtubules whole roots with hairs were preliminary fixed with 2 glutaraldehyde buffered with 0 2 M cacodylate (pH 7 2) in the culture medium at room temperature for 2 hours Some samples were fixed with the addition of 0 2% tannic acid After a 2 hours postfixation in 2% osmium tetroxide all samples were washed in buffer dehydrated in ethanol and flat embedded in Spurr s medium Single hairs still attached to the root were selected under a light microscope on the basis of unaltered morphology relative to fresh hairs and marked with *Petunia* pollen grains Sections were stained with saturated aqueous uranyl acetate for 20 minutes followed by Reynold s lead citrate for 7 minutes

Sections were obtained with a Sorvall Porter Blum ultramicrotome MT 2 or MT 5000 and specimens examined in a Philips EM 201 or EM 300 electron microscope at 60 kV

## 3. Results

### 3 1 Root Hair Growth and Birefringence

The growth rate of root hairs of *Equisetum hyemale* is approximately 40 µm per hour They are not purely cylindrically shaped girth decreases from base to tip (Figs 1 *a*, *b*, *c*) Less than approx 4 days old root hairs proved to be isotropic in plane view in the polarizing microscope (Figs 1 *a* and 2) there is no predominant orientation of the microfibrils in the cell wall In contrast, hairs more than approx 10 days old are positive birefringent (Figs 1 *c* and 2) microfibrils align mainly lengthwise Positive birefringence starts at the base of the root hair between day 3 and day 9 and reaches the tip of the first root hairs at day 6 which coincides with the inflection point on the growth curve (Fig 2) From day 14 on, when almost all hairs are positive birefringent right up to the tip, their growth has stopped They are tawny brown, whereas young hairs are colourless Birefringence is not due to the colouring substance since treatment with $H_2O_2$ HOAc did dissolve the colour while birefringence remained At a root length of approx 70 mm, when nearly all hairs at the base of the root are positive birefringent up to the tip, side roots appear Hairs of up to 8 weeks were examined and were concluded to be alive from the

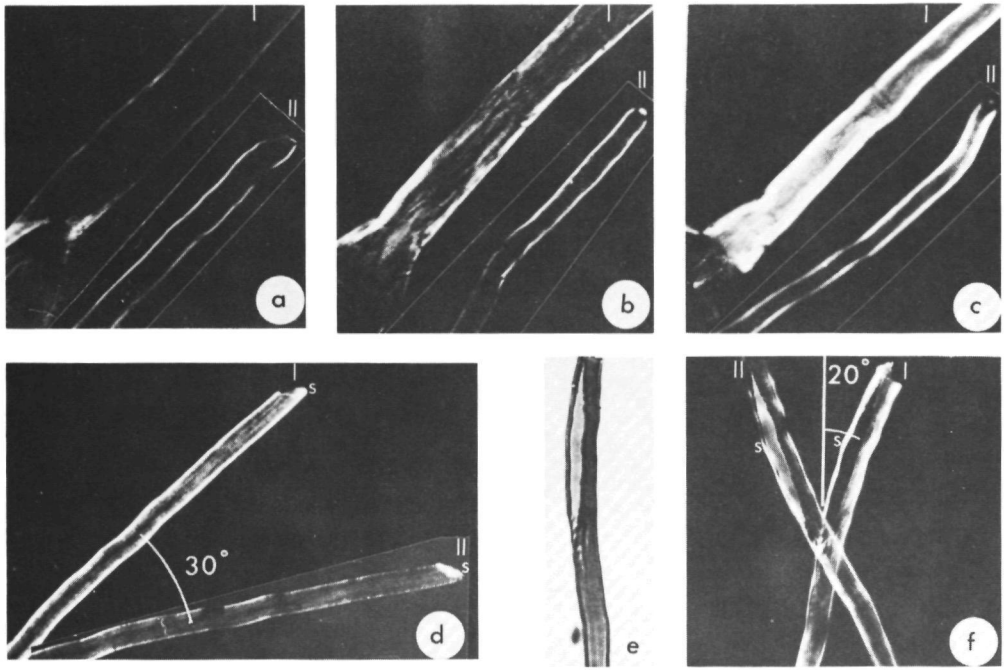



Fig. 1. Birefringence of root hairs of *Equisetum hyemale*. × 800. *a c*: Increase of birefringence with age. All preparations were positioned 45° to the plane of polarized light and photographed under identical conditions. *I*: base of hair, *II*: tip of the same hair. Root hair length: *a*: 2.5mm, *b*: 5.6mm, *c*: 6.2mm of resp. 3, 6, and 11 days old hairs. *d*: Fractured hair with flared end: *I*: positioned 45° to the plane of polarized light: In the complete hair the mean microfibril orientation is lengthwise, effecting the most bright appearance in the black field of the crossed polarizers at an angle of 45°, *II*: Positioned such that the single wall layer (flared end) (*s*) appears most brightly: the angle between *I* and *II* is the same as the angle between the extinction positions of the complete hair (the horizontal) and that of the single wall, which is the mean angle microfibrils in the single wall make with the long axis of the root hair, in this case 30°. *e*: Split hair in normal light. *f*: Same hair as e between crossed polarizers. *I*: single wall layer(s) in extinction position: microfibrils in this layer are oriented parallel to the horizontal. The angle between I and the horizontal (extinction position of complete hair) is the same as the mean microfibril angle with the long axis, in this case 20°. *II*: Rotary stage of the microscope turned such that single wall layer appears brightly

occurrence of cytoplasmic streaming. In the field they remain intact for a much longer period. At the beginning of winter the roots are fully covered with brown thick-walled hairs.

Under the polarizing microscope the extinction position of single wall preparations obtained by fracturing of full-grown hairs and stained with chlorozinc-iodine, makes an angle with the extinction position of complete hairs (Figs. 1 *d*, *f*). This angle is an indication of the general orientation of microfibrils in the single cell wall, being the pitch angle between the cell axis and the tangent to the helical curve. All hairs examined showed a so-called z-helix. The mean pitch angle measured in 52 root hairs, proved to be 26.3 with a standard deviation of 8.3 (Fig. 8 *a*). Without analyser it coulf be detected whether the flared end of the fractured hairs belonged to the front or to the back wall of the hair.

### *3.2. Cell Wall Texture of Young Hairs*

In transverse sections of young growing root hairs the characteristic parabolae of the helicoidal wall are revealed after treatment with $H_2O_2$/HOAc and staining with $KMnO_4$ (Fig. 3 *g*). The cell wall of the growing root hair tip, however, is a primary wall and shows randomly oriented microfibrils. Only this randomly textured primary wall is present in the first 300 μm of the tip of young hairs (Figs. 3 *a*, *b*). Figs. 3 *c*–*f* show that, with increasing distance from the tip, an increasing

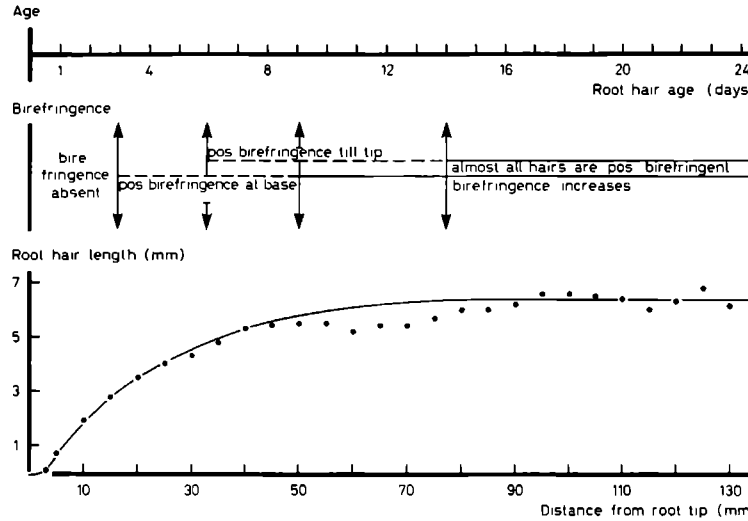



Fig 2 Growth and birefringence of root hairs of *Equisetum hyemale* Mean data from 53 roots Note that the inflection point on the growth curve coincides with birefringence reaching the tip of the first root hairs

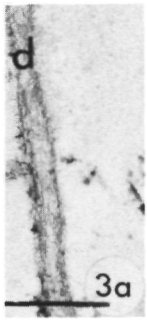
number of helicoidally arranged lamellae becomes visible A shadow-cast preparation of a fractured hair (Fig 3 *h*) shows the outer layer with dispersed texture of the front wall, the inner cell wall layer with helicoidal texture of the back wall (3 superimposed lamellae of the helicoidal wall are visible) and the outer layer with dispersed texture of the back wall The angle between subsequent helicoidal lamellae was measured in 136 shadowcast preparations and proved to be approximately 40 (Fig 5) The rotation of the helicoid is in a clockwise direction Fig 6 represents a schematic drawing of the young growing wall of *Equisetum hyemale* root hairs

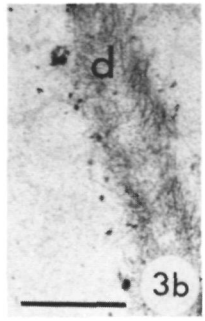
### 3 3 *Cell Wall Texture of Full-Grown Hairs*

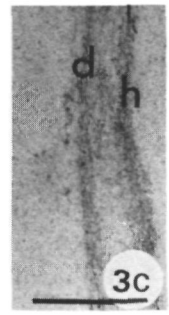
Longitudinal thin sections of full-grown, 40 days old, 7 mm long, root hairs show a thick layer of approximately axially oriented microfibrils on the inside of the helicoidal wall (Figs 4 *a*, *b*) The transition from helicoidally to more or less axially ordered layers, being indistinct in all preparations is probably gradual The fibril angle of this layer, found by means of the polarizing microscope could be verified in shadow-cast preparations The angle of mean microfibril direction with the long axis of the root hair, measured in 116 shadow-cast preparations, proved to be 23 1 with a standard deviation of 12 2 (Fig 8 *b*) Again microfibrils were found to be positioned according to a *Z*-helix (Fig 4 *c*), with considerable displacement, however, of individual microfibrils from the mean helical orientation Fibril layers with two alternating directions with an acute angle to each other seem to be present (Figs 4 *a*, *c*) However, as opposed to the helicoidal wall, no transversely oriented layers occur In the apical dome of full-grown hairs a helicoidal layer is present under the primary wall (Fig 7)

### 3 4 *Cortical Microtubule Direction*

The addition of 0 2% tannic acid to the fixing solution did not yield more or better preserved microtubules At the very tip of the growing root hair, where the primary wall is deposited, no microtubules were observed Transverse sections through the subapical and basal part of young root hairs (isotropic in polarized light[1]) show transverse sections of microtubules only The electronlucent sheath touches the plasma membrane, but no cross-bridges of microtubules to the plasma membrane were observed Tangential sections of young hairs through the cortical cytoplasm show a constant pattern of more or less axially oriented microtubules without changes at the onset of helicoidal texture at approximately 300 μm from the hair tip (Fig 9 *c*)

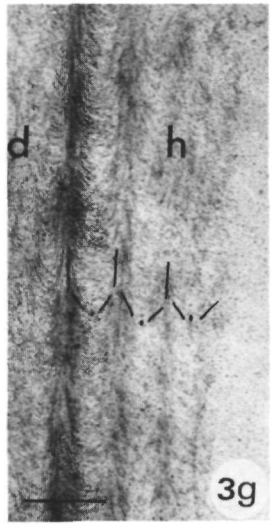


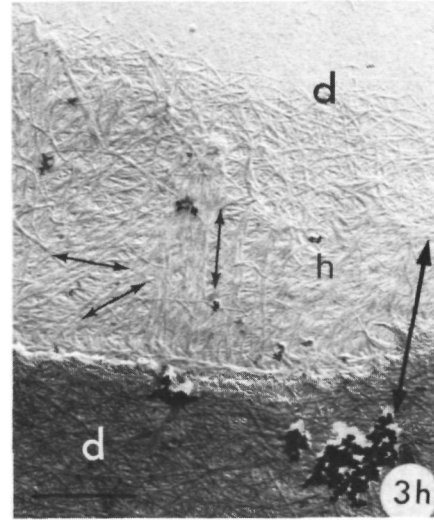



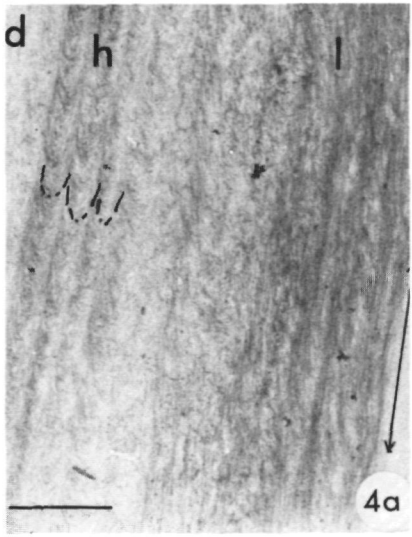



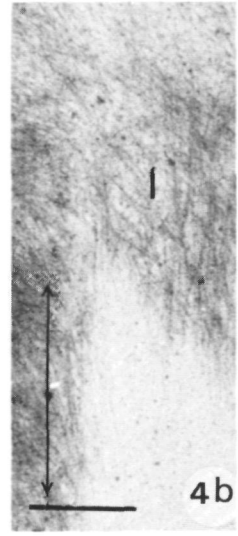


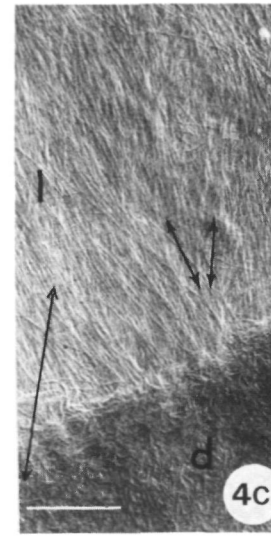





Figs. 3-4

In full grown hairs angles between the individual microtubules and the long axis of the cell were measured by observing sections through the cortical cytoplasm of the back wall only since averaging the measurements of the front wall and the back wall would confuse the results especially when microtubules would align with the helically arranged microfibrils. The angular deviation pattern of microtubules from the long axis is also similar to the pattern in young hairs with a helicoidal wall (Figs. 9*a*, *b* and 10). Hence microtubules are in no instance in mutual alignment with the adjacent microfibrils.

### 3.5. *Vesicles and Plasma Membrane Structures*

Coated vesicles with a diameter of approximately 90 nm, including coat, are present in the cortical cytoplasm and were observed in young as well as in full-grown root hairs (Figs. 9*e*, *f*). Also numerous smooth vesicles are present in the cortical cytoplasm, especially in full-grown hairs (*cf.* Fig. 9*b*). They contain a lightly staining material.

A remarkable feature on the wall-plasma interface was found in tangential sections of full-grown hairs only: randomly distributed electron dense profiles surrounded by a halo, diameters approximately 20 nm without and approximately 50 nm including the halo (Fig. 9*d*).

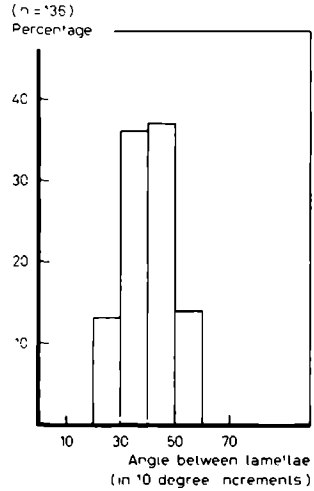



Fig. 5. Angle between helicoidal lamellae of young root hairs of *Equisetum hyemale*.

## 4. Discussion

### 4.1. *Cell Wall Texture*

Root hairs grow at their tips only (Sievers and Schnepf 1981). The apical cell wall is a primary wall with randomly oriented microfibrils. According to Sievers and Schnepf (1981) in the subapical and basal part of root hairs two layers of microfibrils are present in the cell wall "an outer layer similar to the apical cell wall and an inner layer of parallel mainly axially oriented microfibrils". The outer layer of randomly oriented microfibrils is present in the *Equisetum hyemale* root hair. The thickness of this layer increases in the extension zone, but is constant along the side-walls of the hair. The inner layer, however, has a helicoidal texture as in root hairs of *Limnobium stoloniferum* (Pleijmakers 1982) and *Ceratopteris thalictroides* (Sassen *et al.* 1981) both aquatic plants. The

Fig. 3. Cell wall texture of young growing root hairs of *Equisetum hyemale*. Bars: 0.5 μm. *a–f*: Transverse sections at increasing distances from the tip (respectively: 10, 40, 400, 800, 1 600, 1 900 μm) of one 3 360 μm long hair; isotropic in the polarizing microscope. *a*, *b*: Only the dispersed layer (*d*) of the primary wall is present, which increases in thickness with increasing distance from the tip. × 27 300. *c*: At about this distance from the tip (300–400 μm) the first lamella of the helicoidal layer appears. × 27 300. *d*: Half a parabola of the helicoidal wall is shown (*h*). × 27,300. *e*: The first parabola of the helicoidal wall is completed (*h*). × 27 300. *f*: Onset of the second parabola of the helicoidal wall. × 27 300. *g*: Transverse section through base of an isotropic 4 mm long root hair; 3 parabolae of the helicoidal wall are present (*h*). × 22 100. *h*: Shadow cast preparation of a fractured hair showing 3 superimposed lamellae of the inner helicoidal wall (small double arrows) and the dispersed texture (black *d*) of the front as well as of the back (white *d*) wall. Double arrow indicates long axis of root hair. × 24 800.

Fig. 4. Cell wall texture of full grown root hairs of *Equisetum hyemale*. Bars: 0.5 μm. Large double arrows represent long axis of the hair. *a*: Longitudinal section through base of a 7 mm long hair, 40 days old, showing respectively: the dispersed outer layer (*d*), three parabolae of the helicoidal layer (*h*), a less distinct part and the longitudinally inclined helical layer (*l*) near the lumen of the cell. Note acute angled alternating fibril orientations in the helical layer (small double-arrows). *b*: Tangential section through newly deposited helically oriented microfibril layer (*l*) in the proximal part of a 7 mm long, 40 days old root hair. × 27 400. *c*: Shadow cast preparation of a 40 days old fractured hair showing the dispersed outer layer (*d*) of the front wall and the newly deposited helically oriented (*l*) inner microfibril layer of the back wall with a mean microfibril angle with the long axis of the hair of 27°. Acute angled alternating microfibril lamellae can be observed in this layer (small double-arrows).

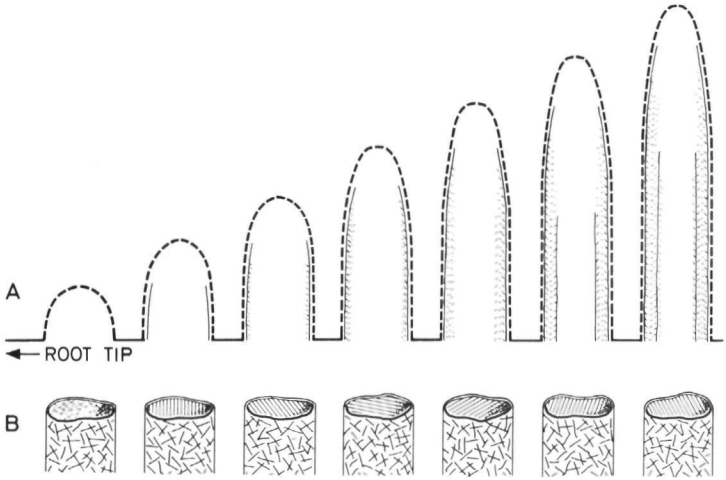



Fig. 6. Schematic drawing of the cell wall texture of young growing root hairs of *Equisetum hyemale*. (*A*) Longitudinal sections of consecutive root hairs showing, within the randomly textured extending primary wall, an increasing number of helicoidally arranged microfibril layers, representing the secondary wall behind the growing tip. (*B*) The same hairs, fractured at their bases, showing the newly deposited microfibril layers

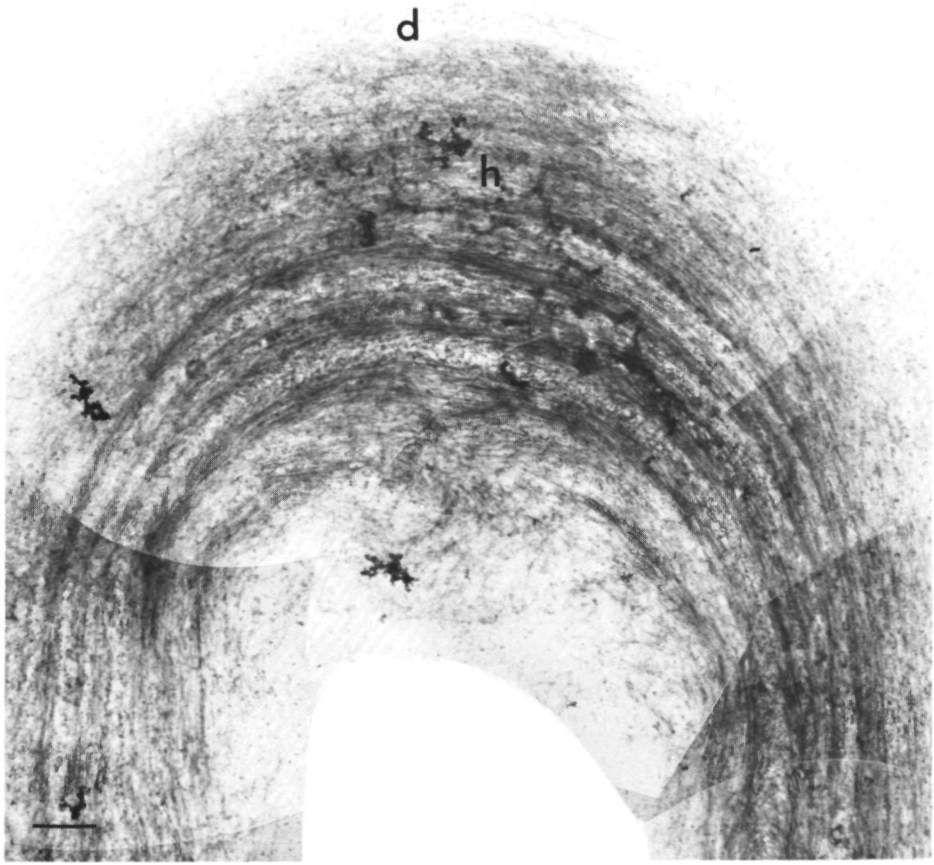



Fig. 7. Longitudinal thin section through the dome of a full-grown, 7mm long, 40 days old, root hair of *Equisetum hyemale*, showing the dispersed outer wall (*d*) and the thick helicoidal inner wall (*h*), made up of successive parallel-fibred lamellae in which the microfibril orientation progressively rotates. Bar: 0.5 μm. × 16,800

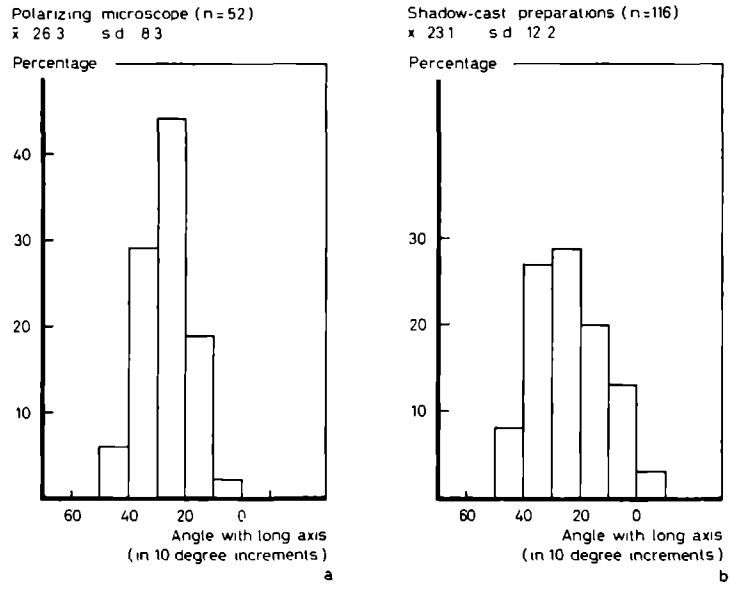



Fig 8 Mean microfibril angle with the long axis of full-grown root hairs of *Equisetum hyemale*, *a* measured in fractured hairs, stained with chlorozinc-iodine by polarizing microscopy 26 3 with a standard deviation of 8 3 *b* measured in shadow-cast preparations of fractured hairs treated with $H_2O_2$ HOAc and shadowed with Pt 23 1 , with a standard deviation of 12 2

parabolae of the helicoidal wall have also been revealed, in *Equisetum hyemale*, by freeze fracturing of untreated hairs (Emons 1982) Additionally, in full-grown hairs of *Equisetum hyemale*, a thick microfibril layer with a helical texture (Z-helix) is present, as has been found in the inner layer of growing root hairs of *Urtica dioica* (Sassen *et al* 1981). In *Urtica*, however, an S-helix has been found For terminology of chirality see Frey-Wyssling (1976)

Although the microfibrils in the apical wall show randomly oriented microfibrils, it cannot be concluded from the results of this study that they are interwoven nor that their deposition occurs indeed randomly. The wall expands during deposition and fibrils within one layer are continually dispersed with the result that the outer side of the primary wall may only appear to consist of a felted mass of microfibrils (Boyd and Foster 1975) In the tip of growing root hairs of *Raphanus sativus* a random texture, similar to that of *Equisetum hyemale*, has been observed (Newcomb and Bonnett 1965) In contrast, in root hairs of *Limnobium stoloniferum* helicoidal layers have been reported to occur within the dome of young growing hairs (Pullymaekers 1982), a difference with *Equisetum hyemale* which we cannot explain. Evidence for microfibrils to be deposited transversely to the cell-axis, according to the multi-net-growth-hypothesis (Roelofsen and Houwink 1953), was not found by means of polarizing nor electron microscopy This would mean that the manner of growth in the tip is such that axial extension does not in any area prevail over transverse extension, just as Houwink and Roelofsen (1954) concluded from their studies on *Zea mays* root hairs.

Deposition of the helicoidal wall occurs along the whole length of the hair Hence, the statement of Bel ford and Preston (1961) that synthesis of cell wall material in root hairs is limited to a short portion behind the tip, does not apply to *Equisetum hyemale*. Autoradiographic observations by Wardrop (1959) on *Avena* root hairs also showed that wall synthesis takes place over the whole cell surface "even in regions where growth has stopped". The helicoidal wall of *Equisetum hyemale* is a real secondary wall laid down in the non-extending part of the growing root hair. When growth stops, a secondary wall, similar in texture—*i.e.*, helicoidal—, is deposited in the apical dome of the root hair

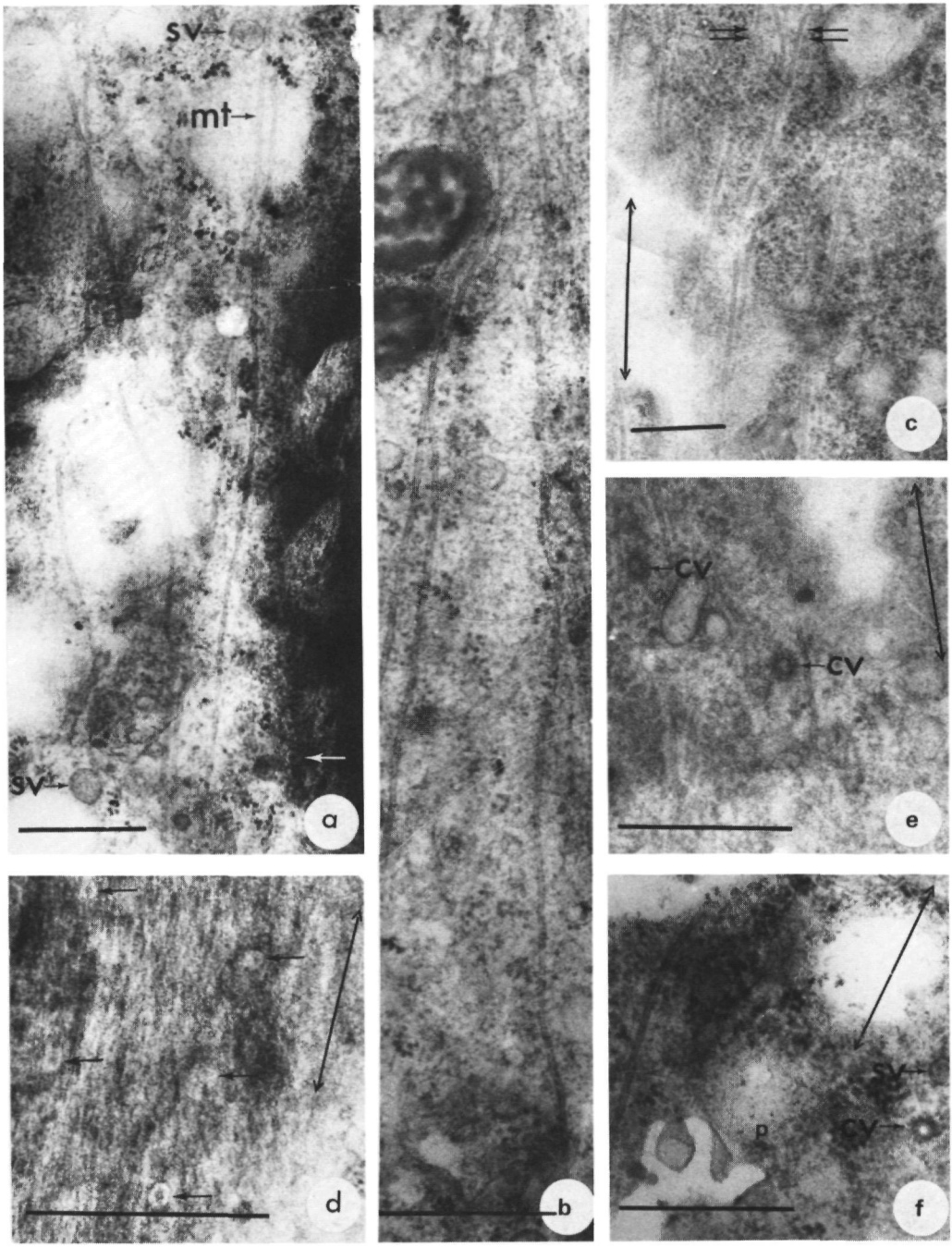



Fig. 9. Cortical cytoplasm of root hairs of *Equisetum hyemale* seen in longitudinal thin sections. Bars: 0.5 μm. Double-arrows indicate long axis of root hair. (*mt*) microtubule, (*cv*) coated vesicle, (*sv*) smooth vesicle. *a*: Axially aligned microtubules in full-grown hairs. × 36,400. *b*: As *a*: note bending of microtubules at organelles; smooth vesicles, containing a lightly staining material are present. × 49,000. *c*: Axially aligned microtubules in young hairs; note anastomosing arrays (arrows). × 25,500. *d*: Grazing section through the wall-plasmalemma interface, showing spots, 20nm in diameter, surrounded by a halo 50nm in diameter (arrows). The striated background putatively represents the microfibrillar pattern of the newly deposited wall layer. × 70,000. *e*: Coated vesicle (*cv*) in young root hair. × 49,000. *f*: Coated vesicle (*cv*) and smooth vesicle (*sv*) in full-grown hair. × 49,000

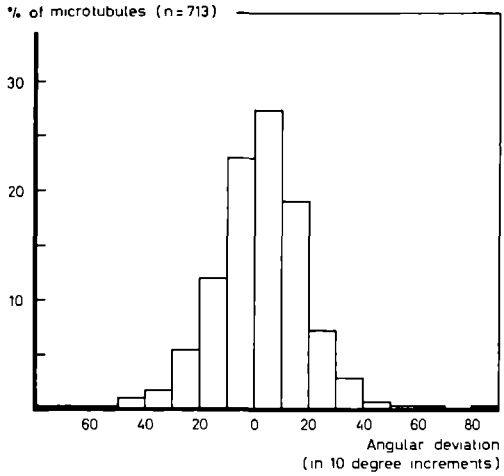



Fig 10 Angular deviation of microtubules from the long axis of full grown root hairs of *Equisetum hyemale* 0 long axis of root hair

Regarding the description of polylamellate cell wall textures, many different names have been used (ROLAND 1981) A crossed-polylamellate wall, which gives herringbone patterns in oblique sections should be distinguished from a helicoidal wall with parabolic patterns in oblique sections, a texture defined by BOULIGAND (1972) NEVILLE *et al* (1976) differentiated between the rotated ply-structure (the helicoid) and the ply-structure with alternation of fiber direction in successive layers (the crossed-polylamellate structure) on account of the supposed difference in behaviour during cell enlargement The crossed-polylamellate textures described by TAKEDA and SHIBAOKA (1981 a) and by ITOH (1979) are possibly helicoidal textures because, beside layers with longitudinal and transverse orientations, they describe layers with oblique orientations The pattern of microfibrils on transverse membranes adjacent to the wall of maize root parenchyma cells might imply a helicoidal wall texture (unlike the pattern on longitudinal membranes), as micrographs of MUELLER and BROWN (1982 a) show TAKEDA and SHIBAOKA (1981 a) describe a polylamellate cell wall texture with microfibril layers in longitudinal, oblique and transverse directions in the outer tangential walls of the epidermal cells of azuki-bean epicotyls (*Vigna angularis*) This wall texture changes, in older cells, to a texture consisting of axially oriented microfibrils They relate the pattern of microfibril deposition to the size of the cell to the extent of further elongation A similar change has been found now in root hairs of *Equisetum hyemale*, though in secondary non-extending walls The change cannot be related to the extent of further elongation but, possibly, to the age of the cell, *i e* , to changes in cell metabolism

The lenticular trellis-like configurations described by BOYD (1975) and hypothesized as evidence for bonding of microfibrils within lamellae and between adjacent lamellae are obvious in shadow-cast preparations of the randomly, the helicoidally and the helically textured walls of root hairs of *Equisetum hyemale*

### *4 2 Role of Microtubules*

If microtubule orientation did control microfibril orientation, the variability in microfibril orientation of the innermost wall layer, the layer adjacent to the plasma membrane, would be expected to reflect the microtubule deviation pattern in the cortical cytoplasm However, during all stages of wall development in root hairs of *Equisetum hyemale*, microtubule orientation does not parallel the orientation of the newly deposited microfibrils and, hence, cannot regulate their ordered deposition Exceptions to the parallelism of microtubules and adjacent microfibrils have been reported before (review HEPLER and PALEVITZ 1974), and universality of the role of microtubules in microfibril orientation has been questioned (SCHNEPF *et al* 1978) But, although not all reports on parallelism of microtubules and microfibrils are equally convincing (O'BRIEN 1972), there is no doubt that the mutual alignment of microtubules and cellulose microfibrils occurs widely, especially in elongating primary walls (review GUNNING 1981) and in secondary walls of tracheary elements and guard cells (review HEPLER 1981)

In the tubular part of tip-growing cells microtubules are aligned predominantly parallel to the long axis of the cell in root hairs (NEWCOMB and BONNETT 1965, SEAGULL and HEATH 1980, EMONS 1982), in pollen tubes (FRANKE *et al* 1972, MIKI-HIROSIGE and NAKAMURA 1982), in fungal hyphae (HOWARD 1981), in *Funaria* caulonema tip cells (SCHMIEDEL and SCHNEPF 1980) Only in the subapical part, from 25 µm behind the tip onwards of root hairs of radish are microfibrils known to align in the same axial direction (NEWCOMB and BONNETT 1965, SEAGULL and HEATH 1980) At 3 25 µm behind the tip of radish root hairs (NEWCOMB and BONNETT 1965), in pollen tubes (SASSEN 1964, CRABBENDAM, personal communication) and in the hyphae of *Schizophyllum commune* (VAN DER VALK

1976) microfibrils show a random pattern The *Phycomyces* sporangiophore resembles root hairs of *Raphanus* in depositing a layer of axially oriented microfibrils (of chitin not cellulose) on the inner surface of the side wall, but no microtubules are present (Newcomb 1969) In all mentioned examples of tip-growing cells, the arrangement of the microtubules is complex, showing considerable heterogeneity in orientation In *Equisetum hyemale* root hairs they form anastomosing arrays as described for subsidiary cells in the cortex of *Phleum pratense* (Palevitz 1981) and bend around vesicles and globules

In intercalarily growing, rapidly expanding walls, microtubules align in parallel and close to each other Their orientation is transverse to the long axis of the cell and parallel to the cellulose microfibrils in the wall Moreover, their orientation alters when lateral organs are initiated, preceding the conversion of the local pattern of microfibrils (Hardham *et al* 1980) This might confirm microtubule involvement in morphogenesis, in cell shaping, but not necessarily their direct control of microfibril orientation In *Closterium acerosum* cells (Hogetsu and Shibaoka 1978) the microfibrils on the inner surface of the expanding walls are deposited in a direction transverse to the cells axis and parallel to the underlying microtubules But when the cells have ceased to elongate an ordered pattern consisting of bundles of 7 11 microfibrils running in directions with marked angles to each other is being laid down, whereas there are no underlying microtubules

Schnepf (1974) suggested that microtubules function as cytoskeletal elements and model the cell surface against turgor pressure and surface tension to form the relief for the wall depositions The cytoskeletal function tallies with axial microtubule orientation in tip-growing cells, with transverse microtubules in expanding walls, with microtubules underlying local thickenings in secondary wall formation of vessels and guard cells and (Domozych *et al* 1981) with their cytoskeletal function presumed in wall-less cells However, microtubular control of microfibril directionality by lifting of the plasma membrane (Schnepf 1974), or via channeling by restricting the fluidity of the plasma membrane (Herth 1980) only holds if microtubule and microfibril orientation match This is not the case in root hairs of *Equisetum hyemale*

Indications of microtubular control of microfibril orientation have been derived from studies utilizing microtubule disrupting agents such as colchicine

Although the orientation of microfibril deposition mostly changes when microtubules have been depolymerized by this treatment, several different, even orderly, textures are deposited randomly oriented microfibrils in the lorica of *Poteriochromonas* (Schnepf *et al* 1975), parallel texture with similar frequencies of cells having transverse, oblique and longitudinal microfibrils in the epidermal cell walls of the epicotyl of *Vigna angularis* (Takeda and Shibaoka 1981 b), bundles of microfibrils in swirls deposited at marked angles to other groups of microfibrils in tracheary elements of *Coleus* (Hepler and Fosket 1971) The green alga *Oocystis solitaria* has been thoroughly investigated with many different microtubule inhibitors by Robinson and co-workers The cell wall has a typical crossed polylamellate texture (Robinson *et al* 1976) Depending on the type of inhibitor and its concentration microtubule inhibitor application induces two forms of aberrant microfibrillar cell wall patterns (Quader *et al* 1978) $10^{-2}$ mM colchicine causes the microtubules to disappear, while parallel oriented microfibrils are being deposited in one preferential direction, in 5 $10^{-3}$ to 10 $^{3}$mM colchicine, however microtubules are present and an intermediate cell wall texture may be obtained in which microfibrils are arranged in bow-shapes (Robinson and Herzog 1977), *i e*, a helicoidal wall texture It might be crucial whether or not these colchinized walls are expanding

In parenchyma cells of pine seedlings helicoidal deposition, similar to that of the wall texture of control cells, continues after microtubules have been depolymerized by colchicine (Itoh 1976) Also in *Pellia* setae (Schnepf and Deichgraber 1979) the disappearance of microtubules after colchicine treatment had no influence on microfibril arrangement Possibly, colchicine exerts two separate effects it depolymerizes microtubules and sometimes changes microfibril orientation c q through the plasma membrane The results of Wunderlich *et al* (1973) indicate the role of colchicine as an inhibitor of membrane fluidity Also Mueller and Brown (1982b) concluded microfibril deposition during colchicine treatment to be much more orderly than has been thought (see Hepler and Palevitz 1974)

### *4 3 Control of Microfibril Orientation*

As there is no congruence in orientation of cortical microtubules and currently deposited microfibrils in root hairs of *Equisetum hyemale*, microtubule direction cannot orient the microfibrils and, hence, another force has to cause their ordered deposition The first-

presumed force was cytoplasmic streaming (CRÜGER 1885). Proponents of this model (WESTAFER and BROWN 1976 and COLVIN 1980) suggest that the mechanism operates through its effect on the plasma membrane of the cell. Microtubules and microfibrils, then, might be often parallel because both are, possibly, oriented by cyclosis. If cyclosis is weak or variable in a particular cell no coïncidence in microtubule and microfibril direction will appear (COLVIN 1980). As a factor affecting cyclosis he mentioned ageing of cells. Cyclosis in *Equisetum hyemale* root hairs will be studied.

Self-assembly of microfibrils has been hypothesized for helicoidal walls (NEVILLE *et al.* 1976). Although self-assembly might explain one specific texture (helicoidal) it cannot equally account for another texture (helical as observed in *Equisetum hyemale* root hairs), if we presume the same chemical structure for the microfibrils of these walls.

Plasma membrane complexes (MUELLER and BROWN 1980) moving in the plane of the membrane (MUELLER and BROWN 1982 a, 1982 b) remain likely candidates for controlling microfibril orientation. Grazing sections through the wall-plasmalemma interface of full-grown root hairs of *Equisetum hyemale* show electron dense profiles (diameter 20 nm) surrounded by a halo (diameter 50 nm).

Whether these structures may be compared with the terminal complexes (diameter 28 nm) or with the particle rosettes (diameter 25 nm) found in *Zea mays* roots (MUELLER and BROWN 1980), or with the 26 nm granules found in leaves and roots of nine species (OLESON 1980) or with the 50 nm pits in *Acanthosphaera* (SCHNEPF *et al.* 1982), all of which have been hypothesized to play a role in microfibril development cannot be concluded from the results of this study. SCOTT *et al.* (1963) reported on pits in the cell wall of different roots hairs, which they suggested to be channels for cuticular substances. The electron dense profiles found in *Equisetum hyemale* root hairs might represent channels transporting material through the plasma membrane. They were not present in abundance, never formed a hint of a pattern and were not found in young hairs. Full-grown hairs are tawny brown, whereas young hairs are colourless. Impregnation of the cell wall of root hairs with a brown substance has been found in more species with thick-walled persistent root hairs and has been called "Metadermisierung" (*cf.*, GUTTENBERG 1968).

A freeze-fracture investigation, which will be undertaken, might elucidate this feature and plasma membrane involvement in microfibril deposition.

### *4.4. Vesicles*

Smooth vesicles, containing a lightly staining material, have already been reported to occur in root hairs of *Equisetum hyemale* by HARRIS (1979). He did, however, not observe coated vesicles. Nevertheless, coated vesicles are present in the cortical cytoplasm of young as well as of full-grown hairs. Although they have been seen in many plant cells (see review by NEWCOMB 1980), their function is still obscure. They might be involved in wall deposition.

## Acknowledgements

The valuable suggestions by Prof. Dr. M. M. A. SASSEN and Dr. M. KROH and the critical reading of the text by Prof. Dr. R. J. CAMPBELL are greatly appreciated.

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# CHAPTER 5

# The Microtubule-Microfibril Syndrome in Root Hairs with Helicoidal Wall Texture

# THE MICROTUBULE-MICROFIBRIL SYNDROME IN A ROOT HAIR WITH HELICOIDAL WALL TEXTURE

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## ABSTRACT

By means of freeze-substitution the dome of the root hair of Equisetum hyemale is shown to contain randomly oriented microtubules. Such an arrangement allows cell expansion in all directions. The pattern is in agreement with the hypothesis that microtubules function in cell morphogenesis. In the hair dome the microfibrils of the cell wall are randomly oriented.

In the non-expanding tube of root hairs of Equisetum hyemale microtubules are axially aligned. Microfibril alignment in the Equisetum hyemale root hair is helicoidal: successive parallel-fibred lamellae with microfibrils in subsequently rotated orientations. From hair tip to base different lamellae are in the process of deposition, thus, different fibril orientations align the plasma membrane. A near transverse orientation occurs from 0.6 mm to 1.2 mm from the hair tip, which is perpendicular to the microtubules, and not in agreement with the hypothesis that microtubules generally act as microfibril orientating structures.

Microfibril growth rate under the conditions of the experiment is calculated to amount to 700 nm/minute.

KEY WORDS; microtubules, microfibrils, helicoidal cell wall texture, Equisetum hyemale, root hairs.

## INTRODUCTION

The mechanism by which microfibrils are assembled is one of the preeminent questions concerning the cytology of plant cell wall formation.

It has been suggested that helicoidal walls self-assemble like liquid crystals in the periplasmic space (Neville et al. 1976, Roland and Vian 1979). However, in a root hair with helicoidal wall texture particle rosettes have been

found on the protoplasmic fracture face of the plasma membrane and terminal globules on the extraplasmic face (Emons 1985). Rosettes together with terminal globules constitute the putative microfibril synthesizing complexes (Brown 1985). The occurrence of particle rosettes and terminal globules agrees with the concept of microfibril tip-growth.

Parallelism between cortical microtubules and nascent microfibrils has been found in the majority of plant cells studied (Robinson and Quader 1982). Experiments with anti-microtubular agents have further strengthened the idea that microtubules determine microfibril orientation (Robinson and Quader 1982), though probably indirectly (Heath and Seagull 1982).

However, this coalignment between cortical microtubules and nascent microfibrils has not been found in root hairs with helicoidal wall texture of *Equisetum hyemale* (Emons 1982, Emons and Wolters-Arts 1983, Emons and Derksen 1986, Traas et al. 1985), *Limnobium stoloniferum* (Traas et al. 1985) *Ceratopteris thalictroides* (Meekes 1985), and *Equisetum fluviatile* (Emons 1986). Net-axial microtubule alignment has been found in thin sections, in dry-cleaved preparations and by immunofluorescence

Lloyd and Wells (1985) have suggested that the buffers (cacodylate and phosphate), used for fixation, may alter the microtubular skeleton e.g. because of osmotic conditions. They thus explain why microtubules and microfibrils do not always coorientate. This suggestion would, however, also invalidate data showing parallelism between these structures if they are obtained by fixation of material in these buffers, which covers the majority of the cases studied. Furthermore, Traas et al. (1985) used Pipes, the buffer Lloyd and Wells (1985) recommended for use instead of phosphate buffer, but obtained results similar to these obtained with phosphate and cacodylate.

In order to avoid the suggested artefacts and because root hairs are particularly susceptible to osmotic conditions, the cortical microtubule orientation in root hairs of *Equisetum hyemale* was reexamined by means of freeze-substitution, where the first step in fixation is purely physical.

For a better understanding of the helicoidal wall texture, the wall was studied by means of dry-cleaving (Sassen et al. (1985). This method is to be preferred to the traditional shadow-casting method because very large stretches (up to 2mm) of inner cell wall are revealed. The meandering of microfibrils within lamellae in dry-cleaved preparations is not seen in freeze-fractured preparations, and is therefore regarded as an artefact. However, dry-cleaving does not influence microfibril orientation per se.

This study reports the pattern of microtubules, and details of the helicoidal wall in *Equisetum hyemale* root hairs: proportion of transverse microfibrils, bending of microfibrils, and density of microfibrils within lamellae.

## MATERIAL AND METHODS

Cuttings from Equisetum hyemale were grown as previously described (Emons and Wolters-Arts 1983). Only young growing hairs were used.

Freeze-substitution was carried out as described by Emons and Derksen (1986), which is an adaptation of the method described by Heath and Rethoret (1982).

Dry-cleaving was performed according to the method described by Sassen et al. (1985).

Preparations were examined in a Philips EM 201.

## RESULTS

### *Microtubules*

Figure 1 shows tangential thin sections of the tip of a root hair. Microtubules are present in the extreme tip and line the hemisphere in random orientations (Fig. 1a). Some 7 micrometers away from the extreme end of the hair, but still in the hemisphere, microtubules are still in various orientations (Fig. 1b). Further from the tip the alignment of the cortical microtubules shows less variation, becoming more and more axial. Outside the hemisphere, in the tube of the hair, microtubules are axially aligned (Fig. 1c).

Figures 2a and b show tangential thin sections in the region of the hair with transverse microfibrils, at approx.

1 mm from the hair tip. Microtubule alignment is axial. Microtubules in this area are less abundant. In the tube of freeze-substituted root hairs of *Equisetum hyemale* an axial alignment was always observed.

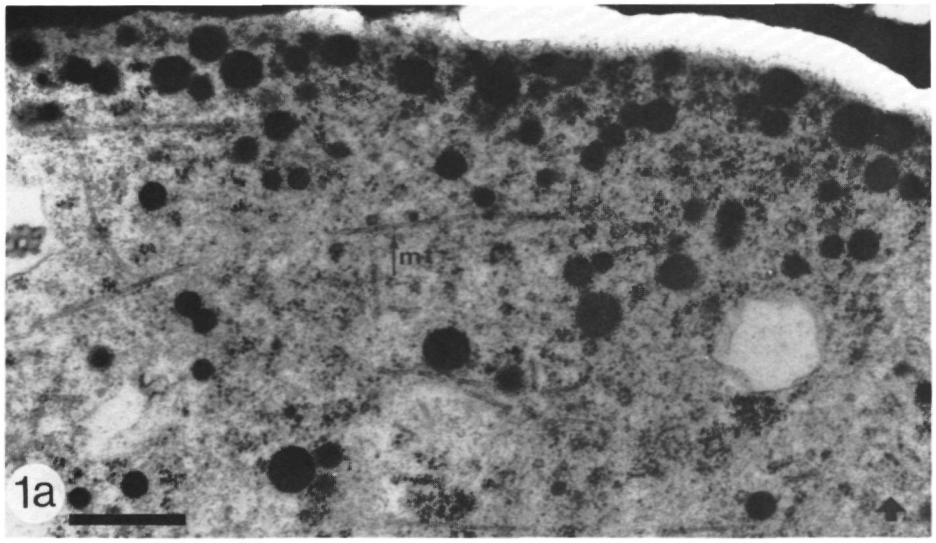
By means of freeze-substitution it was possible to observe cortical microtubules and nascent microfibrils in the same preparation of the trichoblast of *Limnobium stoloniferum*. Microfibrils do not coorientate with the cortical microtubules. The microtubules are oriented parallel to the long axis of the root hair (Fig. 3).

*Microfibrils*

Figure 4 shows the inner cell wall of the hair tip. The cell wall in the hair tip is very thin. Here, in growing hairs, always a random microfibril pattern is observed. Figure 5 shows the inner cell wall at 0.16 mm from the hair tip, composed of a thicker layer of microfibrils than found near the tip. The last deposited microfibrils are oriented more or less longitudinally. Figure 6 shows the inner cell wall at 0.9 mm from the tip of the hair: microfibrils in the last deposited lamella are almost perpendicular to the long axis of the hair. A near transverse orientation is maintained from 0.6 mm to 1.2 mm from the tip.

Figure 7 shows the inner cell wall of one and the same hair at distances from 1.09 mm to 2.35 mm from the hair tip. From tip to base of the hair the microfibrils in the last deposited lamella lie in orientations going from transverse to longitudinal. The wall consists of a stack of fibril lamellae. Looking from the cytoplasm and going from former to later deposited lamellae the progressive rotation in microfibril direction is counter-clockwise.

The angles formed by microfibrils of the three last-deposited lamellae with the long axis of the hair were measured at 60 places of a 1.26 mm long wall area (Fig. 8a). The number of microfibrils in the last two lamellae was counted (Fig. 8b). Fig. 8a shows that microfibrils maintain mean orientation within lamellae and that the number of microfibril lamellae increases from tip (distal part) to base (proximal part) of the hair. The angle between adjacent lamellae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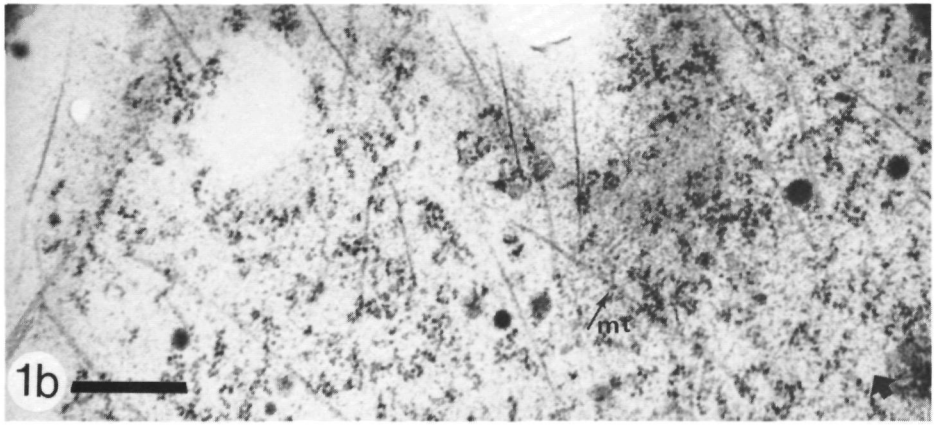
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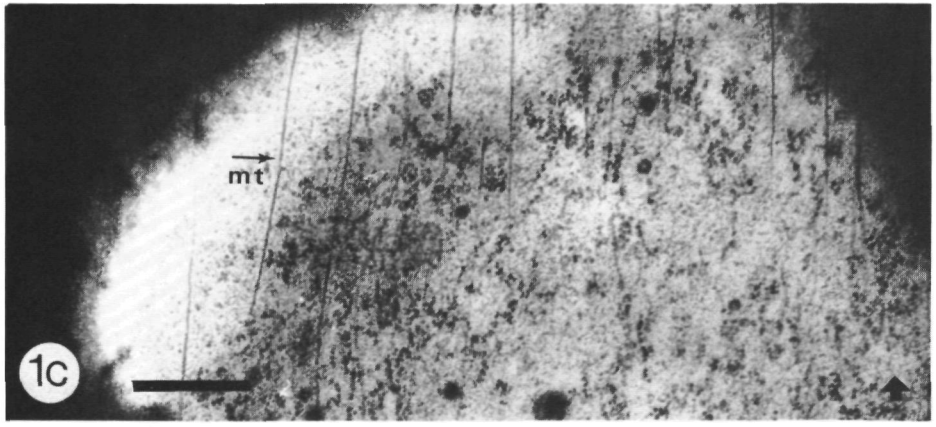
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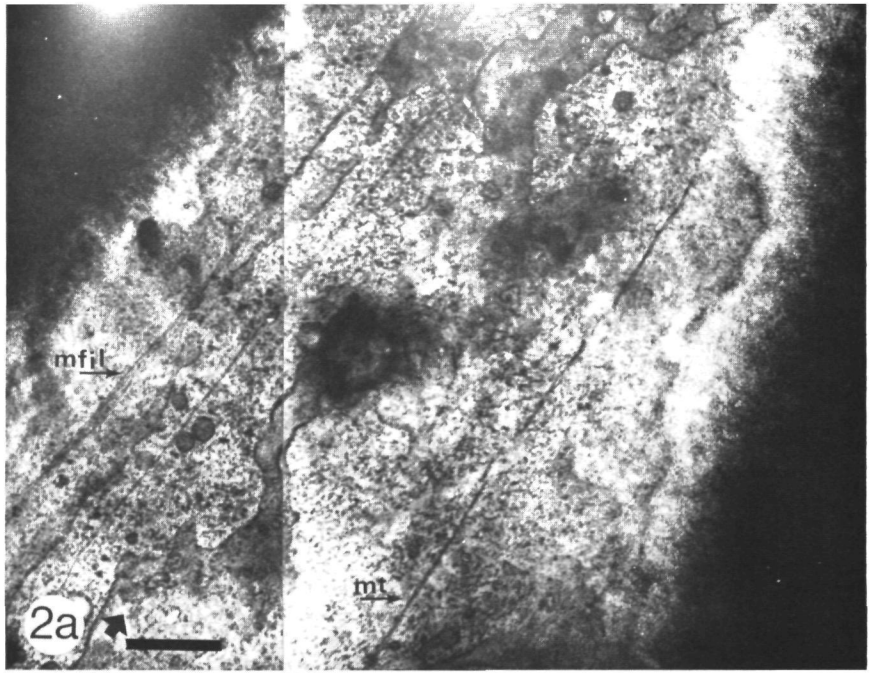
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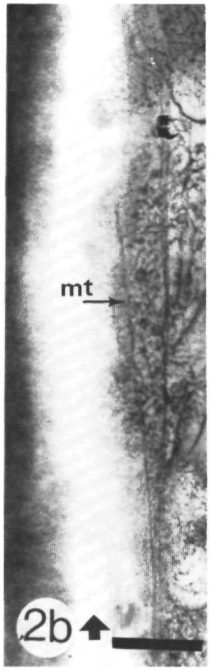
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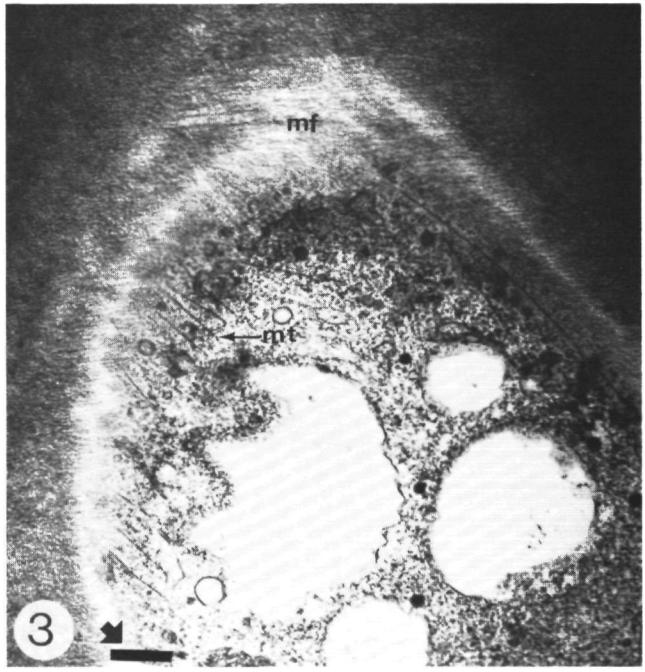
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Figs. 1 a-c: Tangential thin sections of the tip of a root hair of Equisetum hyemale prepared by means of freeze-substitution, showing the cortical cytoplasm at resp. 2 micrometers, 7 micrometers and 18 micrometers from the extreme tip of the hair. Hair diameter was 14 micrometers. Arrow: long axis of hair, mt: microtubule. x26 600. Bar: 500 nm.

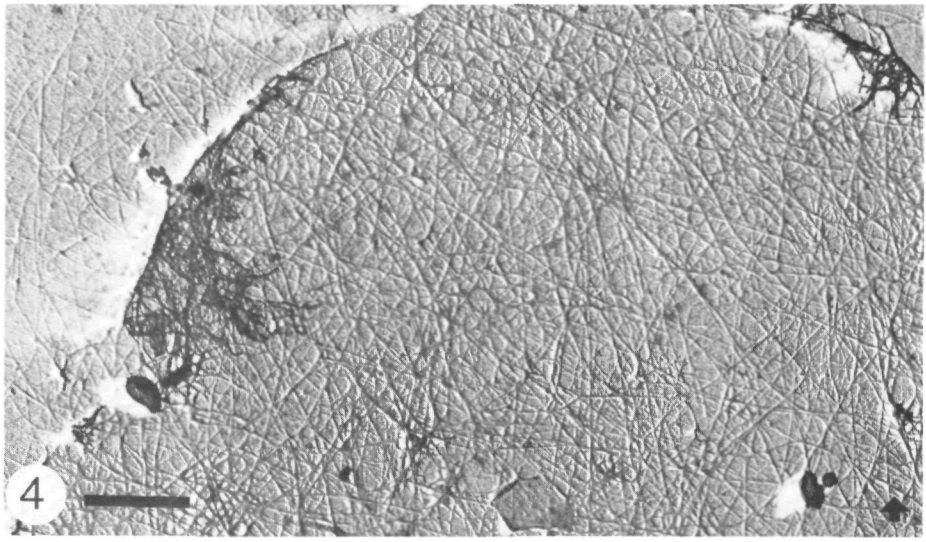
Figs. 2a and b: Tangential thin section at approx. 1 mm from the hair tip of a root hair of Equisetum hyemale. Wall microfibrils, not clearly visible, lie perpendicularly to the axis of the hair, microtubules lie in an axial orientation. Arrow: long axis of hair, mt: microtubule, mfil: microfilament bundle. x21 350. Bar: 500 nm.

Fig. 3: Tangential thin section of freeze-substituted trichoblast (root hair forming cell) of Limnobium stoloniferum, showing microfibrils and microtubules. Microfibrils and microtubules are not in parallel alignment. Arrow: long axis of hair, mt: microtubule, mf: microfibrils. x15 400. Bar: 500 nm

Fig. 4: Shadow-cast preparation of inner cell wall of hair tip of growing root hair of Equisetum hyemale, showing a random pattern of microfibrils. Arrow: long axis of hair.x28 600. Bar: 500 nm.

Fig. 5: Inner cell wall at 0.16 mm from the tip of a root hair of Equisetum hyemale, showing the last-deposited microfibrils in a more or less longitudinal orientation overlying a random texture. Arrow: long axis of hair. x28 600. Bar: 500 nm.

Fig. 6: Inner cell wall at 0.9 mm from the tip of a root hair of Equisetum hyemale, showing the last-deposited lamella with microfibrils in a nearly perpendicular orientation to the long axis of the hair. Arrow: long axis of hair. x28 600 Bar: 500 nm.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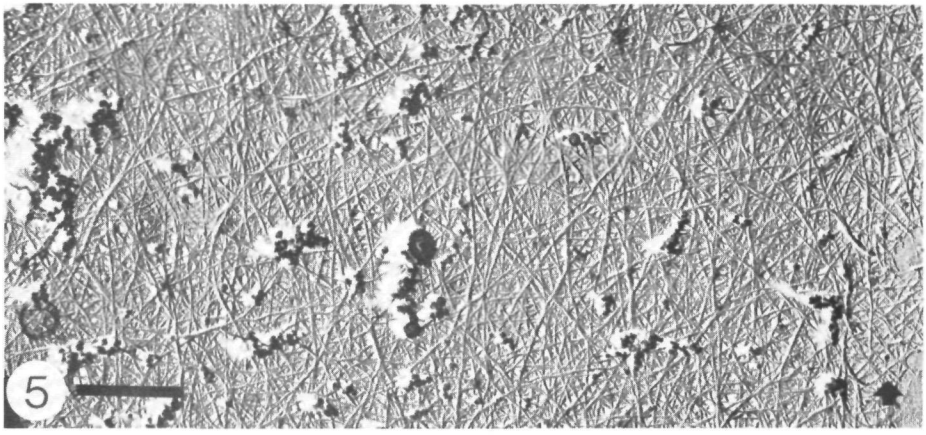
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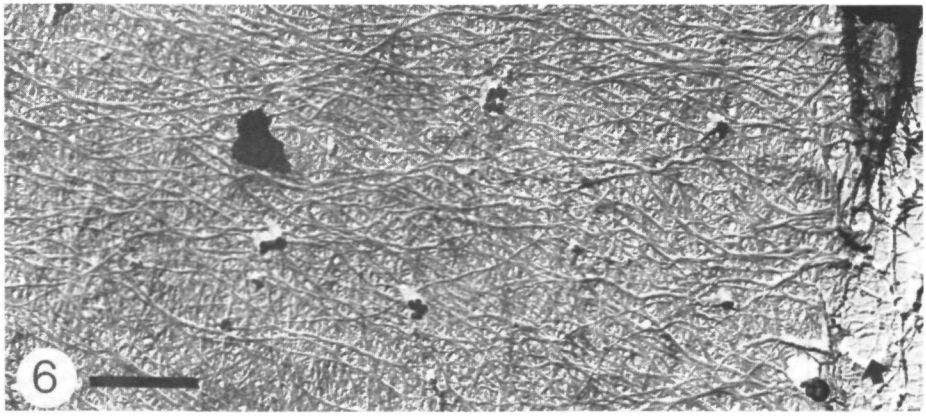
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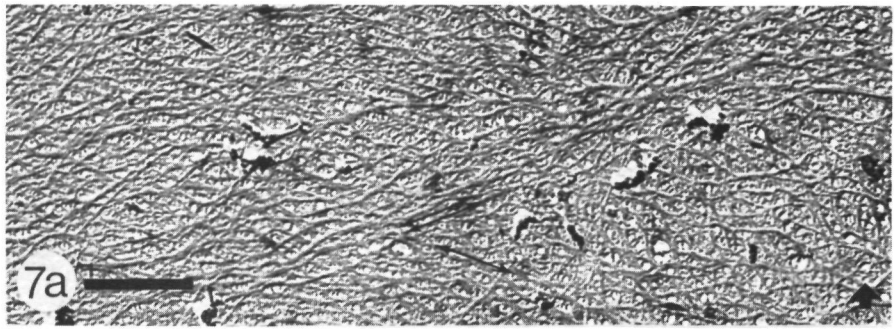
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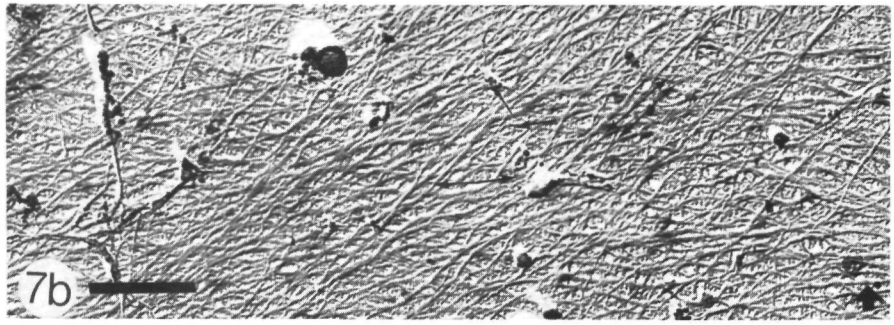
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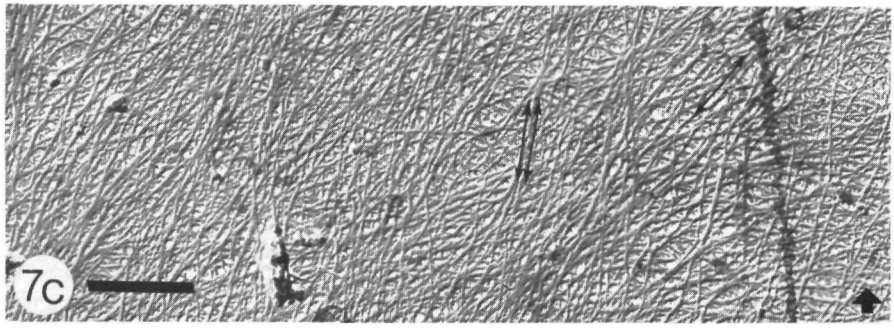
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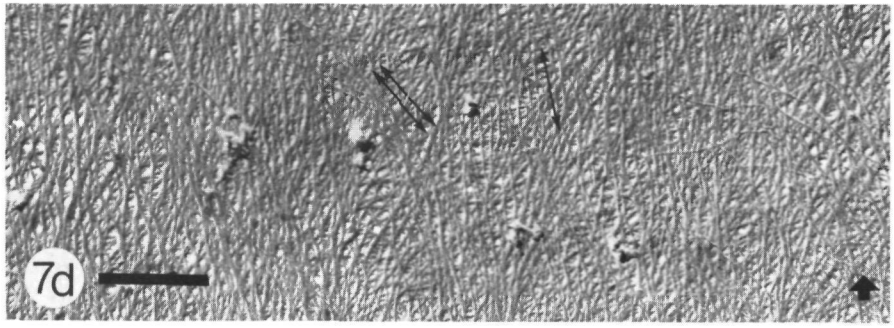
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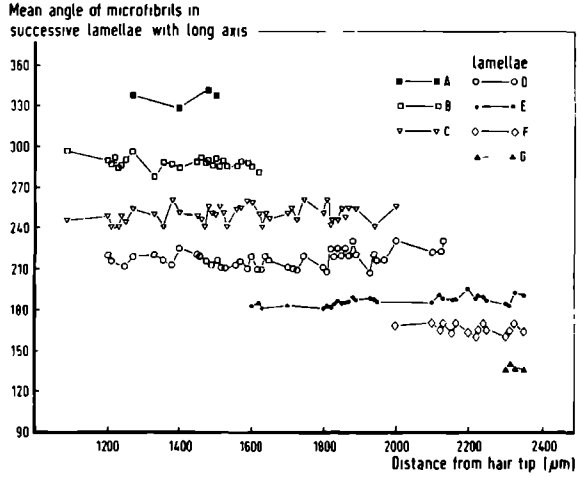
Figs. 7a-d: Inner cell wall of one and the same root hair of Equisetum hyemale at distances of resp. 1.49, 1.825, 2.105 and 2.35 mm from the tip. Microfibril orientation in the last-deposited lamella is from transverse to longitudinal. The rotation in microfibril direction is counterclockwise (looking from the cytoplasm and going from former to later deposited lamellae.). Double arrow: last deposited lamellae, single arrow: last but one lamellae, short arrow: long axis of hair. x28 600 Bar: 500 nm.

Fig. 8a: Mean angle of microfibils with long axis in successive lamellae of the helicoidal cell wall of the root hair of Equisetum hyemale. A to G represent different lamellae. Only the last three lamellae can be seen, but all lamellae are present up to 2.4 mm from the tip (known from thin sections).

Fig. 8b: Number of microfibrils in subsequent lamellae of the cell wall of a root hair of Equisetum hyemale. Lamellae B-G are the same as lamellae B-G in Fig. 8a. At 1.2 mm from the hair tip B and C are completed lamellae, D starts at this point, E,G and F start at resp. 1.58 mm, 1.96 mm and 2.24 mm from the hair tip. Upon lamella completion the number of parallel microfibrils per 4 micrometers perpendicularly is approx. 24.

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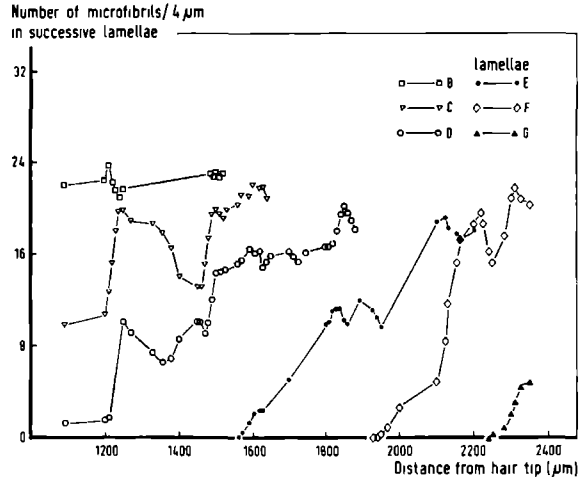



8b

is 30° to 40°. Figure 8b shows that the number of microfibrils within a lamella increases from distal to proximal end of the hair and that microfibril density in a completed lamella is approx. 6 per micrometer, measured by counting the number of microfibrils perpendicularly intersecting 4 micrometers. Figures 9a,b,c are schematical representations of possible microfibril alignment, given a helicoidal wall. The data confirm the schematical representation presented in Fig. 9a.

## DISCUSSION

The dry-cleaving method for cell walls has been described by Sassen et al. (1985). The procedure visualizes large areas of inner cell wall. That the inner cell wall is revealed is apparent from two phenomena: sometimes patches of plasma membrane with microfibril imprints stuck to the microfibrils (Fig. 4) and the clumped cytoplasm did not contain microfibrils.

In studies on the role of microtubules on microfibril deposition it is essential to know that microfibril deposition is actually going on at the moment of cell fixation. There are strong indications that most of the hairs must be in the process of microfibril deposition: wall thickness increases with hair length and hair age (Emons and Wolters-Arts 1983) and particle rosettes and terminal globules, the putative microfibril synthesizing complexes, are present in plasma membranes of young freeze-fractured hairs (Emons 1985).

### *The helicoidal wall*

An interesting feature of a helicoidal wall of a cell with tip-growth is that fibril lamellae are seen not only stacked horizontally as in a multiple plywood but also next to one another vertically in different stages of completion. The root hair, therefore, is an excellent system to study helicoidal wall deposition.

Within a lamella there is a large deviation from the mean orientation. The mean orientation is maintained over the to-

tal length of the lamella. Going from hair tip to hair base the number of lamellae increases as also fibril density within lamellae increases up to a maximum level. It is concluded that microfibrils in root hairs grow from hair base to hair tip.

*Microfibril growth rate*

The consecutive lamellae of the helicoidal wall texture stay behind the growing tip at a certain, more or less constant distance from the tip (Fig. 8a), dependent on e.g. temperature conditions. Lamellae with microfibrils in a near axial direction lengthen at the rate of root hair growth, which, under the conditions of the experiment, is approx. 700 nm/minute. The growth rate of microfibrils found here is well within the range of cellulose microfibril elongation calculated for other objects: Acetobacter: 2000 nm/min (Brown et al. 1976), Acanthosphaera: 500 nm/min (Schnepf et al. 1982), the cotton hair: 1300-2600 nm/min (Willison 1982), Funaria caulonema tip cells: 900 nm/min (Reiss et al. 1984), maize xylem: 931/min (Schneider and Herth 1986), cress xylem: 1283/min (Schneider and Herth 1986). Elongation rate of microfibrils of different cells can only be compared if growth conditions such as temperature and light/dark regime are known. Indeed Sassen (personal communication) found that with decreasing temperature, apart from growth rate of the root hair of *Limnobium stoloniferum*, distances of subsequent lamellae to the hair tip and distances among the onsets of subsequent lamellae decreased.

Assuming that particle rosettes function in microfibril deposition it is possible to calculate the rate of cellulose production per rosette per minute: the number of microfibrils intersecting a $\mu$m perpendicularly in a completed lamella is 5 to 7 (Fig. 8b). Per minute (5 to 7) x 700 nm fibril is deposited on a membrane area of 0.7 x 1 $\mu m^2$. A $\mu^2$ large area of plasma membrane contains 5 to 15 rosettes (Emons 1985). Therefore approx. 600 nm microfibril is deposited per rosette per minute, which means that approx. all rosettes are in the process of microfibril formation.

Microtubules have not been found in sections of chemically fixed root hair tips of *Equisetum hyemale* (Emons and Wolters-Arts 1983). However, freeze substitution clearly demonstrates their occurrence in this area. Also in freeze-substituted hyphae microtubules were encountered in cell tips whereas they had not been seen previously in chemically fixed hyphae tips (Howard and Aist 1979). Lloyd and Wells (1985) found microtubules in root hair tips of radish by immunofluorescence where they had not been seen earlier in thin sections (Seagull and Heath 1980). The exact orientation in the tip cannot be seen clearly in immunofluorescence preparations.

Microtubules line the hairs hemisphere in all different orientations, but attain a net-axial alignment in the transition area of hemisphere and tube, which is maintained throughout the rest of the hair. Microtubules and microfibrils in the expanding wall of the tip have the same distribution pattern.

However, parallelism is absent during secondary wall deposition in the hair tube. Though microfibrils and microtubules were not visualized in the same preparations, the area with almost transverse microfibrils is so large (0.6 mm, Fig. 8) that preparations of the cortical cytoplasm could easily be selected from the same area. Microtubule deviation from the axial orientation in freeze-substituted preparations is even less than in chemically fixed preparations.

The data confirm the hypothesis (Emons and Wolters-Arts 1983, Traas et al. 1985) that microtubules play a role in cell morphogenesis during cell expansion. But a direct channeling of microfibrils by cortical microtubules (Heath and Seagull 1982) seems unlikely because in cases where no cell expansion takes place no parallelism is found (cf. Emons 1982, Emons and Wolters-Arts 1983, Meekes 1985, Traas et al. 1985, Emons and Derksen 1986).

Predetermination of microfibril orientation in the trichoblast by microtubules is refuted by the formation of a helicoidal wall adjacent to unidirectional microtubules (Emons and Derksen 1986).

Mueller and Brown (1982) suggested, on evidence based on the positioning of microfibrils around stationary pit fields, that "membrane flow" might be involved in microfibril orientation. "Directed membrane particle flow" would be a better term for the phenomenon of directed lateral mobility of molecules in the plane of the membrane, because Franke et al. (1971) have determined "membrane flow" as the transfer of biomembranes from one compartment of the endomembrane system to another.

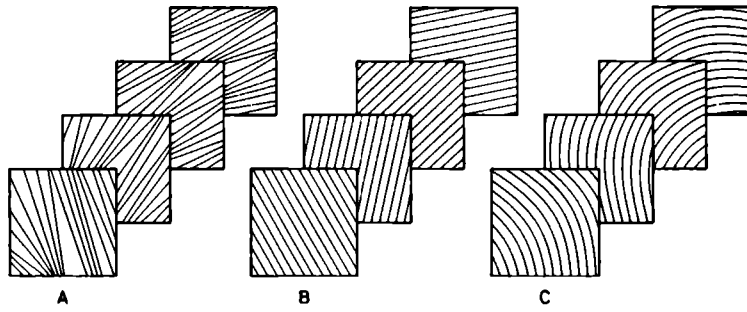
In the "directed membrane particle flow model" the microfibril is free to move in the membrane in the direction of synthesis, the kinetic force of crystallization being sufficient to propel the synthesizing complex through the membrane (Herth 1980).

If directed membrane particle flow is generated by unidirectional microtubules, and supposed that initial microfibril orientation is tranverse, the change in microfibril orientation of subsequent lamellae of the helicoidal wall would cease as soon as the microfibrils in a lamella have adopted the microtubule direction. Then, only half arcs of the helicoid are formed. In *Equisetum hyemale*, however, only full helicoidal arcs occur.

If, however, directed membrane particle flow is generated otherwise, there are two possible models for helicoidal wall formation: the orientating system may change direction in consecutive steps or gradually and constantly. The former model would generate straight microfibrils within lamellae (Fig. 9b), whereas the latter would generate slowly bending microfibrils (Fig. 9c). An abruptly changing directed membrane particle flow is unlikely (Neville and Levy 1984) and Fig. 8a shows that within a lamella microfibrils maintain orientation around a mean orientation. Therefore the hypothesis that directed membrane particle flow is the orientating force for the microfibrils of the cell wall is not supported by the present data.

Figure 9a is a representation of the helicoidal wall texture in root hairs of *Equisetum hyemale*. Though mean microfibril orientation within a lamella does not change, it re-

mains possible that individual microfibrils continually bend.




Figs. 9 a,b and c: Schematical representations of possible microfibril alignments, given a helicoidal texture. Fig. 9a is in accordance with the texture found in root hairs of Equisetum hyemale, In Fig. 9b microfibrils within lamellae are straight, and in Fig. 9c microfibrils within lamellae bend parallelly.

*Self-assembly*

Because of the similarity of architecture between biological helicoids and cholesteric liquid crystals, which are

known to form helicoids spontaneously, self-assembly has been suggested for helicoids of insect cuticles (Neville and Luke 1969) and for plant cell wall (Bouligand 1972, Neville et al. 1976). In this model the inner surface of the previously deposited wall is involved in control of microfibril assembly. A large range of different angles between microfibrils in subsequent lamellae has been found in material from different plants (Neville and Levy 1984, Emons and van Maaren, submitted). In a self-assembly process the difference in texture of different walls must reflect a difference in cellulose polymer molecules, in side chains of the cellulose molecules, or in wall matrix molecules interacting with the cellulose. Indeed, cell walls differ biochemically (Darvill et al. 1985). The gradual transition from helicoidal to helical cell wall texture in full-grown root hairs of *Equisetum hyemale* (Emons and Wolters-Arts 1983) would imply a change in chemical composition of the wall. Though such a change may well occur, the occurrence of two textures in a single cell disputes Neville and Levy's distinction (1984) between cells with helicoidal walls and cells with a smaller number of different microfibril orientations, the latter being under the controle of microtubules, the former not.

A mathematical model for helicoidal wall deposition has been worked out (this thesis, chapter 12).

ACKNOWLEDGEMENT

I thank Prof. Dr. M.M.A. Sassen and Dr. J. Derksen for valuable discussions.

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# CHAPTER 6

## Microfibrils, Microtubules and Microfilaments of the Trichoblast of Equisetum Hyemale

# MICROFIBRILS, MICROTUBULES AND MICROFILAMENTS OF THE TRICHOBLAST OF *Equisetum hyemale*

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Acta Bot. Neerl. 35 (3), 1986

## ABSTRACT

The cell wall of the trichoblastic part of the root hair cell is helicoidal, like the cell wall of the root hair proper. The rotation mode of the helicoid is counterclockwise and the angle between microfibrils in adjacent lamellae is 30° to 40°.

During trichoblast elongation cortical microtubules lie perpendicular to the axis of elongation. During root hair initiation and root hair growth cortical microtubules align according to the long axis of the forming hair, not in parallel with the microfibrils. The freeze-substitution technique reveals microtubules in the apical dome of the forming hair, where they lie in random orientations enabling isodiametric expansion. Microtubules in these cells function in morphogenesis, but not in microfibril orientation.

F-actin cables, microfilaments, are present in the trichoblast; they form a network in the cell interior. They do not coalign with the microfibrils nor with the microtubules.

Key words: Microfibrils, microtubules, microfilaments, Equisetum, trichoblast

## INTRODUCTION

In many plant cells, nascent microfibrils and cortical microtubules have been found in parallel orientations and therefore microtubules have been hypothesized to orientate the nascent microfibrils. It is, however, not known how they do so; and several hypotheses have been proposed (Heath and Seagull 1982).

The coalignment between these structures has especially been studied in enlarging cell surfaces (Hardham 1982, Gunning and Hardham 1982) and in xylem elements, in which local wall thickening occurs (Falconer and Seagull 1985).

Absence of parallelism has been explained by pointing out that microtubules were already in the position of the expected new microfibril orientation (Newcomb and Bonnett 1965, Seagull and Heath 1980).

Recent investigations on root hairs with helicoidal walls, however, did not allow for such an explanation (Emons 1982, Emons and Wolters-Arts 1983, Meekes 1985, Traas et al. 1985). In these cells, microtubules align longitudinally while in time and in place the adjacent microfibrils attain all different orientations with respect to the long axis of the hair. Therefore, the microtubules cannot orientate the microfibrils by their own direction. It has to be emphasized that root hairs are tip-growing cells (Sievers and Schnepf 1981) and that the helicoidal wall is a secondary wall deposited in the non-enlarging hair tube (Emons and Wolters-Arts 1983).

F-actin strands, microfilaments, have recently been shown, by means of the F-actin specific probe rhodamine-phalloidin, to be a normal component of plant cells (Parthasarathy 1985, Parthasarathy et al. 1985, Derksen et al. 1986, Pierson et al. 1986). They may be involved in cytoplasmic streaming (Pesacreta and Parthasarathy 1984, Parthasarathy et al 1985, Derksen et al. 1986) and may interact with microtubules (Pollard et al. 1984).

We studied microfibrils, microtubules and microfilaments in trichoblasts of *Equisetum hyemale* to investigate their possible interactions. A root hair is a protuberance of an epidermis cell. This cell is called a trichoblast. In this paper the term trichoblast is used for the epidermis cell before, during and after root hair formation. As fibril lamellae of the root hair seem to originate from this part of the cell (Emons and Wolters-Arts 1983) a clue for the microfibril orientating mechanism might be found in the trichoblast.

## MATERIAL AND METHODS

The microtubules of the cytoplasm were visualized by immunofluorescence (Wick and Duniec 1983, Traas et al. 1985,).

The microfibrils of the cell wall were visualized by thin-sectioning (Emons and Wolters-Arts 1983) and by dry-cleaving (Sassen et al. 1985).

Microfibrils and microtubules were also visualized with the freeze-substitution procedure. For freeze-substitution, pieces of roots with hairs were placed on pieces of boiled dialysis tubing as large as a grid. These pieces were rapidly frozen by plunging them in liquid propane, cooled by liquid nitrogen. They were transferred to a substitution fluid composed of anhydrous aceton containing 2% $OsO_4$ and 0.1% uranyl acetate precooled at -78° C in a metal vial. The metal vials, containing the specimens were transferred to a freeze-drying device held at a temperature of -80° C (±5° C) by liquid nitrogen during 20 h. The material was brought to room temperature in this apparatus very slowly during another 6 h. 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 cells, showing no evidence of gross ice crystal damage, were selected. The selected hairs were sectioned tangentionally with a Sorvall Porter Blum MT 5000 onto formvar coated grids and stained with uranyl acetate/lead citrate.

Sections were examined with a Philips EM 201 electron microscope.

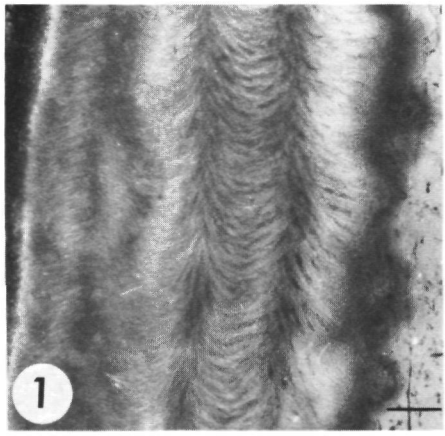
The microfilaments of the cytoplasm were visualized using rhodamine-labelled phalloidin (Derksen et al. 1986).

## RESULTS

Freeze-substitution gave good preservation of the cytoplasm of the root hair, in which the longitudinal microtubule alignment could be ascertained (Emons, submitted). The microfibrils of the cell wall of the root hair were not visualized by this method, but the method did visualize microfibrils in the trichoblast cell wall. Sofar, in this part of the cell we did not succeed to preserve the cytoplasm with cortical microtubules.

The cell wall of the trichoblast is clearly of the helicoidal type (Fig. 1). This is also shown by thin-sectioning of material from which matrix substances had been dissolved (Fig. 2). Dry-cleaving shows the last-deposited microfibrils in surface view (Fig. 3). The angle between microfibril orientations in adjacent lamellae is 30° to 40°. Looking from the cytoplasm and going from former to later deposited lamellae the rotation of the helicoid is counterclockwise. Microfibrils within a lamella are not contiguous.

The three different methods employed show that the cell wall texture of the trichoblast is helicoidal with the same angle between fibril orientations of successive lamellae and with the same rotation of the helicoid as in the root hair cell wall.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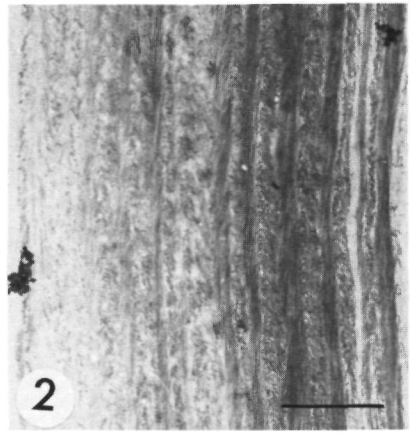
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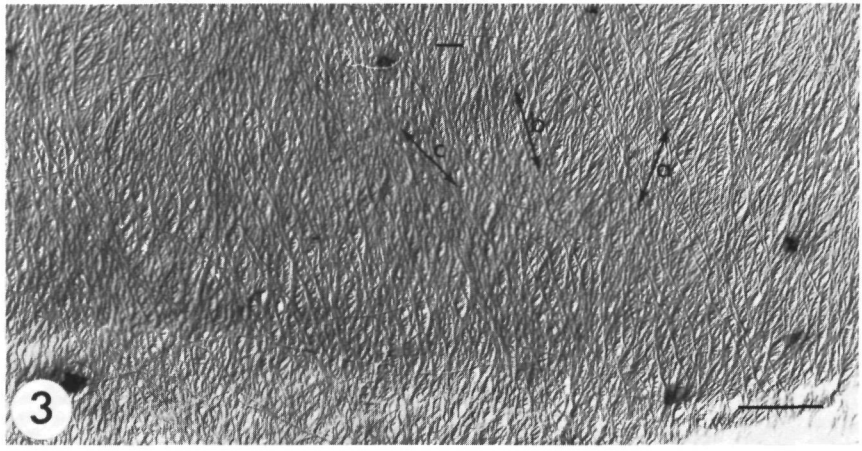
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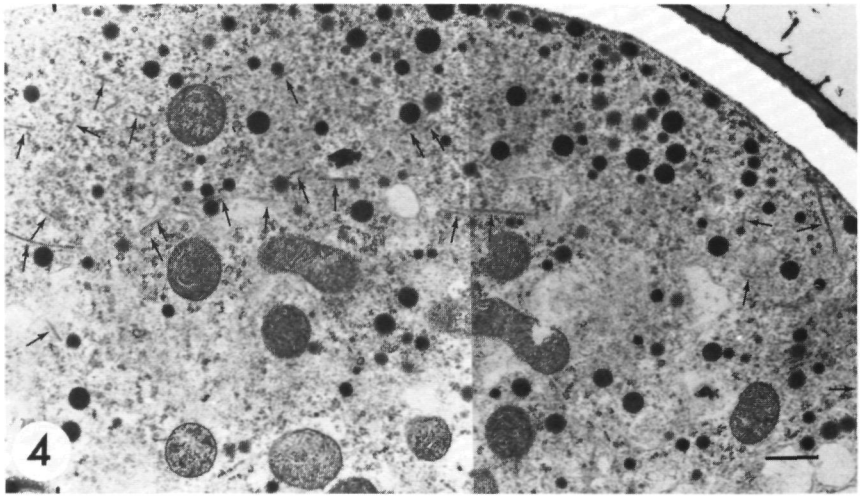
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Fig. 1. Thin section of the cell wall of a trichoblast with short, growing root hair, prepared by freeze-substitution and in-block staining with uranyl acetate, showing the arcs of the helicoidal cell wall. Width of parabolae in a micrograph depends on obliqueness of sections. 13 200 x, bar: 500 nm.

Fig. 2. Thin section of the cell wall of a trichoblast with a long, growing root hair, prepared by dissolving the wall matrix prior to embedding and staining of the sections with permanganate. In cells with longer root hairs more helicoidal arcs are present in the proximal part of the hair than in cells with shorter root hairs. 27 300 x, bar: 500 nm.

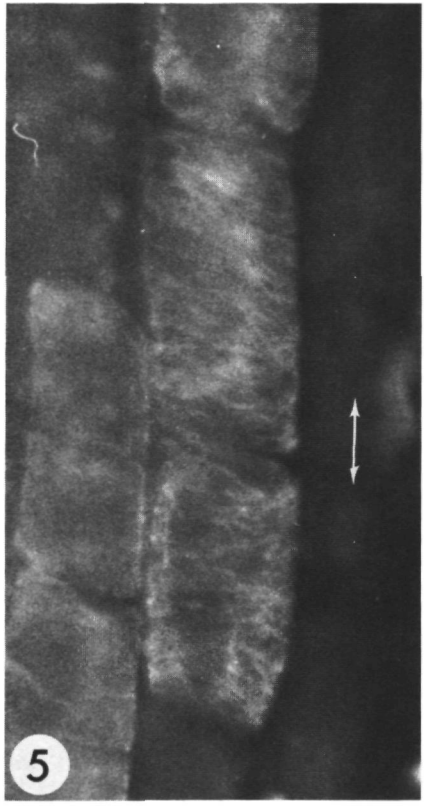
Fig. 3. Surface view of the innermost layer of the cell wall prepared by dry cleaving and shadowing, showing the three last deposited microfibril lamellae. Arrows indicate the direction of the three last-deposited lamellae. Looking from the cytoplasm, going from former (a) to later (b resp. c) deposited lamellae, the rotation mode of the helicoid is counterclockwise. The angle between consecutive lamellae is approximately 40°. 27 000 x, bar: 500 nm.

Fig. 4. Thin section of tip of very young root hair, prepared by freeze-substitution. Small arrows indicate microtubules. Microtubules occur in random orientations. 13 200 x, bar: 500 nm.

Fig. 5. Microtubule alignment of undifferentiated epidermis cells during cell elongation, visualized by immunofluorescence. Cortical microtubules align transverse to the axis of elongation. As to microtubule alignment, trichoblasts and atrichoblasts are the same. Arrow designates long axis of root, which is the axis of elongation of the cell. 1000 x.

Fig. 6 a,b,c. Microtulule alignment in trichoblasts during root hair growth, visualized by immunofluorescence. Microtubules mainly occur in the cortical cytoplasm.
a). trichoblast, which has a short protuberance, b). trichoblast, which has a growing hair, c). trichoblast,which has a full-grown hair. During hair growth microtubules lie in the direction of the long axis of the hair; after growth this alignment is more or less lost in the trichoblastic part of the cell. 960-1000 x.

Fig. 7. Microfilament alignment in a trichoblast, bearing a growing hair, visualized using the F-actin specific probe rhodamine-phalloidin. Microfilaments occur at different levels in the cell, not only in the cortical cytoplasm. 1200 x.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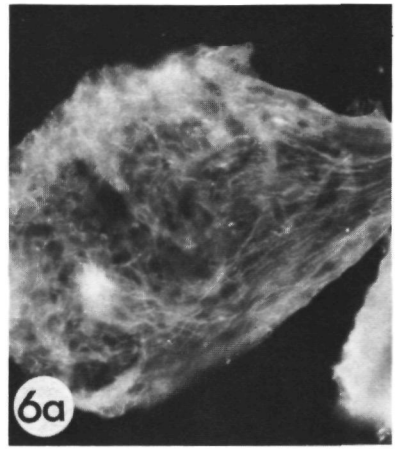
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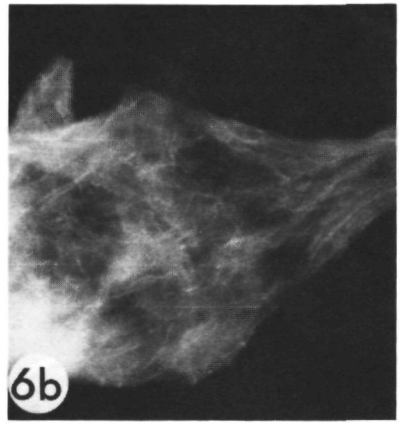
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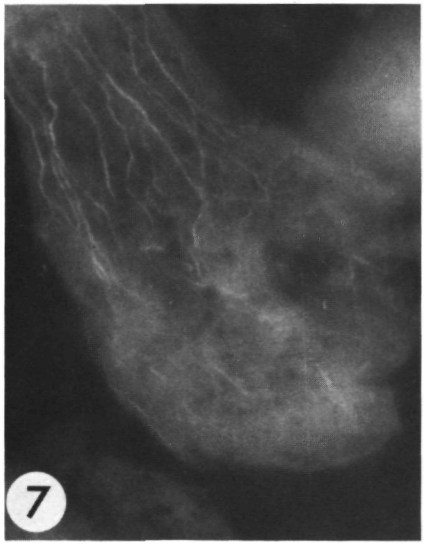
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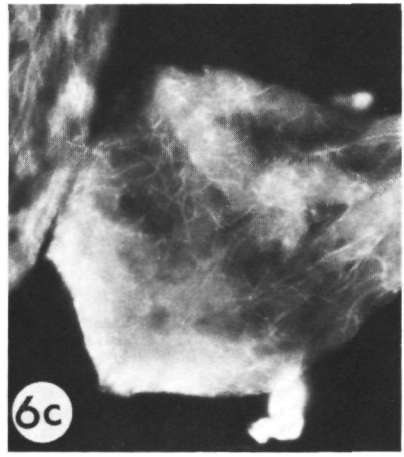
6c

Fig. 5 shows the microtubule pattern in epidermis cells before root hair formation. Figs. 6 a,b,c show the microtubule pattern in a trichoblast with a short protuberance (Fig. 6a), a trichoblast with a growing root hair (Fig. 6b) and a trichoblast with a full-grown root hair (Fig. 6c). In elongating epidermis cells before root hair initiation microtubules align transverse to the axis of elongation of the root and of the epidermis cell (Fig. 5). In a trichoblast with short protuberance (Fig. 6a) the original microtubule alignment in the tangential wall is lost; alignment in the radial walls is still transverse to the axis of elongation of the root (Fig. 6a). Microtubules protrude into the forming hair and lie in the direction of the long axis of this hair. The same microtubule pattern is conserved during root hair growth (Fig. 6b). In Fig. 6c it can be seen that in a trichoblast with a full-grown hair all regular microtubule alignment is more or less lost. Microfibril deposition in the trichoblast goes on during all these stages (Figs. 1 and 2).

Freeze-substitution reveals a random microtubule orientation in the tip of the protuberance (Fig. 4).

Fig. 7 reveals the alignment of F-actin cables, microfilaments, in a trichoblast with a young growing root hair. These microfilaments are situated in the cell interior unlike microtubules, which are present predominantly in the cortical cytoplasm. Throughout hair growth they conserve this alignment. They do not coalign with the microfibrils of the cell wall.

## DISCUSSION

### *Microfibril visualization by means of freeze substitution*

Uranyl acetate has no chemical affinity for cellulose. The image of cell walls after this staining is often unreliable (Neville and Levy 1984). Cox and Juniper (1972) have reported that uranyl acetate stains cellulose microfibrils but that this staining is physical in nature and therefore re-

moved by washing of the material on the grids. In-block staining would reveal microfibrils. The dark threads seen by freeze-substitution of material in-block stained with uranyl acetate (Fig. 1) occur in the same pattern as in thin sections from which matrix material has been dissolved (Fig.2), in shadowed dry-cleaved material (Fig.3) and in freeze-etched material (Emons 1985) and are therefore interpreted as cellulose microfibrils.

In freeze-fractured preparations microfibrils of root hairs of *Equisetum hyemale* measured 8.5 nm (±1.5 nm) including shadow deposit (Emons 1985). In dry-cleaved preparations (Fig. 3) microfibrils measure 8 nm (±1 nm) including shadow deposit. However, in thin sections of material from which the cell wall matrix has been dissolved (Fig. 2) their diameter is much smaller (approximately 3.5 nm). The diameter of microfibrils in the helicoidal layer of the wall prepared with freeze-substitution is much larger (compare Fig. 1 and Fig. 2), which could point at the possibility that a hydrophylic sheath of less-crystalline glucans, which surrounds the crystalline core, is positively stained.

Freeze-substitution of in-block stained material is one of the means to elucidate the debate on microtubule and microfibril coalignment (Emons 1982, Emons and Wolters-Arts 1983, Lloyd and Wells 1985, Traas et al. 1985), because artefacts of chemical fixation are ruled out and microtubules and microfibrils may be visualized at the same time in the same preparation.

*Microtubules and morphogenesis*

In a review article, Cormack (1949) stated that there was general agreement that retardation in vertical elongation of the trichoblasts is a prerequisite for epidermal cells to give rise to root hairs.

It has become known since then that microtubule orientation is at least one of the factors that determine the orientation of cell elongation (Gunning and Hardham 1982, Hardham 1982, Busby and Gunning 1984). In cells expanding uniformly in one direction, microtubules lie perpendicular to the axis of elongation (Gunning and Hardham 1982, Hardham 1982, Busby and Gunning 1984, Sassen and Wolters-Arts 1986). This can

also be seen in epidermis cells before root hair initiation (Fig. 5). In isodiametrically expanding cells, as protoplasts, microtubules lie in random orientations (Marchant 1979, Lloyd et al. 1980, van der Valk et al. 1980). The random microtubule orientations occur also in the expanding tip of *Equisetum hyemale* root hairs (Fig. 4).

Cells, which have completed elongation, never form hairs (Cormack 1949). In these cells microtubules occur in random orientations (data not shown). It seems that a microtubule alignment perpendicular to the axis of elongation of the trichoblast, i.e. according to the long axis of the protuberance, is one of the prerequisites needed to form a hair, but it is not the root hair initiating factor as atrichoblasts have the same microtubule pattern and do not form hairs. In *Equisetum hyemale* the trichoblast stops elongating at the onset of root hair formation.

Our observations clearly indicate a morphological role of the cortical microtubules: at the time that the protuberance forms, microtubule alignment along the tangential wall of the trichoblastic cell changes (Fig. 6). Microtubules loose alignment around the cell, but along the radial walls they lie in the direction of the long axis of the protuberance. At the tip of the protuberance they span the hemisphere and lie in random orientations allowing isodiametric expansion. The crucial question therefore is: "What determines microtubule orientation?"

It further remains to be explained how transverse hair expansion is inhibited in tip-growing cells, which have axial microtubule alignment.

*Microtubules and microfibril orientation*

Cortical microtubules lie against the plasma membrane. Across the membrane, microfibrils are deposited. During root hair growth, microfibril deposition occurs along the whole *Equisetum hyemale* root hair and the cell wall of the trichoblast thickens also (compare Figs. 1 and 2). As in the hair, microfibrils in this basal part of the cell are deposited according to a helicoid, showing parabolae in thin sections

(Figs. 1 and 2). Thus, lamellae with microfibrils in subsequently differing orientations are deposited while microtubules remain aligned in one direction only. This corroborates the statement that microtubules do not orientate the nascent microfibrils in a helicoidal cell wall (Emons 1982, Emons and Wolters-Arts 1983).

Lloyd and Wells (1985) have suggested that phosphate buffer somehow would disorientate transversely oriented microtubules in root hairs. Traas et al. (1985), however, did not find any differences in microtubule pattern between cells processed in phosphate buffer and cells processed in Pipes buffer recommended by Lloyd and Wells (1985).

Microtubules and microfibrils influence plant cell morphogenesis. Microtubules constitute the inner skeleton, with a role in maintenance and alteration of cell shape, comparable to their role in animal cells (erythrophores: Ochs 1982, amoebae: Uyeda and Furuya (1985). Microfibrils constitute the outer skeleton of a plant cell, with a role in more permanently shaping cells. This outer skeleton is transient in growing cells or cell parts and permanent in full-grown cells or cell parts. An enlarging cell in a transient wall needs the microtubules as the inner cytoskeleton and to alter the axis of elongation. Absence of coalignment of microfibrils and microtubules has also been shown in Valonia (Itoh and Brown 1984). Mizuta and Wada (1982) concluded that microtubules do not orientate the nascent microfibrils, because anti-microtubule agents did not affect microfibril orientation in Boergesenia. Hahne and Hoffmann (1985) have found that in mesophyll cells microtubules are present during cell division and cell enlargement but not during cell wall deposition.

As microtubules and microfibrils have some functions in common, microtubules and microfibrils lie in parallel in enlarging cells, but microtubules do not directly orientate the nascent microfibrils.

*Microfilaments*

The orientation of F-actin cables, microfilaments, visualized with rhodamine-labelled phalloidin differs from the orientation of microtubules and is not correlated to the

orientation of nascent microfibrils. We conclude that the actin cables observed are not involved in cell morphogenesis nor in microfibril orientation.

In freeze-substituted root hairs of *Equisetum hyemale* microfilaments are often seen along the microtubules (Emons, submitted). These filaments are not visualized with the rhodamine labelled phalloidin. They may interact with microtubules (Pollard et al. 1984).

ACKNOWLEDGEMENT

We thank Dr. T. Wieland (Heidelberg) for his generous gift of rhodamine-phalloidin and Professor Dr. M.M.A. Sassen for support in various ways.

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# CHAPTER 7

# The Influence of Colchicine on Root Hair Wall Texture

# THE INFLUENCE OF COLCHICINE ON ROOT HAIR WALL TEXTURE

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in preparation

## ABSTRACT

Roots of Equisetum hyemale and Raphanus sativus were grown in a concentration of colchicine that slowed down root hair growth without stopping growth completely. The treatment effected depolymerization of microtubules and increase in root hair diameter. In root hairs of Raphanus sativus the deposition orientation of microfibrils changed to oblique and even transverse orientations, whereas untreated hairs show axial microfibril alignment. In root hairs of Equisetum hyemale a helicoidal wall was deposited, which is the same as without treatment. It is concluded that microtubules function in determining cell shape, but do not directly control orientation of nascent microfibrils.

KEY WORDS: microtubules, microfibrils, colchicine, helicoidal wall texture

## INTRODUCTION

In root hairs different types of wall textures have been found: axial, helical, helicoidal (Sassen et al. 1981) and crossed polylamellate (Lloyd and Wells 1985). The helicoidal wall is built of a stack of parallel-fibred lamellae. Microfibril orientation in every subsequent lamella is rotated with respect to the previous lamella. In general, cortical microtubules in root hairs are axial or steeply helical (Seagull and Heath 1980, Emons 1982, Lloyd and Wells 1985, Traas et al. 1985), though more complicated configurations have been reported for Allium (Lloyd and Wells 1985, Traas et al. 1985). Thus, in root hairs with axial or helical wall

texture microtubules and nascent microfibrils are often in parallel, while in root hairs with helicoidal wall texture such a coorientation has not been found. In young root hairs of *Equisetum hyemale*, which have a helicoidal wall texture, (Emons 1982, Emons and Wolters-Arts 1983) axial microtubule alignment was found consistently by means of different methods: thin-sectioning of chemically fixed material (Emons 1982, Emons and Wolters-Arts 1983), dry cleaving (Traas et al.), immunofluorescence (Traas et al. 1985, Emons and Derksen 1986) and freeze-substitution (this thesis chapter 5).

Colchicine depolymerizes microtubules and has various effects on cell wall texture (review: Robinson and Quader 1982). To study the effect of colchicine on root hairs, we used *Equisetum hyemale* and *Raphanus sativus* with well known root hair wall texture: helicoidal in *E. hyemale* (Emons and Wolters-Arts 1983) and axial in *R. sativus* (Sassen et al. 1985).

## MATERIAL AND METHODS

Root hairs of Equisetum hyemale were taken from stem cuttings (cf. Emons and Wolters-Arts 1983). Root hairs of Raphanus sativus were taken from seedlings germinated on moist filter paper and grown in bidest in petri dishes. Plantlets were grown in special cuvettes in which adherent roots of stem cuttings and intact seedlings could be examined under a light microscope to follow hair initiation and growth, and in which the growth medium could be changed without disturbing the roots. Only hairs growing in the medium were used. Roots were fixed in these cuvettes. Concentrations and treatment times were: $10^{-2}$ M colchicine during 7 h and $10^{-3}$ M colchicine, overnight.

Microtubules were studied by means of immunofluorescence (Traas et al. 1985). Cell wall texture was examined by means of the dry-cleaving technique (Sassen et al. 1985).

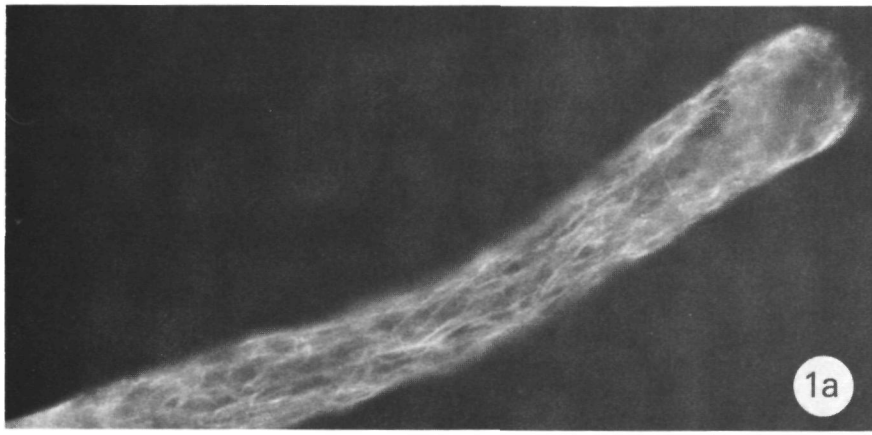
## RESULTS

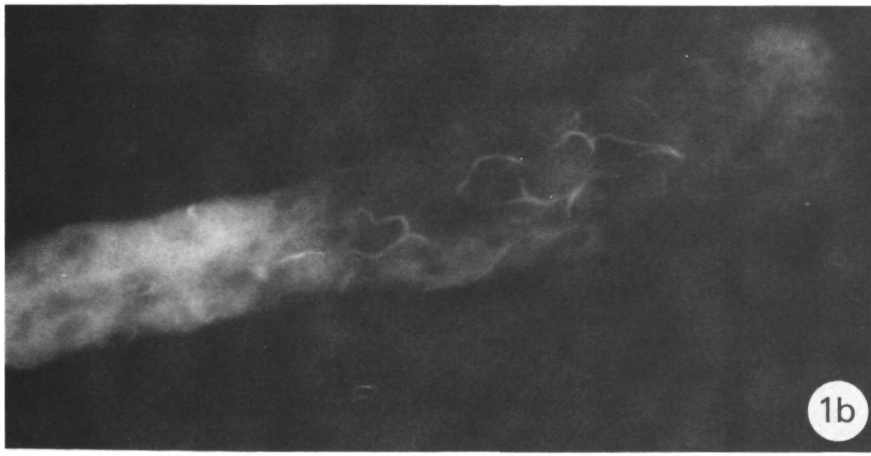
Fig. 1a shows microtubules in an untreated root hair. Fig. 1b shows a root hair of *Equisetum hyemale* grown in colchicine: most of the microtubules are depolymerized, the orientation of the remaining microtubules deviates more from the axial direction than microtubules in untreated hairs do.

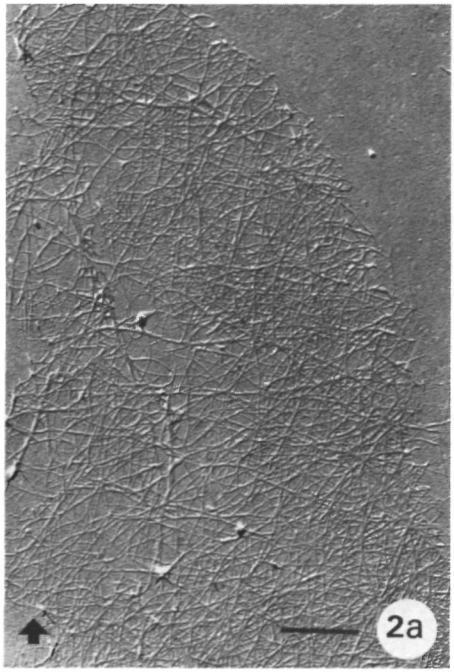
Figs. 1a,b: Microtubules in root hairs of Equisetum hyemale visualized by immunofluorescence a: control, b: hair treated with $10^{-2}$ M colchicine, during 7h. 1250x.

Figs. 2a-d: Surface views of inner cell wall of root hair of E. hyemale deposited during colchicine treatment: Fig. 2a: random texture at the hair tip. Fig. b-d: the last-deposited lamellae have different orientations according to the long axis of the hair. 20 160x, bar: 500 nm.

Figs. 3a-d: Surface views of inner cell wall of root hair of Raphanus sativus deposited during colchicine treatment: the last deposited microfibrils have different orientations. 20 240x, bar: 500 nm.

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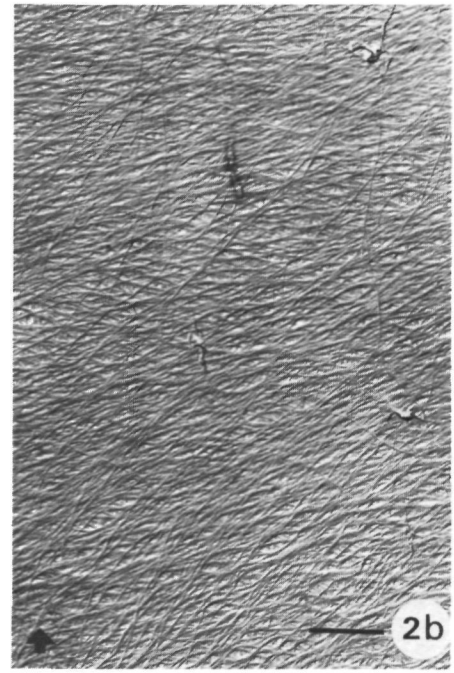
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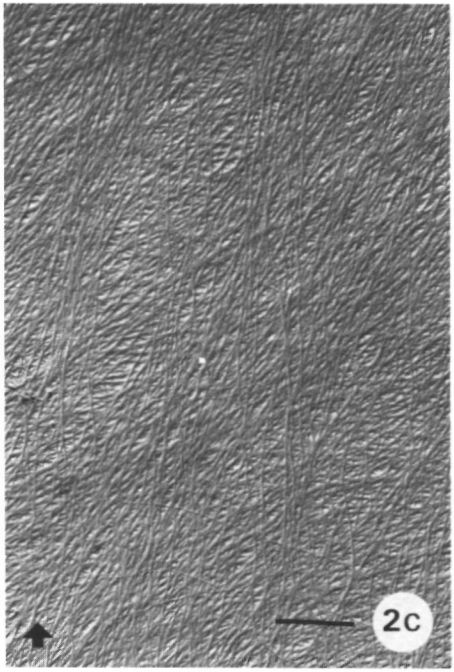
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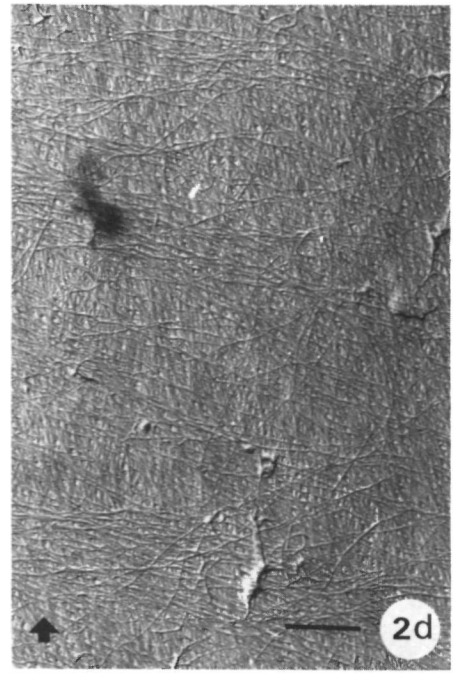
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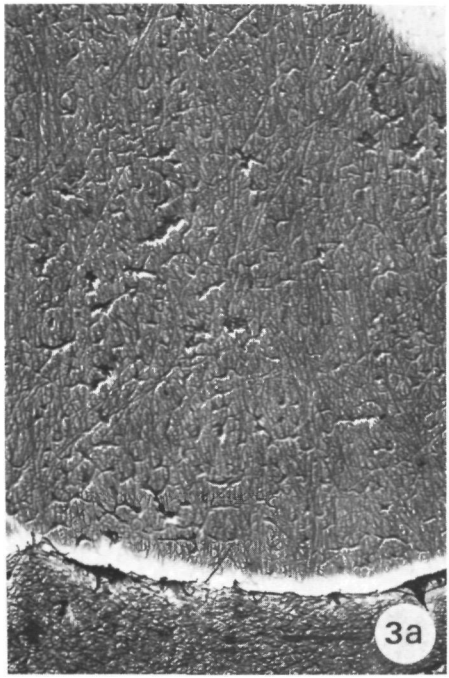
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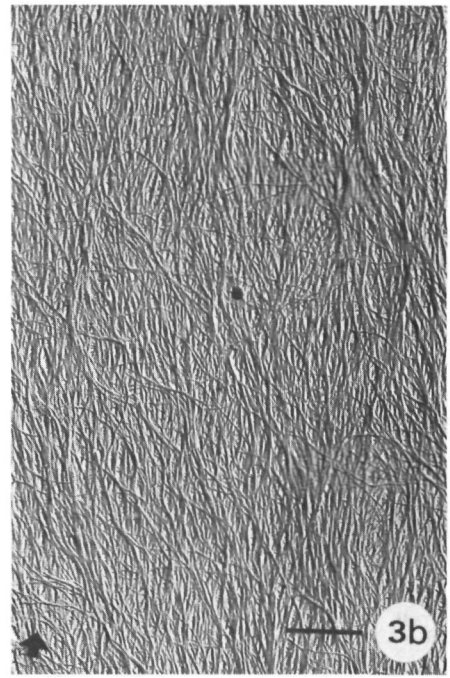
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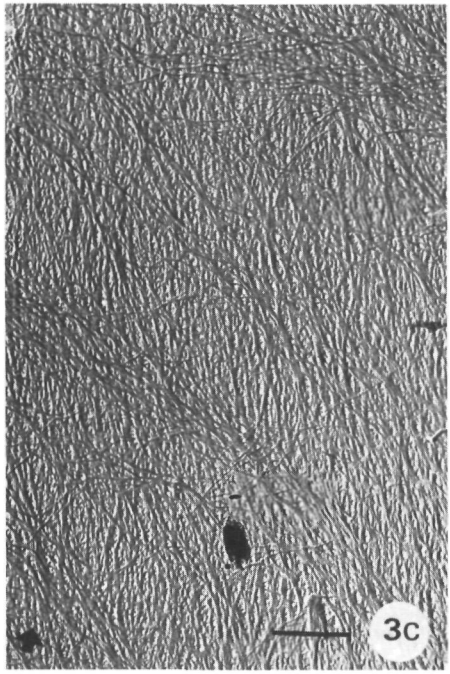
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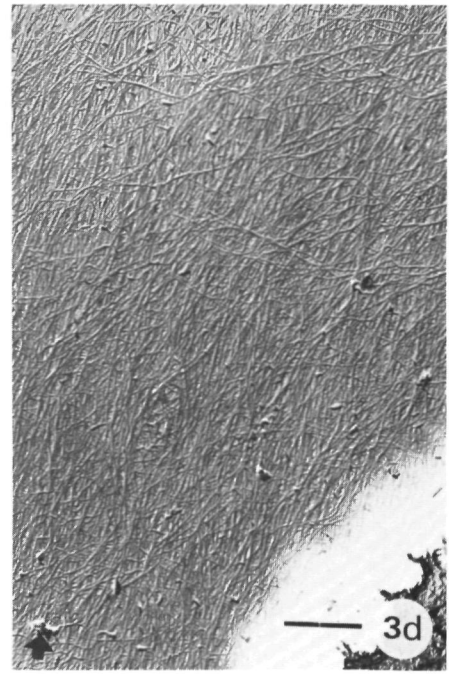
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The cell wall texture of root hairs of *Equisetum hyemale* deposited during colchicine treatment was clearly helicoidal (Fig. 2 b,c,d), with a random texture at the hair tip (Fig. 2a). The microfibrils in the last-deposited lamella showed different orientations at different places along the hair, as is found in control preparations (this thesis, chapter 5). The rotation mode of the helicoid is counterclockwise. The angle between microfibrils in adjacent lamellae, 30° to 40°, did not differ significantly from the control.

The effect of colchicine, in the same concentrations, on microtubules of *Raphanus sativus* root hairs was similar, though less prominent, as on *Equisetum. hyemale* root hairs. More microtubules remained after treatment.

However, the generic axial microfibril deposition pattern changed: microfibrils deposited in different orientations (Figs. 3 a,b,c,d) and wall texture became helicoidal-like.

## DISCUSSION

It has been proposed that helicoidal microfibril deposition is controlled by shifting microtubular helices (Roberts et al. 1985), or alternatively originates from self-assembly like in liquid crystals (Neville 1986).

The concentration of colchicine used did not result in a complete loss of cell polarity, though hair width increased. It has been postulated that the pattern of microfibril deposition is related to the shape of the cell (Takeda and Shibaoka 1981) and that the helical angles of various wall layers correlate with cell dimensions (Preston 1974).

A mathematical model has been worked out according to which a helicoidal wall can be formed without any orientating force of microtubules (Emons 1986). In this model the wall texture is determined by cell shape, polysaccharides adherent to microfibrils and density of microfibril initiation points (Emons 1986, this thesis chapter 12). The model predicts that, given a root hair with axial wall texture, sufficient increase in cell diameter will result in the formation of a helicoidal wall texture.

The colchicine treatment we applied to root hairs resulted in depolymerization of microtubules, increase in hair width, persistence of a helicoidal cell wall texture in *Equisetum hyemale*, but deposition of microfibrils in oblique and transverse orientations in *Raphanus sativus* root hairs.

Further research, especially by means of thin-sectioning, is needed to establish the nature of the cell wall of root hairs of *Raphanus sativus* deposited after colchicine treatment. We therefore present these results as preliminary.

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# CHAPTER 8

## Plasma-Membrane Rosettes in Root Hairs of Equisetum Hyemale

Planta (1985) 163:350–359

Planta
© Springer Verlag 1985

# Plasma-membrane rosettes in root hairs of *Equisetum hyemale*

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**Abstract.** Particle arrangement in the plasma membrane during cell wall formation was investigated by means of the double replica technique in root hairs of *Equisetum hyemale*. Particle density in the protoplasmic fracture face of the plasma membrane was higher than in the extraplasmic fracture face. Apart from randomly distributed particles, particle rosettes were visible in the PF face of the plasma membrane. The rosettes consisted of six particles arranged in a circle and had an outer diameter of approx. 26 nm. No gradient in the number of rosettes was found, which agrees with microfibril deposition taking place over the whole hair. The particle rosettes were found individually, which might indicate that they spin out thin microfibrils as found in higher-plant cell walls. Indeed, microfibril width in these walls, measured in shadowed preparations, is 8.5±1.5 nm. It is suggested that the rosettes are involved in microfibril synthesis. Non-turgid cells lacked microfibril imprints in the plasma membrane and no particle rosettes were present on their PF face. Fixation with glutaraldehyde caused, probably as a result of plasmolysis, the microfibril imprints to disappear together with the particle rosettes. The PF face of the plasma membrane of non-turgid hairs sometimes showed domains in which the intramembrane particles were aggregated in a hexagonal pattern. Microfibril orientation during deposition will be discussed.

**Key words:** Cellulose microfibril – *Equisetum* (root hair) – Plasma membrane rosette

## Introduction

Synthesis and ordered deposition of microfibrils in the cell wall is one of the important events determining plant cell morphogenesis. The hypothesis that microfibrils are synthesized by enzyme complexes in the plasma membrane was formulated by Preston (1974). The putative enzymes, however, have only seldom and poorly been revealed in ultrathin sections (Mueller and Brown 1980, Olesen 1980). The freeze-fracture technique has allowed the visualization of a morphologically recognizable complex, embedded in the plasma membrane, presumed to represent the cellulose-synthesizing enzyme (for review see Mueller 1982). The putative synthesizing structure manifests itself on the extraplasmic fracture face (EF) of the plasma membrane as a globular, approximately 25 nm wide terminal complex (Mueller et al. 1976) and on the protoplasmic fracture face (PF) of the plasma membrane as a particle rosette (Mueller and Brown 1980), consisting of six circularly arranged intramembranous particles. Rosettes measure about 24 nm in diameter, their constituting particles 8 nm (Mueller and Brown 1980). Since, in fully turgid cells, they are attached to the most recently deposited microfibrils (Mueller 1982), the complexes might spin out microfibrils into the wall as they move through the membrane (Lloyd 1980).

Single particle rosettes have been identified in protonemata of the fern *Adianthum* (Wada and Stachelin 1981), in corn, bean and pine seedlings (Mueller and Brown 1980), during primary wall formation in *Micrasterias* (Giddings et al. 1980) and, recently, in *Funaria* caulonema tip cells (Reiss et al. 1984). Hexagonal arrays of rosettes have been found during secondary wall deposition in the algae *Micrasterias* (Giddings et al. 1980), *Closterium* (Stachelin and Giddings 1982), and *Spirogyra* (Herth 1983). In contrast, the PF of plasma membranes of isolated cell-wall-regenerating plant protoplasts from different origin exhibit arrays of closely hexagonally packed particles (Robenek and Peveling 1977, Davey and Mathias 1979, Schnabl et al. 1980, Wilkinson and Northcote 1980).

*Abbreviations*: EF = extraplasmic fracture face; PF = protoplasmic fracture face

Previous studies on the *Equisetum hyemale* root hair (Emons 1982, Emons and Wolters-Arts 1983) have shown that in young growing hairs, as well as in full-grown hairs, microfibrils are deposited along the whole hair tube and that the cortical microtubules and the nascent microfibrils are not in parallel. Thus it was concluded that the microtubules cannot exert, at least not by their direction, control of microfibril orientation.

The present study was undertaken to investigate the membrane-mediated control of microfibrillar order in cell walls of *E. hyemale* root hairs.

## Material and methods

Stem cuttings of *Equisetum hyemale* (L.), cultivated in the botanical garden containing several nodes, were grown in wide test-tubes in tap water, at room temperature under normal light conditions. This study deals foremost with young growing hairs but full-grown hairs are also considered. For freeze-fracturing, a monolayer of root hairs, still attached to the root and remaining under water, was placed between two thin copper specimen holders. Without any prior treatment with fixative or cryo-protectant, root hairs were rapidly frozen by two jets of liquid propane, one on either side, in a cryojet (CF D 101 Balzers Liechtenstein), and transferred to liquid nitrogen for storage. Other samples were fixed in 2% gluteraldehyde in cacodylate buffer (0.2 M, pH 7.2) for 1 h and processed in the same way. Keeping them under liquid nitrogen, specimens were transferred to the hinged double-replica device (Balzers) and transferred to the specimen stage. A Balzers freeze-etch apparatus was used at −108° C. The material was etched for 30 s, 60 s or 2 min, shadowed with platinum and carbon and coated with carbon at an initial vacuum of $3 \cdot 10^{-6}$ Torr. Replicas were cleaned with 40% chromic acid, washed in aqua bidest and mounted on formvar-coated copper grids. The replicas were examined and photographed with the model EM 201 electron microscope (Philips, Eindhoven, The Netherlands). The terminology of Branton et al. (1975) is used for designating the fracture faces.

## Results

*1 The freeze-fracture technique.* Although initially designed for monolayers of unicellular organisms, propane-jet-freezing proved to be very useful with these cells of a higher plant, since the hairs could be spread in a monolayer on the copper plate, while the root itself rested in the groove between the copper plates. The fracture plane seldom went through the inner part of the cell, which would have exposed the different cell organelles, never through the cortical cytoplasm so as to disclose the microtubules, often the fracture plane was through the wall only and many cells fractured within the plasma membrane. Etching for 2 min uncovered the microfibrils, as in Fig. 1 and 3. The plasma membrane, however, was better preserved with an etching time of 30 s. By etching for 60 s the membranes were well preserved and the adjacent wall texture could be observed too.

*2 The cell wall.* The architecture of the cell wall, observed by freeze-fracturing, was similar to that observed by shadow-casting and thin-sectioning (Fig. 1). The outer primary wall, deposited in the growing tip, has randomly arranged microfibrils against which, in the lateral wall of young hairs, a helicoidal microfibril texture is deposited from approx. 300 µm behind the growing tip onwards. Figure 2 shows that lamellae with a transverse microfibril orientation also occur in the helicoidal wall. Later, in full-grown hairs, an additional inner helically oriented layer is deposited, starting at the base of the hair (Emons and Wolters-Arts 1983). The transition from random to helicoidal is very gradual (data not shown). In freeze-fracture micrographs, microfibrils of the cell wall of *E. hyemale* measured 8.5 ± 1.5 nm in width. Microfibril width was measured with a calibrated 10× ocular on microfibrils lying perpendicular to the direction of shadowing from replicas of 50 different, 2-min-etched, cells with a magnification ranging from 30000 to 70000× (Fig. 3).

*3. Particle rosettes in turgid hairs.* Replicas of turgid hairs were obtained from young, untreated, growing hairs. Figure 4a shows the EF adjacent to the helicoidal part of the wall with imprints – seen as elevations on the EF – of wall microfibrils in two superimposed differently oriented lamellae. Terminal globules were only seldom observed (Fig. 4b).

Protoplasmic fracture faces of the plasma membrane of *E. hyemale* root hairs clearly reveal intramembrane particle rosettes (Fig. 4c, d), consisting of six intramembrane particles arranged in a circle. Microfibril imprints were either slight (Fig. 4c) or obvious (Fig. 4d), in the latter case rosettes were sometimes seen at the ends of microfibril imprints. In a high magnification of the PF of the plasma membrane, the helicoidal nature of the adjacent wall does not appear clearly because, between the interspaces of microfibril imprints of the last-deposited lamella, imprints of the previous lamellae show up. The density of the rosettes varied from 5 to 15 $\mu m^{-2}$. The diameter of the rosettes, measured from the edges of particles on either side, was 26 ± 5 nm. The large variation in rosette diameter appears to be independent of shadowing characteristics and of the size of the constituting particles, but to depend rather on the compaction of the particles of a rosette. The constituting particles are either more or less tightly packed as if

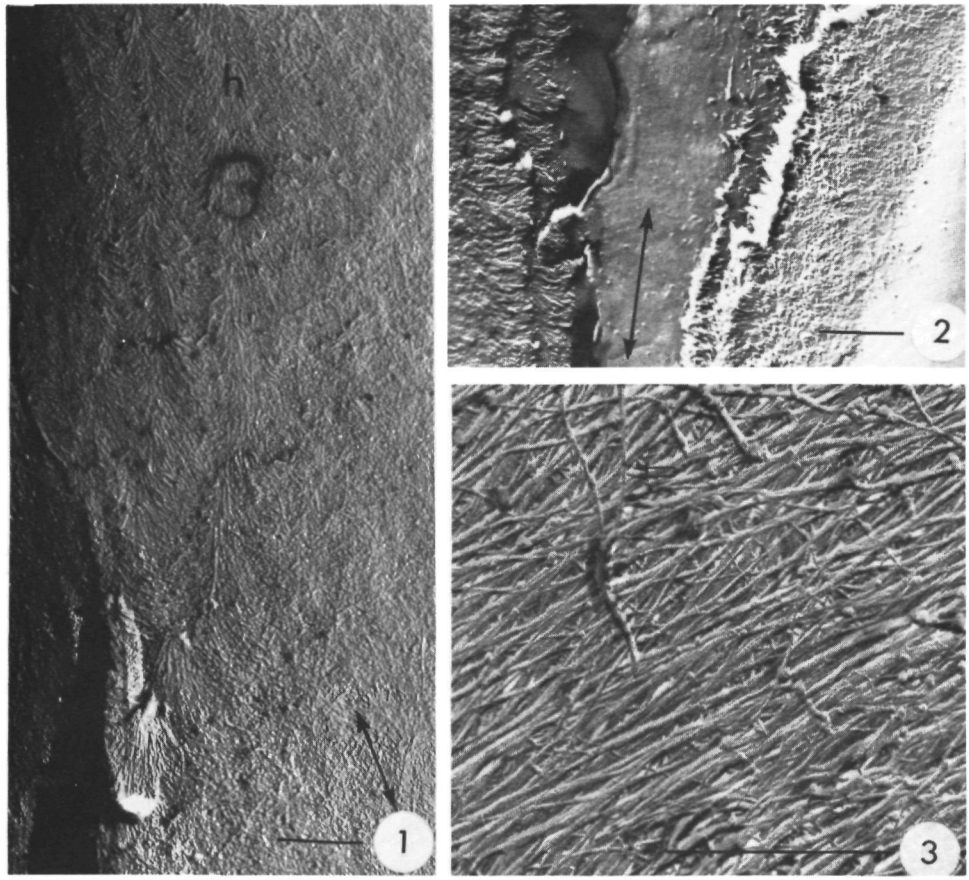



**Fig. 1.** Oblique fracture through the cell wall of a full-grown root hair of *Equisetum hyemale*, showing the dispersed texture of the outer layer (*d*), three parabolae of the helicoidally textured layer (*h*), the helically arranged microfibrils of the inner layer (*l*). *Arrow* indicates long axis of the hair; 2-min etch; ×23000

**Fig. 2.** Lengthwise slit-open cell wall of young *Equisetum hyemale* root hair, showing several microfibril lamellae, some of them transverse to the hair. *Arrow* indicates long axis of the hair; 60-s etch; ×22000

**Fig. 3.** Oblique fracture through cell wall of *Equisetum hyemale* root hair, showing two microfibril lamellae of the helicoidal part of the wall. Microfibril width was measured on microfibrils (*double arrow*) lying in the direction of shadowing; 2-min etch; ×60200. *Encircled arrows* indicate the direction of shadowing; bars = 0.5 μm

rosettes are aggregating or disintegrating. Figure 5 shows micrographs of differently sized particle rosettes, taken from one and the same hair. Rosettes are randomly distributed over the membrane. They are not arranged in distinct clusters or in rows and differently shaped rosettes are randomly interspersed.

Only rarely did the hair fracture through the cap proper. Figure 6a shows an EF near to the hemisphere with microfibril imprints in random di-

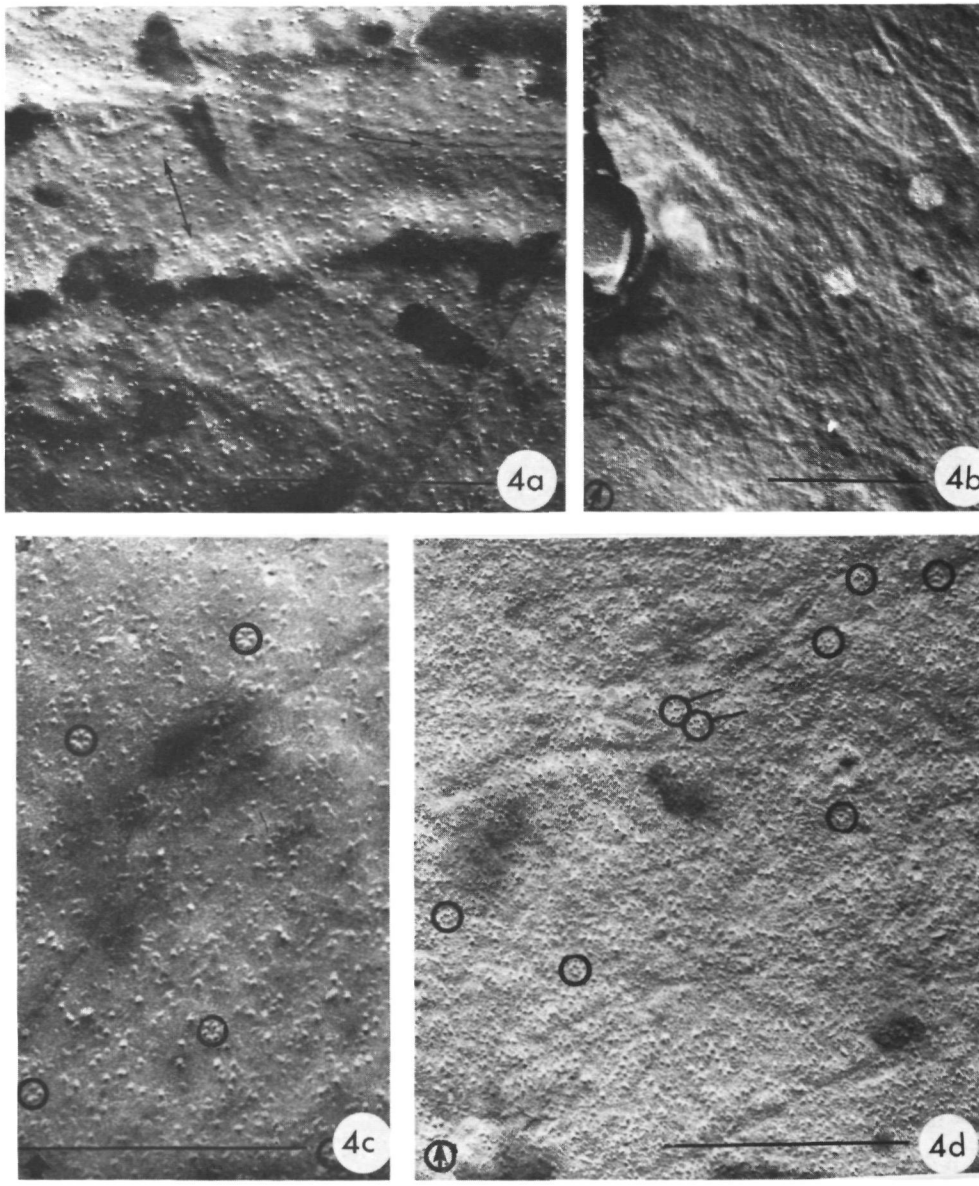



**Fig. 4a–d.** Plasma membrane against helicoidal wall of young untreated root hair of *Equisetum hyemale*. **a** The EF with microfibril imprints of two subsequent lamellae (*arrows*); 60-s etch; ×64400. **b** The EF with microfibril imprints and some terminal globules at the ends of imprints (*arrows*); 60-s etch; ×42000. **c** The PF with slight microfibril imprints and with particle rosettes (26±5 nm in diameter); 60-s etch; ×96600. **d** The PF with clear microfibril imprints and particle rosettes sometimes at one end of an imprint; 60-s etch; ×64400. *Encircled arrows* indicate the direction of shadowing; bars = 0.5 μm

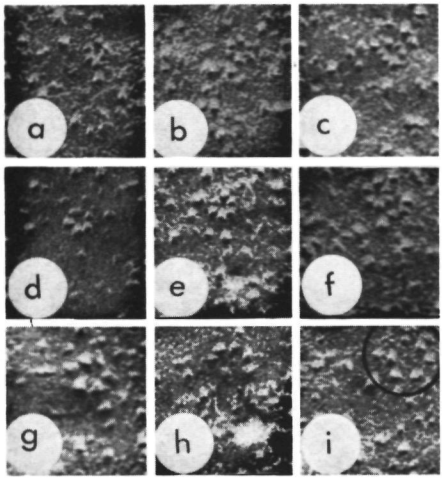
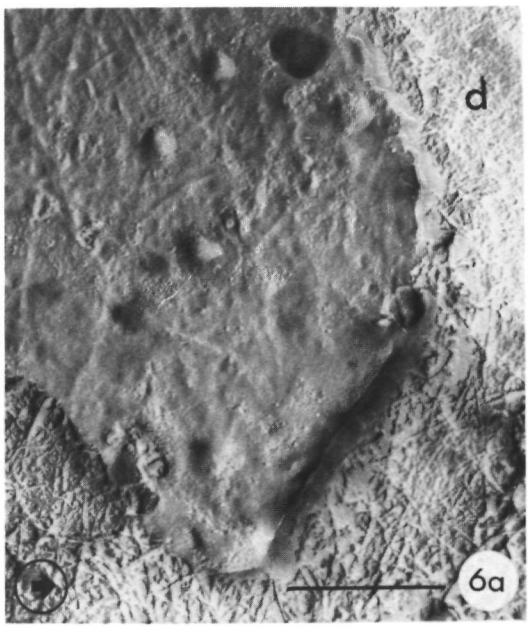



**Fig. 5a–i.** Particle rosettes of different sizes, taken from one and the same hair; the particle arrangement is as in Fig. 10. The arrangement in *i* is possibly accidental and these rosettes were not, therefore, included in rosette-diameter measurement; 60-s etch; *encircled arrow* indicates the direction of shadowing; bar = 0.5 μm; × 147000

**Fig. 7a, b.** Plasma membrane of young untreated non-turgid root hair of *Equisetum hyemale*. **a** The EF with regular intramembrane-particle distribution and no microfibril imprints, taken as proof that the cell is not turgid; 30-s etch; × 64400. **b** The PF with regular intramembrane particle distribution, no microfibril imprints and no rosettes; 30-s etch; × 64400

**Fig. 8a, b.** Plasma membrane of full-grown untreated root hair of *Equisetum hyemale*. **a** The EF with regular intramembrane particle distribution and no microfibril imprints; 30-s etch; × 64400. **b** The PF with regular intramembrane particle distribution, without microfibril imprints and without particle rosettes; 30-s etch; × 64400

**Fig. 9a, b.** Plasma membrane of young glutaraldehyde-fixed root hair of *Equisetum hyemale* with less-regular particle distribution than in unfixed hairs. **a** The EF without microfibril imprints; 30-s etch; × 64400. **b** The PF without microfibril imprints and without particle rosettes; 30-s etch; × 64400. *Encircled arrows* indicate the direction of shadowing; bars = 0.5 μm



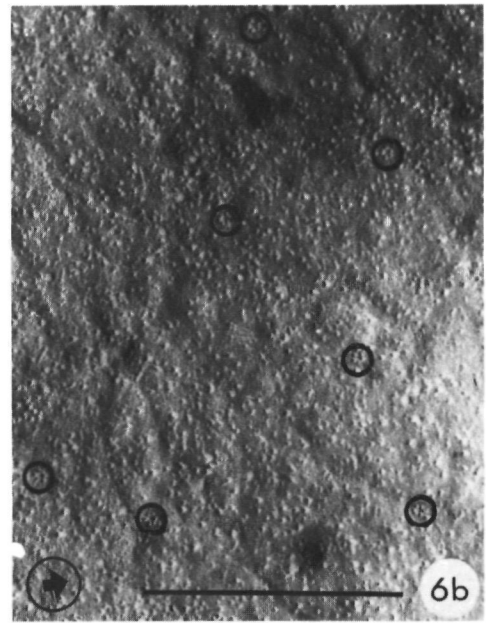
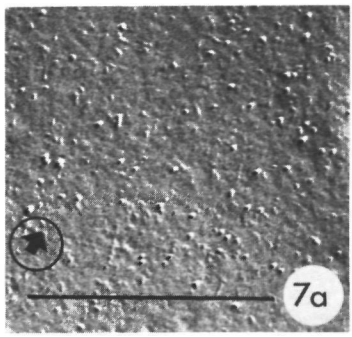




**Fig. 6a, b.** Plasma membrane against randomly textured wall of the tip near the hemisphere of an untreated young *Equisetum hyemale* root hair. **a** The EF of a turgid hair with microfibril imprints in random directions, lying on the dispersed microfibrils of the primary cell wall. *d*, Near-transverse fracture of this wall; *arrow*, long axis of the hair; 60-s etch; × 42000. **b** The PF of a turgid hair with microfibril imprints in random directions and with particle rosettes; 60-s etch; × 70000. *Encircled arrows* indicate the direction of shadowing; bars = 0.5 μm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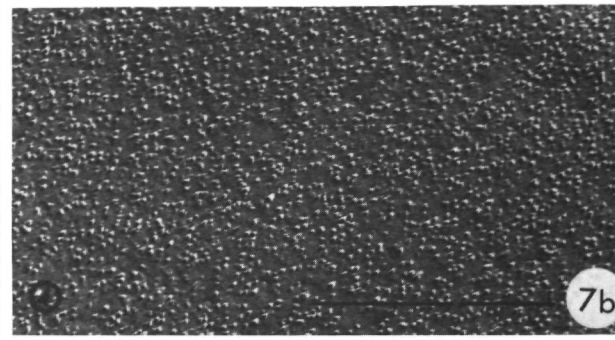
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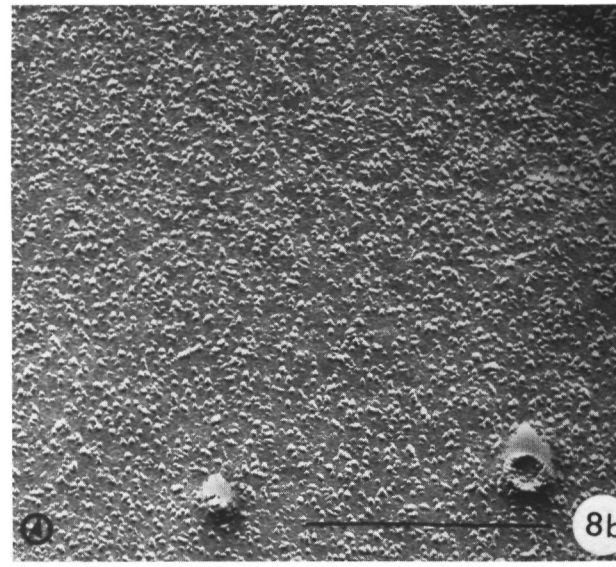
8a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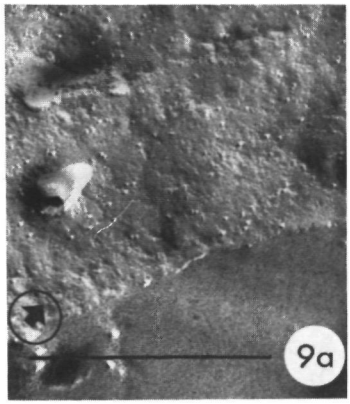
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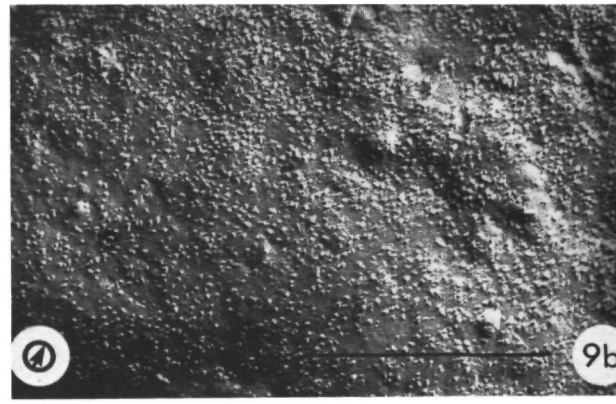
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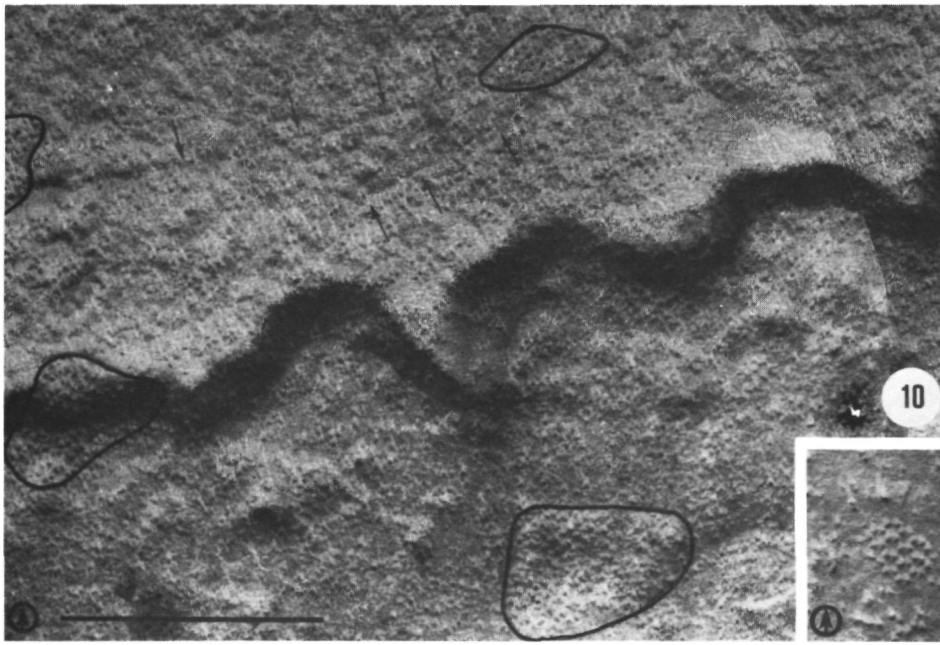


**Fig. 10.** The PF of the plasma membrane against a randomly textured wall near the hemisphere of the tip of an untreated young root hair of *Equisetum hyemale*. In the upper part of the micrograph, the plasma membrane is appressed to the microfibrils of the cell wall and shows microfibil imprints (*arrows*): the lower part of the plasma membrane is retracted from the wall and has no microfibril imprints. Both parts as well as the transition area have hexagonal particle arrays (*encircled areas*). 60-s etch; × 70000. *Inset*: a clear hexagonal particle array, 60 s etch, × 70000. *Encircled arrow* indicate the direction of shadowing; bar = 0.5 μm

rections. The PF of the plasma membrane in this area shows a pattern and density of particle rosettes (Fig. 6b) similar to those found in the area against the helicoidal wall.

*4. The plasma membrane of non-turgid and glutaraldehyde-fixed hairs.* The absence of microfibril imprints on the plasma membrane was taken as proof that the plant cell was not fully turgid, either physiologically or artefactually (Fig. 7a). On the PF of the plasma membrane of non-turgid hairs, particle rosettes were absent (Fig. 7b). Note that, as is common in plant cells, the EF has fewer intramembranous particles than the PF. Possibly as a consequence of their length and fragility, full-grown hairs were never observed to be turgid by means of this freezing method, which may account for the absence of particle rosettes in plasma membranes of full-grown hairs (Fig. 8a: PF, 8b: EF).

Also, young hairs fixed in glutaraldehyde in buffer showed no microfibril imprints, and particle rosettes were not seen on the PF of the plasma membranes (Fig. 9a: PF, 9b: EF). Furthermore, particles were less regularly distributed in fixed hairs. Amongst the usually randomly distributed particles of the PF of the plasma membrane of young unfixed hairs, membrane domains showing a regular hexagonal array of particles were sometimes interspersed. The hexagonal particle arrays were seen in non-turgid cells and in cells which were in the process of loosing turgidity, that is, in which the plasma membrane was still partly appressed to the wall microfibrils but had in another part retracted from the cell wall (Fig. 10).

## Discussion

The idea that microfibrils are synthesized at the plasma membrane has recently been corroborated by work on freeze-fractured membranes adjacent to cellulosic cell walls (for review see Mueller 1982). Indications have been found that specialized particle rosettes play a role in microfibril synthesis. The presence of such rosettes is confirmed now for young growing root hairs of *E. hyemale* by freeze-fracturing of unfixed, uncryoprotected, rapidly frozen hairs.

*Particle rosettes in the plasma membrane.* In the *E. hyemale* root hair, the density of rosettes is the same as has been found in other studies (approx. 10 $\mu m^{-2}$) (Mueller 1982). Recently, Herth (1984 lecture "Third Cell Wall" Meeting, Fribourg) has found 160 rosettes $\mu m^{-2}$ in cress xylem cells. The difference may be explained by a difference in turn-

over rate or, more probably, by a difference in proximity of the nascent microfibrils Also from dry-cleaved preparations (data not shown) it can be deduced that there may be a considerable distance between nascent microfibrils

In *Adianthum* (Wada and Staehelin 1981) and in the cells of higher plants investigated by Mueller (1982) the particle rosettes in the PF of the plasma membrane do not form rows or any geometrical pattern In the PF of the plasma membrane against the secondary wall of some algae, hexagonal patterns of rosettes have been found in *Micrasterias* (Giddings et al 1980), *Closterium* (Staehelin and Giddings 1982) and *Spirogyra* (Herth 1983) Giddings et al (1980) suggest that each of the rosettes accounts for one thin microfibril as found in higher-plant cell walls, whereas rows of rosettes would lead to lateral aggregation of microfibrils into the wider microfibrils of these algae Microfibrils in the cell wall of *E hyemale* are $8\,5 \pm 1\,5$ nm wide, measured in shadow cast preparations, as is common in higher plants (for discussion of microfibril width and microfibril width measurement see Preston 1974)

Herth (1983) presumes that each of the six units of a rosette might synthesize six glucan chains, which then co-crystallize into a 36-glucan-chain elementary fibril Microfibrils in *Equisetum* root hairs are $8\,5 \pm 1\,5$ nm in diameter One rosette might synthesize one crystalline core, the width of an elementary fibril which is then surrounded by a paracrystalline cortex, consisting of an admixture of cellulose and hemicellulose (Preston 1974) In the wider fibrils of *Spirogyra* (Herth 1983) this cortex possibly surrounds the whole wide fibril, but not the constituting elementary fibrils

However, since the biochemical analysis of the microfibril synthesizing complex has not yet been accomplished, the synthetic activities of the particle rosettes remains a matter of speculation Rosettes might also represent condensation centers, crystallizing the already-synthesized glucan chains, or extrusion sites through which the cellulose precursors are excreted or as suggested by Willison (1983), rosettes might function in fixing enzymes in the membrane or in connecting microfibrils to guiding structures Willison (1983) argues that the rosettes are not the enzyme complexes for microfibril biosynthesis and assembly because their density in root tissues and cotton hairs is lower than that of terminal globules and because they are not always microfibril-terminal In the *E hyemale* root hairs, however terminal globules in the EF of the plasma membrane were seldom seen, while rosettes in the PF occurred with a frequency of $5\ 15\ \mu m^{-2}$

In addition the individual glucan chains will not leave imprints on the membrane only after crystallization of the glucan chains at the outside of the plasma membrane will a microfibril imprint be visible Indeed, experiments with calcofluor white and congo red have shown that the steps of polymerization and crystallization are not simultaneous (Haigler et al 1980, Herth 1980, Herth and Hausser 1984)

*Turgidity and presence of particle rosettes* Mueller (1982) has already emphasized the importance of cellular turgor for visualizing the membrane complexes In the root hairs of *E hyemale* turgidity was indeed required Also, cells fixed in glutaraldehyde were never observed in their turgid state and displayed no particle rosettes

The absence of particle rosettes in other plant tissues (Crevecoeur et al 1982, Sjolund and Shih 1983) is probably a consequence of gluteraldehyde fixation The particle aggregates seen in fixed, freeze-fractured cress seedlings (Volkmann 1983) show a different morphology from the particle rosettes shown here

So far, particle rosettes have not been observed in full-grown hairs of *E hyemale*, which are known to continue microfibril deposition (Emons and Wolters-Arts 1983) Full-grown hairs, however, were never turgid, and thus, like young non-turgid hairs, lacked particle rosettes The full-grown hairs were longer than the copper plates between which they were sandwiched and therefore more susceptable to mechanical damage than the younger shorter hairs Whether non-turgid hairs can synthesize microfibrils is not known For *Tradescantia*, microfibril deposition in plasmolysed root hairs has been reported (Schroter and Sievers 1971)

In young non-turgid hairs and in hairs engaged in the process of loosing turgidity, hexagonal arrays of particles occur Similar arrays have been found in microfibril-forming protoplasts isolated from higher plants (for review see Willison and Klein 1982) These particle arrays have been argued to function in microfibril synthesis (Robenek and Peveling 1977), in solute transport (Schnabl et al 1980) or to have no function at all, being plasmolysis-induced (Wilkinson and Northcote 1980) Indeed, the absence of microfibril imprints reveals the non-turgidity of the wall-regenerating protoplasts It is quite conceivable that the uncoupling of the plasma membrane from the cell wall leads to alterations in the arrangement of particles within the membrane, in which case, the hexagonal array would be a stress phenomenon Recently similar hexagonal particle arrays have been found

in glutaraldehyde- glycerol- or sucrose pretreated *Lepidium* root hairs (Volkman 1984)

*Control of microfibril orientation* The cytoplasmic agents governing microfibril deposition are not yet known Cortical microtubules have been proposed to control microfibril orientation (for review see Robinson and Quader 1982) In *E hiemale* root hairs however the microtubules are not in parallel with the nascent microfibrils (Emons and Wolters Arts 1983) Recently a discrepancy in orientation between microtubules and nascent microfibrils has also been reported for the alga *Valonia* (Itoh and Brown 1984)

If particle rosettes represent the cellulose-microfibril-synthesizing enzymes their movement in the plasma membrane determines microfibril orientation The energy for the propulsion of the rosettes might be delivered by the formation of the microfibril itself (compare Herth 1980) and if no forces from the cell interfere propulsion will continue in the same direction The propulsion of the rosettes however is influenced by the medium the fluid membrane The directed lateral mobility of molecules within the plasma membrane is currently attributed to the actin-myosin filaments of the cytoskeleton possibly conducted by microtubules (Berlin et al 1979 Heath and Seagull 1982) Plasma-membrane-bound filaments have recently been revealed in plant cells (Traas 1984) Microfibril orientation thus would be subjected to two forces the kinetic force of fibril formation and the cell directed movement of the plasma membrane (compare Mueller 1982) Microfibril orientation then will be the resultant of these two forces and need not lie in the direction of the microtubules Therefore with the present assumption that the force generated by the microtubules is insufficient in some instances to channelize the microfibrils the discongruity in microtubule microfibril orientation can be explained Whether or not a helicoidal wall can thus be made is under further investigation

I am greatly indebted to Professor M M A Sassen and I thank Dr J W M Derksen for helpful discussions I also thank Professor W Herth (Zellenlehre Universitat Heidelberg FRG) for reading of the manuscript and for providing useful criticisms

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Received 2 June accepted 25 August 1984

# CHAPTER 9

# Hexagonal Patterns of Particles in the Plant Plasma Membrane

# HEXAGONAL PATTERNS OF PARTICLES IN THE PLANT PLASMA MEMBRANE

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## ABSTRACT

Intramembranous particles in the plasma membranes of pollen tubes and generative cells of pollen grains of Lilium longiflorum and of root hairs of Equisetum hyemale and Lepidium sativum were studied by means of freeze-fracturing. Two hexagonal particle configurations were observed in these higher plant cells: a single rosette, containing six particles and a regular pattern of hexagonally ordered particles. The hexagonal pattern differs from the hexagonal pattern known to occur in yeast cells. The hexagonal pattern in yeast is one of the five Bravais-lattices described in crystallography. It is proposed to use the term "honeycomb pattern" for hexagonal patterns in higher plant cells to differentiate between the two types of hexagonal patterns.

It is hypothesized that the honeycomb particle configuration functions in transport of solutes through the membrane, the single rosette constituting a channel for transport of microfibril precursors.

KEY WORDS: freeze-fracture, hexagonally ordered particles, plant cell, plasma membrane rosettes

## INTRODUCTION

Freeze-fracturing has disclosed a variety of particle patterns in plasma membranes. Plasma membranes of plant cells exhibit randomly dispersed intramembranous particles (IMPs), unequally distributed between the protoplasmic (PF) and extraplasmatic (EF) fracture faces. However, IMPs occur also in specific patterns: a) particle rosettes (Emons 1985, review: Brown 1985), b) hexagonal arrays of rosettes (Giddings et al. 1980, Herth 1983, Hogetsu 1983); c) hexagonally ordered particles (Willison and Klein 1982, Volkmann 1984, Emons 1985, Kroh and Knuiman 1985); d) hex-

agonal patterns such as found in yeast cells (Moor and Mühlethaler 1963); e) granule bands (Robinson and Preston 1972, Brown and Montezinos 1976) and f) parallel strings of particles (Robenek and Peveling 1977).

Particle rosettes (a), hexagonal arrays of particle rosettes (b) and granule bands (e) have been hypothesized to function in microfibril synthesis (review: Brown 1985). Two functions have been attributed to the hexagonally ordered particles (c): microfibril synthesis (Robenek and Peveling 1977, Volkmann 1984) and solute transport (Schnabl et al. 1980, Kroh and Knuiman 1985). It has, however, also been hypothesized that hexagonally ordered particles are an effect of plasmolysis (Wilkinson and Northcote 1980, Emons 1985).

Volkmann (1984) reported the presence of hexagonally ordered particles in *Lepidium* root hairs and the absence of particle rosettes in this plant material during active growth and microfibril synthesis. In *Equisetum* root hairs, however, particle rosettes were regularly observed, hexagonally ordered particles were seen during retraction of the plasma membrane from the cell wall (Emons 1985).

To investigate whether the different particle patterns are related, we studied intramembranous particle distribution in the plasma membranes of: *Lilium longiflorum* pollen tubes and generative cells of *Lilium longiflorum* pollen grains, *Lepidium sativum* root hairs and *Equisetum hyemale* root hairs.

## MATERIAL AND METHODS

### Plant material:

Pollen grains and tubes of Lilium longiflorum were obtained as described for tobacco by Kroh and Knuiman (1985). Root hairs of Equisetum hyemale were obtained from stem cuttings grown as described by Emons and Wolters-Arts (1983). Lepidium sativum seeds were sown in petri-dishes in moist air. After germination, they were grown in small dishes with bidest. Root hairs grown in water were examined.

### Methods:

Freeze-fracturing of pollen grains and tubes was as described by Kroh and Knuiman (1985) and of root hairs as described by Emons (1985).

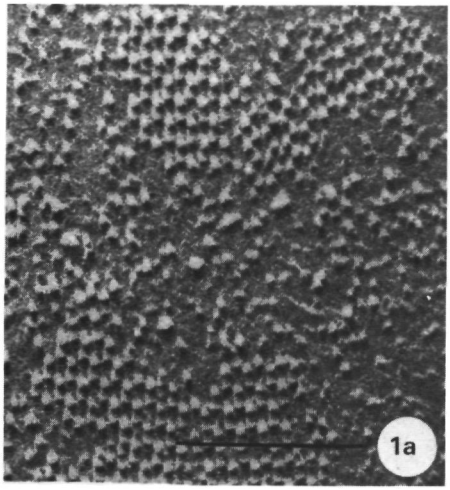
*The freeze-fractured membrane*

Apart from regions with randomly scattered single particles, the PF-face of the plasma membrane of lily pollen tubes shows hexagonally ordered particles (Fig. 1a). Sometimes imprints of hexagonally ordered particles are visible on the EF-face of the plasma membrane (Fig. 1b), which proves that they really exist in the intact membrane. In the lily pollen tube single particle rosettes also occur on the PF-face of the plasma membrane. The generative cell is enclosed by two membranes: the plasma membrane of the generative cell itself and the plasma membrane of the vegetative cell. The PF-face of the plasma membrane of the generative cell of lily pollen grains as well as the PF-face of the plasma membrane of the vegetative cell, which surrounds the generative cell, contain hexagonally ordered particles (Figs. 2a,b).

In *Equisetum hyemale* root hairs particle rosettes were regularly observed, but hexagonally ordered particles may also occur in plasma membranes. Hexagonally ordered particles occur in regions with and without microfibril imprints (Figs. 3 a,b). Microfibril imprints are caused by a close apposition of the membrane to the cell wall microfibrils and, therefore, are seen only in fully turgid cells. Plasma membranes with hexagonally ordered particles may also contain single particle rosettes (Fig. 3c), and pairs of rosettes, sharing a pair of adjacent particles (Fig. 3d).

*Lepidium sativum* root hairs grown in bidest contained particle rosettes on the PF-face of the plasma membrane (Fig. 4); hexagonally ordered particles were seldom observed, a situation comparable to the situation in *Equisetum hyemale* root hairs.

Fig. 6a shows the particle pattern in yeast. The pattern is different from the pattern found in pollen tubes and root hairs. Figure 6b is a schematic representation of the lattice found in yeast. The basic unit of this lattice is a triangle (Fig. 6b), whereas the basic unit of the pattern found in higher plant cells is a hexagon (Fig. 7).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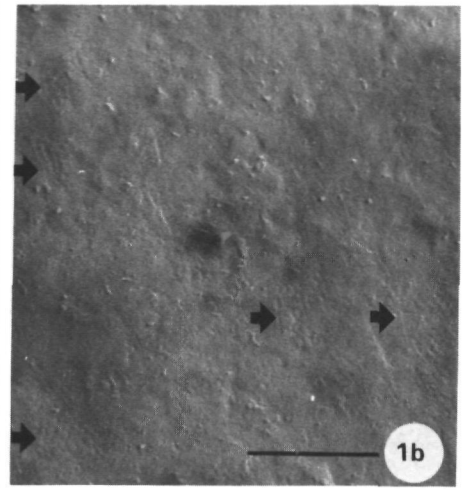
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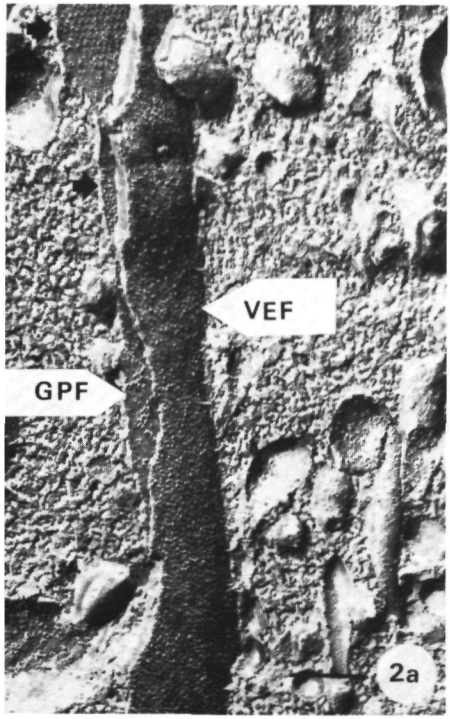
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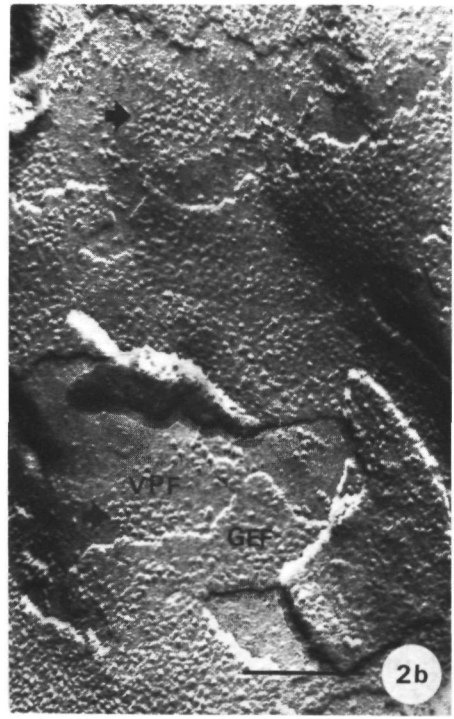

VPF
GPF
2b

Fig. 1: Plasma membrane of pollen tube of Lilium longiflorum, showing hexagonally ordered particles (honeycomb pattern) on the PF-face of the membrane (Fig. 1a) and imprints of hexagonally ordered particles (honeycomb pattern) on the EF-face of the membrane (arrows) (Fig. 1b).
Bars: 200 nm, a: x 144 000, b: x 92 200.

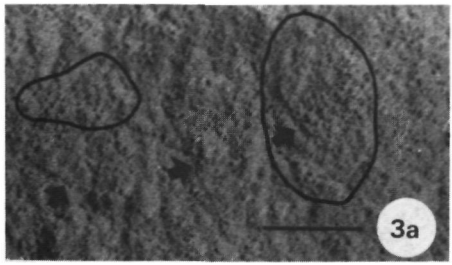
Fig. 2a: Cytoplasm of pollen grain of Lilium longiflorum. PF-face of the plasma membrane of the generative cell (GPF) containing the honeycomb particle pattern (arrows), and the EF-face of the plasma membrane of the vegetative cell (VEF). Being an EF-face, the plasma membrane of the vegetative cell has many particles.
Bar: 200nm, x 51 100.
Fig. 2b: EF-face of the plasma membrane of the generative cell (GEF) showing relatively many particles, and PF-face of the plasma membrane of the vegetative cell (VPF) showing a honeycomb particle pattern (arrow).
Bar: 200 nm, x 80 500.

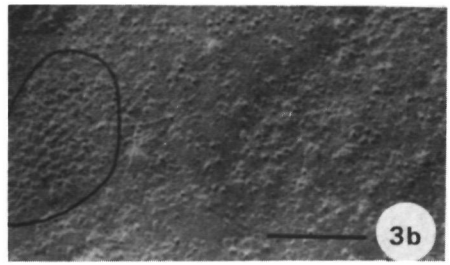
Fig. 3: PF-face of plasma membrane of root hair of Equisetum hyemale showing hexagonally ordered particles (honeycombs) in cell regions with microfibril imprints (Fig. 3a) and without microfibril imprints (Fig.3b). Figs. 3a and 3b are from one and the same cell. Single rosettes (Fig. 3c) and pairs of rosettes are present in membranes containing honeycomb patterns.
Bars: 200 nm, 3a,b: x 64 400, 3c,d: x 126 000.

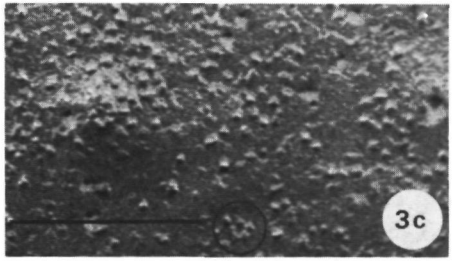
Fig. 4: PF-face of plasma membrane of root hair of Lepidium sativum, frozen in bidest, showing particle rosettes (encircled).
Bar: 200 nm, x 147 000.

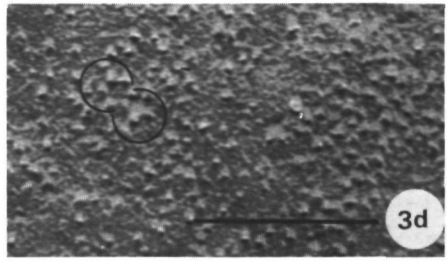
Fig. 5: EF-face of the plasma membrane of young Equisetum hyemale root hair showing a terminal globule (arrow).
Bar: 200 nm, x 64 400.

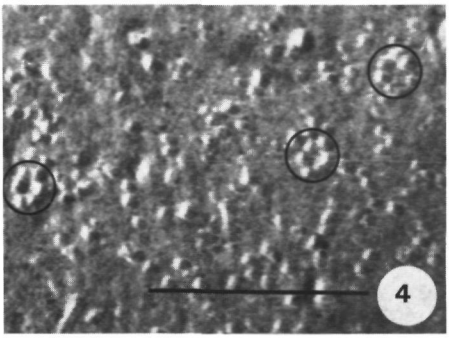
Fig. 6a: PF-face of plasma membrane of yeast, showing a hexagonal pattern of intramembranous particles (not honeycombs). Bar: 200 nm, x 64 400.
Fig. 6b: Schematic representation of the particle configuration found in the plasma membrane of yeast cells. The basic unit of this lattice is a triangle.

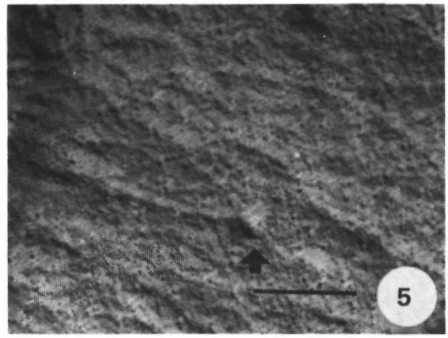


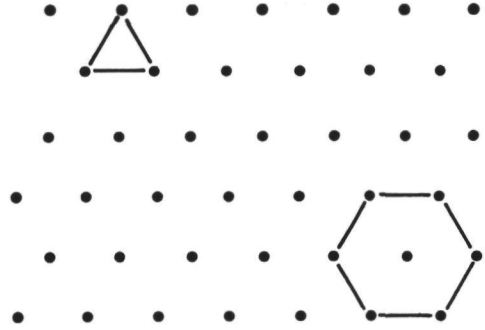







6b

*Hexagonally ordered particles*

The particle pattern found in yeast cells (Figs. 6a and b) has been called an hexagonal array of particles (Moor and Mühlethaler 1963). In this view, "hexagonal" is the term used in crystallography. The hexagonal lattice has been described as one of the five possible two-dimensional Bravais lattices. Mathematically, it is trigonal (Fig. 6b). The pattern found in higher plant cells has also been called a hexagonal particle pattern (Wilkinson and Northcote 1980). Being built of hexagons, mathematically it is a hexagonal pattern. It is, however, not one of the Bravais lattices of crystals. In crystallography, the five two-dimensional Bravais lattices constitute modes in which globular subunits pack regularly in a plane. Wilkinson and Northcote (1980) have shown that both types of hexagonal particle configurations may occur in plant protoplasts. There is need to distinguish the two patterns. We propose that the pattern found in yeast be called a hexagonal pattern, because in animal cell literature the term is widely in use for this pattern, and the pattern described in the present paper be called a honeycomb pattern.

*The honeycomb pattern*

The honeycomb pattern of particles found in lily pollen tubes has also been found in pollen tubes of tobacco (Kroh and Knuiman 1985). Reiss and coworkers found only particle rosettes in lily pollen tubes. They used short germination tubes (length= 0.5 x pollen grain diameter); we used longer tubes (length = 6 x pollen grain diameter).

Volkmann distinguished three zones in the PF-face of the plasma membrane of growing root hairs of *Lepidium sativum* with respect to the distribution and frequency of intramembranous particles. In 60% of the root hairs the plasma membrane of the vacuolation zone of the hair revealed areas of hexagonally ordered particles, honeycomb patterns. The honeycomb pattern of particles was absent from the apical and the basal zone of the hair. He hypothesized that hexagonally or-

dered particles (the honeycombs) are involved in the synthesis of cellulose microfibrils.

We, however, find particle rosettes in *Lepidium sativum* root hairs as in root hairs of *Equisetum hyemale* (Emons 1985). The difference in procedure between our preparation and Volkmann's is that we used uncryoprotected roots in bidest and Volkmann (1984) used glycerinated or sucrose incubated roots. We rarely found honeycomb patterns of particles in Lepidium and Equisetum root hairs.

Culture conditions have been argued to induce hexagonally ordered particles (honeycombs) (Wilkinson and Northcote 1980). Culture media for plant protoplasts always contain sucrose. Pollen tube culture media also contain sucrose. The germination conditions used for lily pollen tubes by Reiss and coworkers (1985) were not reported.

Pearce (1985) has found patterns of particles, which are clearly honeycombs, on PF-faces from wheat leaf bases dehydrated over 8 hours to a water content of 11% of that of the fully turgid tissue. In the same membrane, particle-free patches occurred. The particle-free patches were hypothesized to be induced by cell dehydration (Pearce 1985). No explanation was given for the occurrence of honeycomb patterns of particles.

Thus, in plant plasma membranes honeycomb patterns of particles are present if the medium contains sucrose or glycerol and during plasmolysis and dehydration.

In animal cells, particles of gap junctions have been shown to occur under both polygonal and linear form. After 10 minutes exposure to 20 mM glucose, 51% of the recognizable gap junctional particles (GJP) of isolated perfused rat pancreas were present in polygonal form (In 't Veld et al. in press). The remaining particles were in linear arrays and the total number of GJP was identical. The glucose induced formation of polygonally packed particles from linear arrays was concluded to be a reversible calcium and glucose dependent process.

Just as the linear particle array of gap junctions is easily transformed into a hexagonal array (In 't Veld in press), rosettes of plant cell membranes might easily trans-

form in single particles (Emons 1985) or in honeycomb patterns of particles and vice versa. The occurrence of rosettes with varying diameters (Emons 1985) and rosettes missing a particle (Reiss et al. 1984) have been seen to be an indication for rosette formation or disintegration.

*Particle rosettes:*

Particle rosettes have been found in a variety of plant cell membranes (review: Brown 1985), and have been regularly found in the PF-face of the plasma membrane of *Equisetum hyemale* root hairs (Emons 1985).

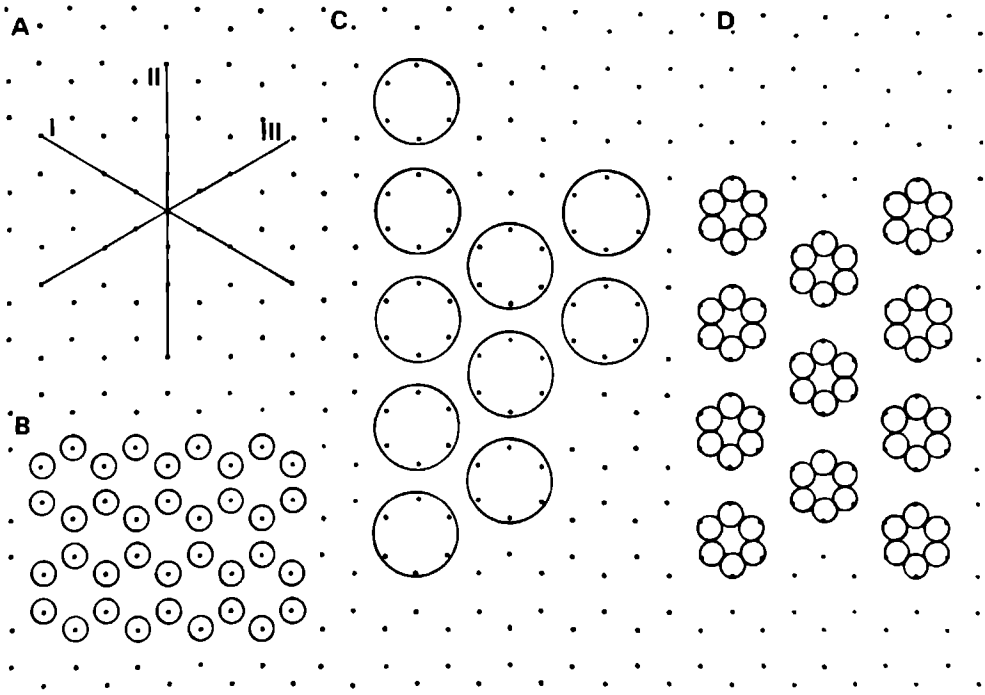
Particle rosettes have also been found in animal cells. Miller and Pinto da Silva (1977) found particle rosettes preferentially associated with the cytoplasmic leaflet (PF) of the periaxonal Schwann cell membrane. Rosettes had a diameter of approximately 13 nm and consisted of 6 particles in a hexagonal pattern comparable to the particle rosettes of plant cell membranes, which, however, measure approximately 25 nm in diameter. In the periaxonal Schwann cell often two or more rosettes were joined, sharing a pair of adjacent particles, as is also seen in the *Equisetum hyemale* root hair plasma membrane, that contains hexagonally ordered particles (Fig. 2d). The function of rosettes in the Schwann cell membrane is not clear (Miller and Pinto da Silva 1977).

*Relation between rosettes and honeycombs*

Figure 7a is a schematic representation of a honeycomb pattern, I,II and III are the symmetry axes. Fig. 7b represents the honeycomb pattern of particles, Fig. 7c shows that single particle rosettes are sufficient and necessary for the construction of this pattern, fig. 7d shows an hexagonal pattern of particle rosettes, obtained by converging the particles of the hexamers, that constitute the building rosettes of the honeycomb pattern (Fig. c). The hexagonal pattern of particle rosettes thus obtained is similar to the pattern found in some algae (Giddings et al. 1980, Herth 1983, Hogetsu 1983).

Thus Fig. 7 shows the relation between the single rosette, the honeycomb pattern of particles and the hexagonal pattern of rosettes.

Fig. 7: Schematic drawing of a honeycomb pattern:
A: I, II and III are symmetry axes, B: honeycomb pattern of particles, C: honeycomb pattern constructed out of single particle rosettes., D: hexagonal pattern of particle rosettes, obtained by converging the hexamers that constitute the building rosettes.



*Function of honeycomb patterns of particles*

The hexagonal pattern of particle rosettes in algae has been hypothesized to be the microfibril synthesizing complex (Giddings et al. 1980, Herth 1983, Hogetsu 1983). And indeed the complexes are seen at the ends of microfibril imprints and microfibril width is correlated to the number of rosettes in line with the fibril (Staehelin and Giddings 1982, Herth 1983). Single particle rosettes -single hexagons- have also been hypothesized to be involved in microfibril synthesis (review: Brown 1985). Schnepf et al. (1985) showed that site and intensity of growth were related closely to the distribution and frequency of particle rosettes in the PF-face of the plasma membrane.

Rosettes seem to function in microfibril synthesis, though they are not necessarily the microfibril synthesizing enzymes (Willison 1983, Emons 1985).

Honeycomb patterns of particles seem to be related to solute transport, whether or not in combination with loss of turgor. The fact that plasma membranes of generative cells of pollen grains contain the honeycomb pattern, indicates that honeycombs are not merely plasmolysis induced, because plasmolysis in plant cells is the retraction of the plasma membrane from the cell wall, and the lily generative cell lacks a cell wall (Fig. 2a, Sassen and Kroh 1974). It is possible that honeycomb patterns of particles function physiologically in transport of small molecules through the membrane.

Figure 7 shows a close relationship between the three honeycomb patterns. During cell wall synthesis molecules have to pass through the plasma membrane. Cell wall matrix is thought to be brought through the membrane by insertion of golgi-derived smooth vesicles (Sievers and Schnepf 1981) containing the matrix material. Oligomers for the synthesis of microfibrils might pass the membrane via the particle rosettes. It is known that membranes can regulate the passage of ions and/or small molecules by ordering membrane proteins, thus creating channels in the membrane for this purpose. Regulation is achieved by conformational changes of the protein in response to chemical and electrical sti-

muli. An example of such a protein is the one which forms the gap junction in animal cells (Unwin and Ennis (1984). A particle of the gap junction is composed of six identical rod-shaped units and spans the bilayer, creating a channel along its central hexad axis (Unwin and Ennis 1984).

Honeycomb patterns of particles as well as particle rosettes might function in solute transport, rosettes being sites in the membrane where cellulose precursors pass the membrane. Precursors would pass through the protein particle, the arrangement of particles in a rosette allowing individual glucan-chains to co-crystallize into a cellulose microfibril.

In this hypothesis, terminal globules of the EF-face of the plasma membrane (Fig. 5) might be the microfibril synthesizing enzymes. To understand cellulose microfibril deposition in higher plants the microfibril synthesizing complexes need to be characterized biochemically.

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# CHAPTER 10

# Coated Pits on the Plasma Membrane of Plant Cells

European Journal of Cell Biology 41, 57 64 (1986) © Wissenschaftliche Verlagsgesellschaft mbH Stuttgart

# Coated pits and coated vesicles on the plasma membrane of plant cells

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Received December 30, 1985
Accepted March 14, 1986

*Coated pit dry-cleaving — freeze fracturing — plant cell — plasma membrane*

The cytoplasmic surface of the plasma membrane of root hairs and cortical root cells, both growing and full grown, of several plant species and of plant protoplasts, is shown to contain numerous coated pits Specimens were prepared by the dry cleaving method Freeze substitution and freeze fracturing were used as control methods

The density of coated pits in different cells varies greatly Pits are more numerous in root hair tips and in tubes of tip growing hairs than in full-grown hairs, and are also more numerous in meristematic and expanding root cells than in elongated cortical root cells

Planar pits with protruding chains measure 60 to 170 nm in diameter and are primarily built up of hexagons but often planar pits, irrespective of size, contain a pentagon Clearly curved pits, which range in diameter 92 to 124 nm, contain hexagons and some pentagons Coated vesicles measure 72 to 96 nm in diamter including coat, a size typical for plant cells The vesicle in the coat has a unit membrane structure The center-to-center distance of the polygons in the pits is 23 to 25 nm, the sides of the polygonal networks are 7 to 8 nm in width

Coated pits and coated vesicles on the plasma membrane are suggested as functioning in membrane turnover

## Introduction

Coated pits and coated vesicles are two related structures found in all eukaryotic cells Their cytoplasmic sides are constituted of polyhedral coats of clathrin and associated polypeptides [25] According to a widely accepted hypothesis, coated vesicles on the plasma membrane in animal cells derive from coated pits by a budding process by which the polyhedral coat structure rearranges [16] Upon budding, the coats are shed and the vesicles fuse with their target membranes, whereas the clathrin molecules are thought to return to the plasma membrane to reassociate with an incipient coated pit [14] The coated structures in animal cells thus function in several ways [4] they bring macromolecules into the cell and transport them across the cell, they retrieve membrane from the plasma membrane and thus function in membrane turnover

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Coated vesicles are also present in plant cells [23] where they have been observed near the plasma membrane and near dictyosomes Little is known, however, about their functioning in plant cells Franke and Herth [12], working on growing suspension culture cells of Haplopappus gracilis, have suggested that they play a role in exocytosis Accordingly, from studies on other plant cells it has been proposed that coated vesicles derive from dictyosomes and fuse with the plasma membrane [8, 27, 33]

However, Joachim and Robinson [17] and Tanchak et al [30] showed that cationic ferritin is endocytosed by protoplasts through coated pits

To put constraints on models regarding dynamics and functioning of coated membranes of plant cells, quantitative data on occurrence, distribution, density, dimension and ultrastructure are necessary Detailed quantitative data from plant cells have only been reported from preparations of protoplasts [11, 33] We have therefore studied a variety of plant material growing as well as full-grown tip-growing cells, and uniformly expanding as well as elongated cells of the root cortex and plant protoplasts Coated structures were visualized with the dry-cleaving method To identify possible artefacts of the procedure, the dry-cleaved material was compared with freeze-substituted and freeze-fractured specimens

The results are discussed with respect to a possible function of coated pits and coated vesicles in the retrieval of excess plasma membrane during cell expansion

## Materials and methods

Plant cells from different origins were used growing root hairs of Equisetum hyemale and Raphanus sativus, full-grown root hairs of Equisetum hyemale, meristematic and elongating cortical root cells of Raphanus sativus and Allium cepa, elongated cortical root cells of Raphanus sativus, Limnobium stoloniferum and Ceratopteris thalictroides Furthermore, protoplasts of suspension culture cells of Nicotiana plumbaginifolia were used

Roots of Equisetum hyemale were taken from stem cuttings growing in tap water, roots of Ceratopteris thalictroides and Limnobium stoloniferum of plantlets obtained by cutting buds from the mother plants and growing them in an aqueous soil extract [19] and of Raphanus sativus and Allium cepa from seedlings grown on moist filter paper

Protoplasts were prepared by incubating suspension culture cells in 0.4 M mannitol with 4% cellulase and 0.5% hemicellulase during 3 h at 50 rpm, 28 °C.

The cytoplasmic side of the membrane of root cells was investigated with the dry-cleaving method described by Traas [32]. Protoplasts were fixed in 2% glutaraldehyde in 0.4 M mannitol in Pipes buffer (50 mM) during 1 h. After fixation they were rinsed in buffer and treated with tannic acid (1% in Pipes) during 30 min. Subsequently, the cells were rinsed in the buffer and allowed to settle on poly-L-lysine coated grids. They were successively postfixed in 1% osmium tetroxide in bidest, stained in uranyl acetate 1% in bidest and critical-point-dried after dehydration in ethanol. Subsequently, the cells were cleaved on adhesive tape as described by Traas [32].

In order to identify possible artefacts of the chemical fixation, the dry-cleaved preparations were compared to material obtained with freeze-substitution. Freeze-substitution was carried out as described by Tiwari et al. [31] and references in that paper.

Furthermore, the plasmatic (PF) and extraplasmatic (EF) faces of the plasma membrane were studied with freeze-fracturing. The freeze-fracture procedure was carried out as described by Emons [10].

The material was photographed in a Philips EM 201. Density and dimensions of the coated pits were measured from photo-

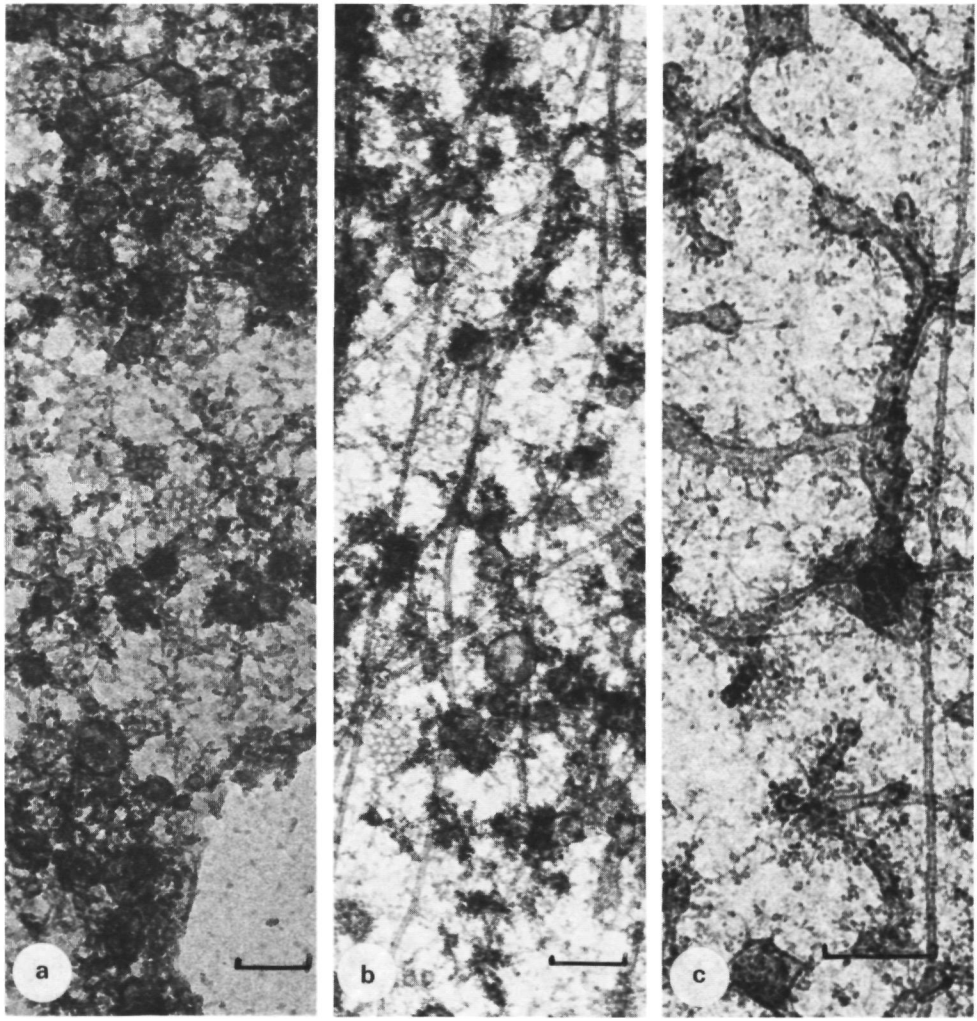


**Fig. 1.** Dry-cleaved preparation of root hairs of Equisetum hyemale. — **a.** Tip-region of growing hair. — **b.** Tube of tip-growing hair. — **c.** Tube of full-grown root hair. Pit density in the growing hair is higher than in the full-grown hair. — Bars 250 nm.

graphs, printed at a final magnification of 35000 to 40000. Density of pits is given as numbers/surface area.

## Results

### *Density and distribution of coated pits on the plasma membrane*

Figures 1a to c show the cortical cytoplasm of: Equisetum hyemale root hair tip of growing hair, Equisetum hyemale root hair tube of growing hair and Equisetum hyemale root hair tube of full-grown hair. The density of coated pits is high in growing hairs, tips (2.5–4.5 pits/$\mu m^2$) as well as tubes (0.3–3.4 pits/$\mu m^2$), but low in full-grown root hairs (0.1–0.6 pits/$\mu m^2$) (Tab. I). Pit densities tend to be higher in the extreme tip.

The number of coated pits/$\mu m^2$ on plasma membranes of cortical root cells of different plant species and of protoplasts of Nicotiana plumbaginifolia is also given in Table I. The density of coated pits varies greatly. The density in meristematic and elongating cells of Raphanus and Allium is higher than the density in elongated cells. Elongated cortical root cells of Raphanus, Limnobium and Ceratopteris all show values within the same range.

Also clearly curved pits, either budding from or fusing with the plasma membrane, may be seen: in one particular area of 180 $\mu m^2$ of Equisetum hyemale root hair plasma membrane 15 coated vesicles, 25 curved pits and 78 more planar pits were counted. Figure 2 is a stereomicrograph of a curved pit.

The distribution of pits does not show a special pattern; sometimes two or three pits are found in close proximity (Fig. 3f).

**Tab. I.** Density of coated pits on the plasma membrane of different plant cells. Surfaces of 20 to 203 $\mu m^2$/cell were measured.

| Species | Cell type | Number of cells | Density of pits/$\mu m^2$ |
|---|---|---|---|
| Equisetum | root hairs, growing, tip | 4 | 2.5–4.5 |
| Equisetum | root hairs, growing, tube | 18 | 0.3–3.4 |
| Equisetum | root hairs, full-grown | 11 | 0.1–0.6 |
| Raphanus | root hairs, growing, tube | 6 | 4.0–8.0 |
| Raphanus | meristematic/elongating | 6 | 0.5–1.3 |
| Raphanus | cortical, elongated | 7 | 0.5–0.6 |
| Allium | meristematic/elongating | 2 | 1.6–6.6 |
| Limnobium | cortical, elongated | 3 | 0.7–1.2 |
| Ceratopteris | cortical, elongated | 5 | 0.3–0.9 |
| Nicotiana | protoplasts | 15 | 0.6–2.0 |

### *Ultrastructure and dimensions of coated pits and coated vesicles*

Apart from single clathrin timers and very small pits, all stages of pit formation and all stages between pits and vesicles were observed (Figs. 3a–g).

Planar pits have protruding chains of the clathrin molecules around them (Figs. 3a–d). They measure 60 to 170 nm in diameter, the smaller consisting of some polygons, the larger pits of up to 25 complete polygons. Planar pits usually contain hexagons but also small pits containing a pentagon are seen (Fig. 3b).

The size of clearly curved pits depends on the degree of curvature and ranges from 72 to 124 nm in diameter. They consist of hexagons and on top of the curved coat usually a pentagon occurs (Fig. 3e). Strongly curved pits sometimes seem to have a ring-like boundary, due to the fact that the protruding chains of the curved pit are below the pit and cannot be seen anymore.

Figure 4 shows the size range of pits given as the number of polygons/pit. Sometimes pits with a diameter above 170 nm were observed, but these clearly represent confluent pairs (Fig. 3f).

Coated vesicles measure 72 to 96 nm in diameter including the coat (Fig. 3g), which corresponds with the size of

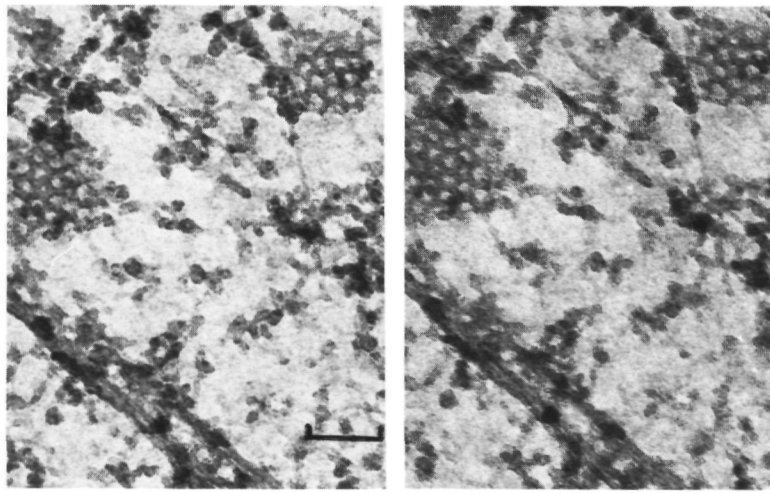

**Fig. 2.** Stereo micrograph of the cytoplasmic side of a protoplast of Nicotiana plumbaginifolia prepared by dry-cleaving showing two curved pits with a pentagon at the top. — Note filaments attached to the pit. — Bar 100 nm.

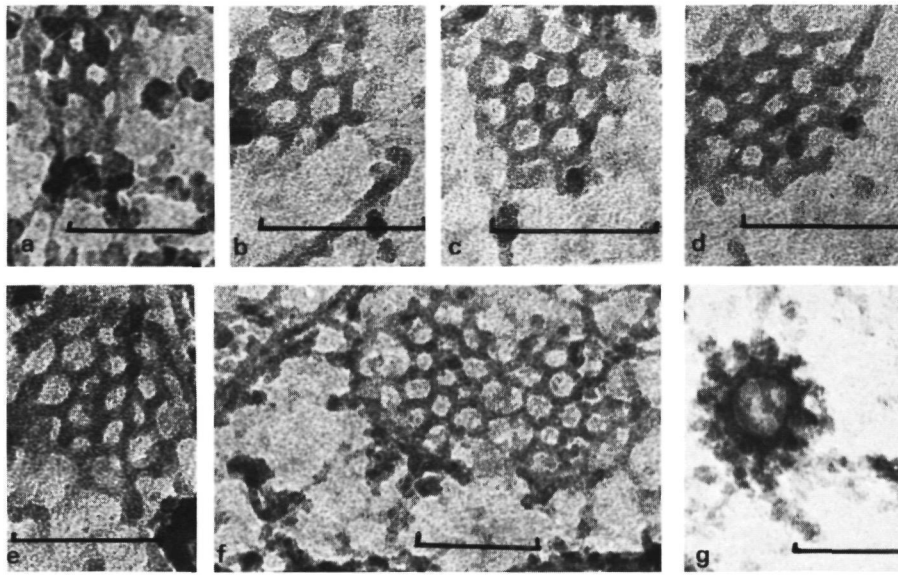


**Fig. 3.** Different stages of coated pit formation in protoplasts of Nicotiana plumbaginifolia. — **a.** Small pit with a complete hexagon. — **b.** Small pit with a pentagon in the center of the pit. — **c, d.** Large pits with protruding clathrin chains around them. — **e.** Curved pit with pentagon. — **f.** Confluent pairs of coated pits. — **g.** Coated vesicle; the coat contains a vesicle with unit-membrane structure. — Bars 100 nm.

the largest planar pits. A sphere of 85 nm in diameter has a surface of 170 $nm^2$. A small vesicle with unit-membrane structure is present within the coat.

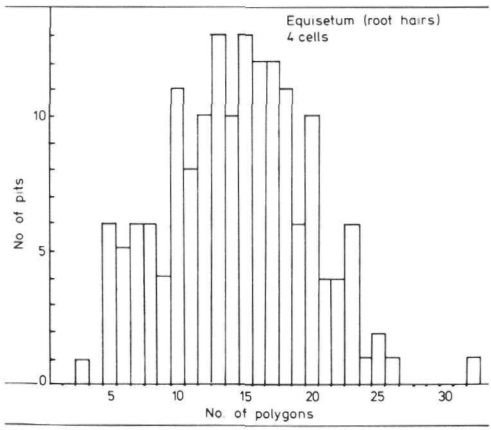


**Fig. 4.** Distribution of coated pit sizes in dry-cleaved root hairs of Equisetum hyemale, given as the number of polygons per coated pit.

The center-to-center distance of the polygons in the coated pits is 23 to 25 nm, the sides of the polygonal networks measure 7 to 8 nm in width.

*Coated pits in freeze-substituted and freeze-fractured preparations*

The density of coated pits in oblique sections of freeze-substituted young Equisetum hyemale root hairs (Fig. 5) is comparable to the density observed with dry-cleaving. Also size and morphology do not differ from dry-cleaved material.

Freeze-fracturing reveals the plasmatic, PF face, and the extra-plasmatic, EF face, of the plasma membrane. Indentations are visible in the PF face of the plasma membrane (Figs. 6a, b) and elevations in the EF face (Figs. 6c, d). Density and size of indentations and elevations are comparable to coated pit density and size in dry-cleaved material.

## Discussion

The dry-cleaving method permits the visualization of large areas of the cytoplasmic side of the plasma membrane with clearly visible coated pits. In all cells various stages of pit formation were observed.

A distinct variety of coated vesicle, the "spiny coated vesicle" [21], which occurs in clusters and is variable in size and shape, was not found in our material.

*Density of coated pits on the plasma membrane*

The density of pits varies greatly between cells (Tab. I). The plasma membrane areas in this study may be categorized as follows: *a)* rapidly expanding membrane of root hair tips, *b)* membrane of the non-expanding tubes of hairs growing at the tip, *c)* uniformly expanding membrane of root cells of the meristematic and elongating zone, *d)* membrane of elongated cortical root cells, *e)* non-expanding membrane of full-grown root hairs, *f)* membrane of protoplasts before microfibril deposition takes place.

From Table I it is clear that plasma membranes of growing cells contain more pits than plasma membranes of full-grown cells, even in the root hair tube, where no cell expansion takes place.

In thin-sectioned root hairs of white clover [27] the number of coated pits was about 20 times larger at the tip of root hairs (1.7 coated pits/$\mu m^2$) than the number towards the base of the hair (0.1 coated pits/$\mu m^2$). Bonnett and Newcomb [3] did not record a higher frequency of coated vesicles at the tip of the growing root hair in Raphanus. We found a high frequency in growing hairs, in the tip (Equisetum hyemale: 2.5–4.5 pits/$\mu m^2$) as well as in the tube (Equisetum hyemale: 0.3–3.4 pits/$\mu m^2$, Raphanus sativus: even up to 8 pits/$\mu m^2$) of growing hairs, but a smaller frequency in full-grown hairs (Equisetum hyemale: 0.1–0.6 pits/$\mu m^2$). Similarly, pit frequencies found in expanding cortical root cells are higher than those found in elongated cortical root cells. Meristematic cells with a clear plasma membrane were difficult to obtain; often an unidentified precipitate obscured the coated pits.

Density of coated pits in freeze-substituted cells resembles the pit densities found by dry-cleaving. The correspondence between these values indicates that coated pits are neither artefactually induced by chemical fixation nor by the other steps of the dry-cleaving method.

Freeze-fractured plasma membranes of hair tubes show indentations in the PF face (Figs. 6a, b) and elevations in the EF face of the membrane (Figs. 6c, d). The size of indentations and elevations agrees with the coated pit size. They are not caused by exocytosis of smooth vesicles. Smooth vesicles are exocytosed at the hair tip [29]. The indentations and elevations seen in the hair tube are most probably from coated pits. Size and density of them corresponds to size and density of coated pits.

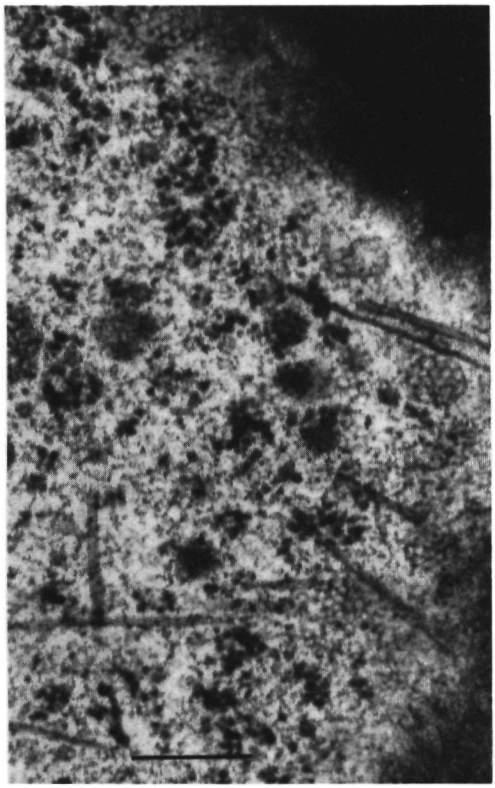

**Fig. 5.** Thin oblique section of cortical cytoplasm of freeze-substituted root hair of Equisetum hyemale, taken near the tip of the young growing hair with abundant coated pits on the small stretch of membrane visible in thin section. — Bar 250 nm.

*Ultrastructure of coated pits and coated vesicles*

Coated pits on the plasma membrane of the plant cells studied are primarily built up of hexagons and are 60 to 170 nm in diameter. Even small pits with protruding clathrin chains often contain a pentagon. The clearly curved pits consist of hexagons and one or more pentagons, a requirement for the formation of a globular structure [14]. The maximum pit size, 170 nm, is the size required to form vesicles in the range of 72 to 96 nm.

The coated vesicles measure 72 to 96 nm, a size typical for plant cells [23]. In animal cells these organelles range more widely in size, from about 60 to 160 nm. Croze et al. [7] classified clathrin-coated vesicles in rodent liver into three distinct populations: 60 to 80-nm vesicles, almost exclusively found within the Golgi apparatus region, 100 to 160-nm vesicles within 100 to 500 nm of the cell surface and 90-nm vesicles present both at the Golgi membranes and at the cell surface. Preliminary studies on Equisetum hyemale root hairs indicate that coated vesicles near dictyosomes are different in size and morphology from these organelles near the plasma membrane (data not shown).

The center-to-center spacing of the polygons (23–25 nm) and the diameter of the sides (7–8 nm) is in the range of values calculated from coated vesicles of animal cells [16].

*Mechanism of coat and vesicle formation*

The mechanism of coat formation is still poorly understood. For animal cells it has been proposed that coats at the plasma membrane surface gradually increase by addi-

tion of coat subunits rather than by fusion of small coated pits [16]. Accordingly, in our preparations of Equisetum hyemale root hairs a smooth gradation in pit sizes was found (Fig. 4).

The mechanism of vesicle formation originally postulated by Kanaseki and Katoda [18] envisages a rearrangement of clathrin molecules by which hexagons turn into pentagons. This conversion of hexagons into pentagons could create sufficient strain into the lattice to introduce curvature [16]. However, the properties of clathrin in vitro suggest that pentagons and hexagons form simultaneously [6, 14]. Indeed, we found very small planar pits, that con-

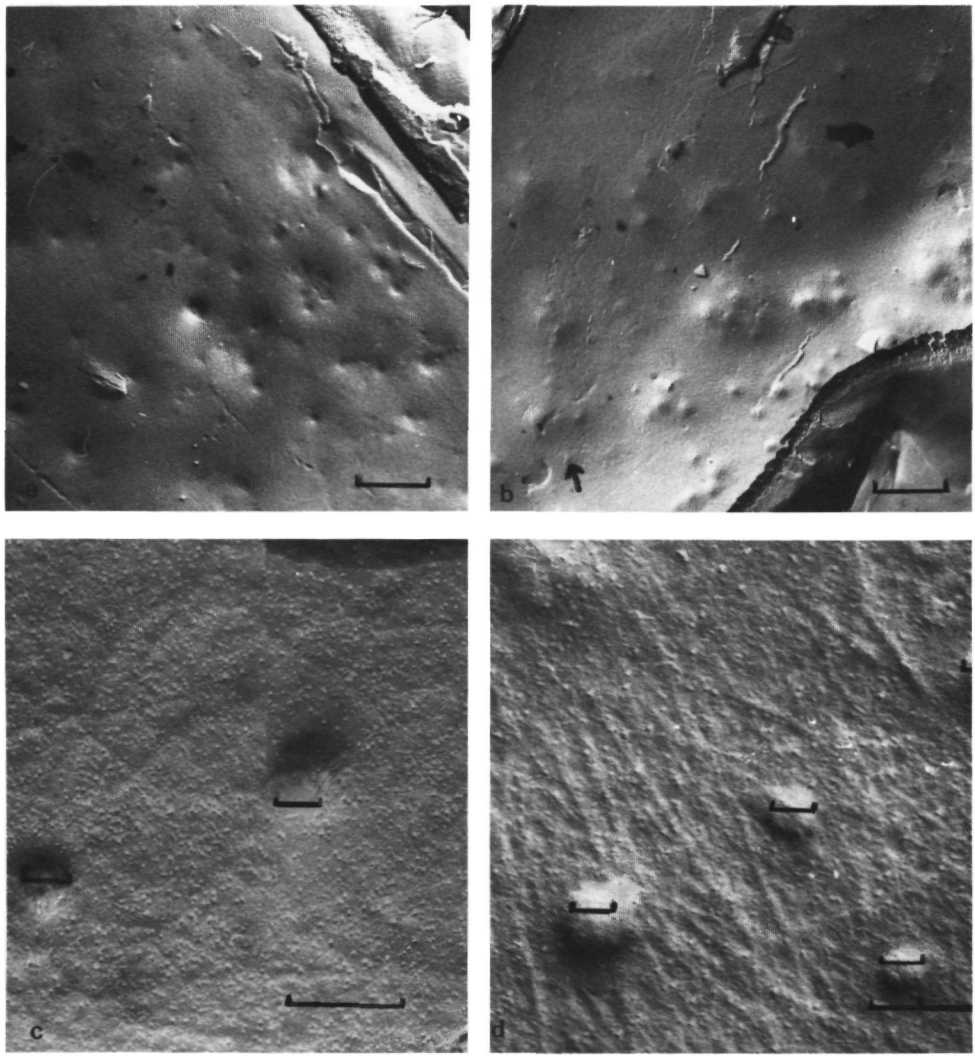


**Fig. 6.** Freeze-fractured root hair of Equisetum hyemale showing the plasmatic fracture face (PF) (**a, b**) and the extraplasmatic fracture face (EF) (**c, d**) of the plasma membrane. — **a, c.** At low magnification. — **b.** Detail of PF face with indentations. — **d.** Detail of EF face with elevations. — *Bar* through indentations and elevations is 90 nm, *arrow* indicates direction of shadow. — Bars 1000 nm (**a, c**), 250 nm (**b, d**).

tain pentagons (Fig 3b) Thus, curvature and closure are built into the geometry and bonding properties of the clathrin trimer

Results obtained with both animal and plant cells [25, 30] indicate that a coated pit has a lifetime of less than 60 s Given the pit density and vesicle diameter and assuming that each coated pit is internalized within 60 s, the rate of entire plasma membrane internalization can be calculated Assuming that all pits are involved in a budding process, that each vesicle is formed in 30 to 60 s and given the mean diameter of the naked vesicles (about 60 nm), the entire plasma membrane of a growing cell with a density of two pits per square micron (root hair tubes, meristematic cells, protoplasts) is internalized in 20 to 40 min Given a density of 0 7 pits per square micron (nongrowing cells) internalization is achieved in 60 to 115 min

*Function of coated structures at the plasma membrane*

In plant cells, coated pits have been suggested as originating from dictyosomes and to function in the exocytosis of cell wall precursors and in the transfer of membrane to the plasma membrane [3, 20, 21, 23, 33]

Recent observations, however, argue for an endocytotic role of coated pits protoplasts internalize ferritin via coated pits [17, 30] Coated vesicles appear to shed their coat immediately after formation before they fuse with their target membranes [17, 25, 30] Coated vesicles associated with dictyosomes of plant protoplasts are smaller than coated vesicles associated with the plasma membrane, suggesting that they represent two different classes [30, 33] A role of coated pits in endocytosis has also been concluded by O'Neill and La Claire [24] from the induction of coated pits and coated vesicles through mechanically wounding cells of the giant alga Boergesenia Furthermore, if a coat inhibits fusion while uncoated membranes rapidly fuse with other membranes, as has been convincingly argu mented by Altstiel and Branton [2], the visible clathrin coat must always be in the process of budding, of retrieving membrane from another membrane, rather than being in the process of fusing

We have found low pit-frequencies in non-expanding full-grown root hairs, which are actively depositing microfibrils [9] This is a further argument against a role of coated pits in exocytosis of cell wall precursors In our view the clathrin coat provides a structural framework necessary for the invagination of a vesicle and is thus a site for membrane retrieval after exocytosis of wall material by smooth vesicles It is known that during release of secretory products by exocytosis, smooth vesicles are inserted at the tip of the growing root hair [29] The insertion of membrane at the tip and retrieval of excess membrane along the whole root hair causes a constant flow of membrane Also Picton and Steer [26] found that excess membrane is inserted by smooth vesicles in the plasma membrane of growing pollen tubes Wall formation might be expected to require more vesicle fusion than does plasma membrane growth In this hypothesis the abundance of coated vesicles found in cell plates [20, 21] might also be explained as the retrieval of excess membrane from the forming plate

Whether coated pits in plant cells also function to internalize substances into the cell cannot be concluded from our data Substances thus brought into the cell could be the wall oligosaccharins, or substances released by them, which Albersheim et al [1] have suggested to be regulating substances for many plant cell functions

Our suggestion for an endocytotic role of coated structures associated with the plasma membrane does, however, not exclude an exocytotic process by which cell wall material is extruded through the plasma membrane by insertion of Golgi-derived coated vesicles But when fusing, their coats must have been shed

This implies that, if rosettes, the putative microfibril synthesizing complexes, visualized by freeze-fracturing [5, 10, 15], are inserted in the plasma membrane by vesicles [28], these vesicles are not the coated vesicles

*Acknowledgements* We wish to thank Prof Dr M M A Sassen for various support, Dr J W M Derksen for helpful discussion and H Kengen for the preparation of the protoplasts Cell suspension cultures were kindly provided by Dr A de Laat, ITAL Wageningen

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CHAPTER 11

# Cytoplasmic Organization in Root Hairs of Equisetum and Limnobium and Cytoplasmic Streaming in root Hairs of Equisetum

# CYTOPLASMIC ORGANIZATION IN ROOT HAIRS OF *Equisetum* AND *Limnobium* AND CYTOPLASMIC STREAMING IN ROOT HAIRS OF *Equisetum*

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## ABSTRACT

The ultrastructure of root hairs of Equisetum hyemale and Limnobium stoloniferum was studied by means of freeze-substitution. Special attention was given to the Golgi apparatus and the cytoskeleton. Rate of cytoplasmic streaming in developmental stages of root hairs of Equisetum hyemale was measured.

The structural polarity of the dictyosomes is in keeping with the membrane flow concept. Polarity is shown in staining properties of the membranes and in inter- and intra-cisternal width; the cis-face may be juxtaposed to the endoplasmic reticulum. The vesicles at the outside of the trans-face of the dictyosomes are similar in appearance and size to the "secretory vesicles". Secretory vesicles, smooth vesicles primarily located in the hair tip, measure approx. 300 nm.

Coated pits occur in abundance at the plasma membrane and are occasionally seen on the trans-cisterna of the dictyosomes.

Besides microtubules, two distinguishable classes of filaments can be identified in the root hairs. Single filaments occur in the cortical cytoplasm, and are in association with microtubules. Bundles of filaments are found throughout the cytoplasm with the exception of the tip region of the hair. This region is characterized by the absence of the central vacuole, and decreases in length during hair growth. Bundles of microfilaments stained with rhodamine-labelled phalloidin and are presumably F-actin.

At a temperature of 25° C cytoplasmic streaming is absent from the tip region of short hairs, but gradually increases up to 3.5 micrometers/sec in the base of the hair. In long hairs cytoplasmic streaming reaches up to the tip and is up to 7 micrometers/sec in the tube of the hair.

## INTRODUCTION

Root hairs develop from trichoblasts, epidermal cells which are densely cytoplasmic (Harris 1979, Meekes 1985). They lengthen by tip-growth (Sievers and Schnepf 1981). Detailed ultrastructural and cytochemical analyses have been published (review: Sievers and Schnepf 1981) and have shown a polar organization of the cytoplasm. These analyses were based on conventional glutaraldehyde/osmium tetroxide fixations, which have their limitations (Howard and Aist 1979, Howard 1981, Heath et al. 1985). Hoch and Howard (1980) have discussed these limitations and have shown in a study on fungal hyphae that they can be overcome by means of freeze-substitution. Especially "secretory vesicles" and microfilaments are better preserved by means of freeze-substitution (Heath et al. 1985).

Secretory vesicles in tip-growing cells appear to be produced from Golgi bodies and to be transported to the cell tips, where they are thought to deliver their contents to the wall after exocytosis (Sievers and Schnepf 1981).

Microfilament bundles have been shown by means of rhodamine-labeled phalloidin to occur in plant cells (Parthasarathy et al. 1985, Derksen et al. 1986), among which root hairs (Emons and Derksen 1986). Microfilaments are hypothesized to be related to cytoplasmic streaming (Pesacreta and Parthasarathy 1984). They can also be visualized by freeze- substitution (Tiwari et al 1984).

The objective of this paper is to give a description of the Golgi system and its secretory vesicles and to correlate the presence of microfilaments with the occurrence of cytoplasmic streaming.

## MATERIAL AND METHODS

Roots were taken from stem cuttings of Equisetum hyemale, grown as described by Emons and Wolters-Arts (1983) and of young plantlets of Limnobium stoloniferum (Sassen et al. 1985). Freeze-substitution was carried out as described by Emons and Derksen (1986), which is an adaptation of the method described by Heath and Rethoret (1982). Freeze-fracturing was carried out as described by Emons (1985). Preparations were examined with a Philips EM 201 electron microscope.

For determination of cytoplasmic streaming roots with hairs of Equisetum hyemale were placed on viewing slides in a drop of the same incubation medium in which they grew, and examined in a light microscope. By means of a flask, filled with 0.1% copper sulphate placed between light source and condensor lens, care was taken that a more or less constant temperature was conserved during examination. Temperature was 25° C ± 2.5° C. The movement of granules (granule diameter approx. 2.5 micrometers) was recorded on video-tape and analysed in slow-motion to calculate velocity of cytoplasmic streaming.

## RESULTS

### *Cytoplasmic organization and ultrastructure of organelles*

Longitudinal sections through the root hair reveal the polar distribution of the cytoplasm (Fig. 1).

The plasma membrane in freeze-substituted preparations is not undulated and there is no periplasmic space between the plasma membrane and the cell wall (Fig. 2a). Coated pits are present abundantly on the plasma membrane (Fig. 2b). No coated vesicles clearly free from the plasma membrane were observed. Microtubules line the plasma membrane of the tip in random orientations (Fig. 3). The apex is filled with a cluster of smooth vesicles: the "secretory vesicles" (Fig. 3), which have circular profiles 150-300 nm in diameter . These vesicles have intensely stained contents. Similar vesicles are seen at the trans- face of Golgi cisternae. The secretory vesicles measure around 300 nm in diameter, the smaller profiles being sections out of the centre of the vesicle. This is known from dry-cleaved preparations which yield only whole vesicles (data not shown).

Secretory vesicles lie minimally 10 nm from each other and from the plasma membrane (Fig. 3).

Along the sides of the hair, secretory vesicles are present and are often observed in association with microtubules with a minimum spacing of 10 nm between vesicles and microtubules (Fig. 4).

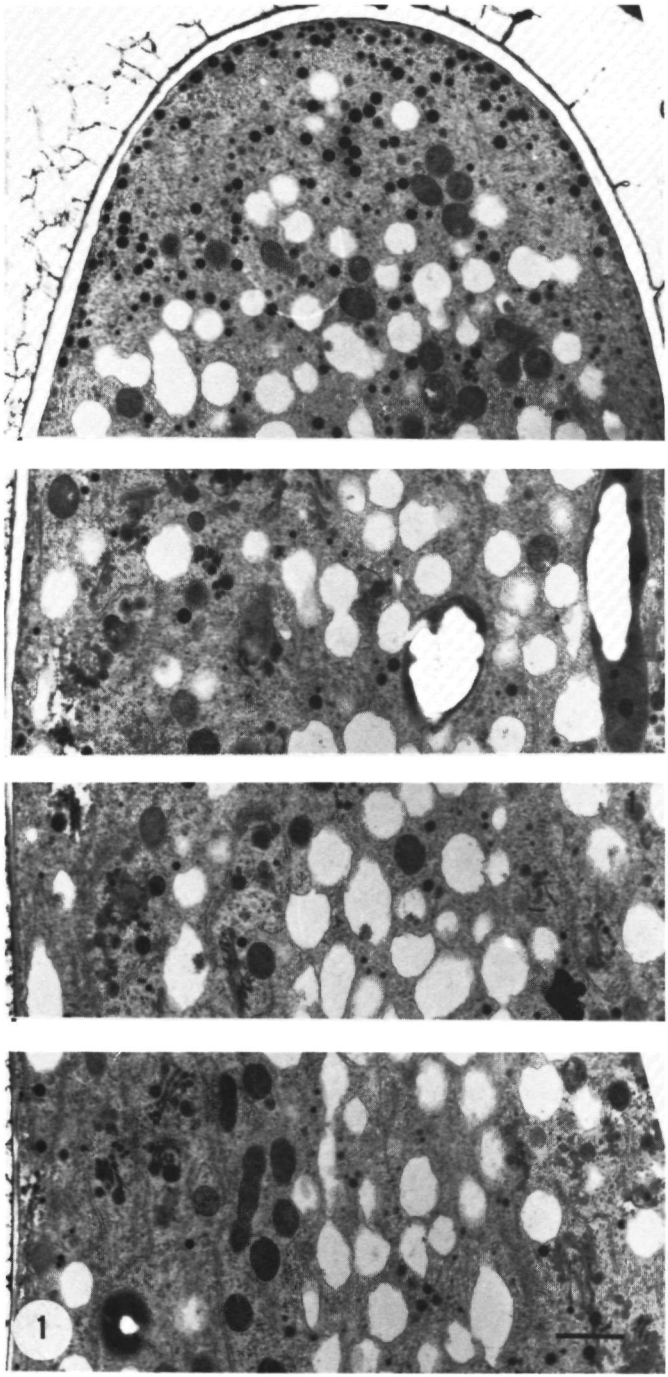
Besides secretory vesicles, (poly)ribosomes (Fig.5a) and, cisternae of the endoplasmic reticulum (Fig.5b) occur in the tip of the root hair. The latter are especially abundant in

*Limnobium*.

The subapical region of the cytoplasm contains many dictyosomes: in thin sections 16 dictyosomes/micrometer$^2$. Five to six cisternae are stacked together. The central cisternae measure 800 nm to 1200 nm in diameter, including the vesicles. There is a clear structural gradation from the cis- to the trans-face of the dictyosome: the inter-cisternal width increases, while the intra-cisternal width decreases. The intensity of staining of the membrane is another indicator of polarity. The procedure used stains trans-cisternae darker than cis-cisternae. The latter are comparable in staining to the endoplasmic reticulum. The size of attached vesicles increases across the stacked cisternae from cis- to trans-face, and the substance contained within the vesicles is darker in the larger trans-face vesicles than in the smaller cis-face vesicles (Fig. 6). Polarity of dictyosomes is also observed in freeze-fractured preparations (Fig. 7). (for procedure cf. Emons 1985). The cis-face of the dictyosome may be adjacent to the endoplasmic reticulum (Fig.6a). Sometimes dictyosomes lie close together in all kinds of juxtapositions. Always numerous vesicles lie at the trans-face of the dictyosome (Fig. 6 b). The trans-face sometimes contains coated pits (Fig.6c).

Mitochondria are located basipetal from the region with secretory vesicles. Along with cisternae of the endoplasmic reticulum they are oriented primarily parallel to the long axis of the hair. Longitudinal sections near the hair tip show short or even globular mitochondria while in the subapical region mitochondria are more elongate (Fig. 1).

Vacuoles in the subapical zone increase in volume from hair tip to hair base (Fig. 1). In longer hairs a vacuole largely fills the subapical part of the hair. In young hairs of *Equisetum hyemale* the nucleus lies in the trichoblast, the root hair forming cell. In longer hairs the nucleus moves into the basal part of the hair (Fig. 8). Especially in *Equisetum*, many plastids occur in the root hair. From tip to base of the hair they increase in length (Fig.1).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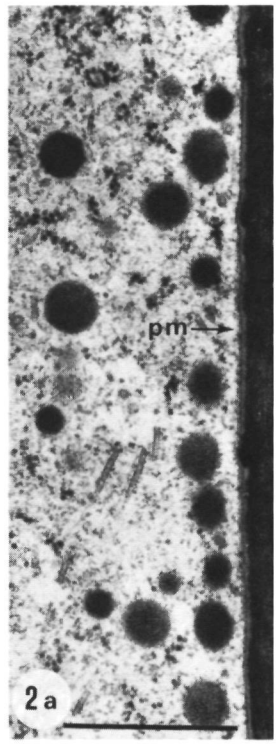
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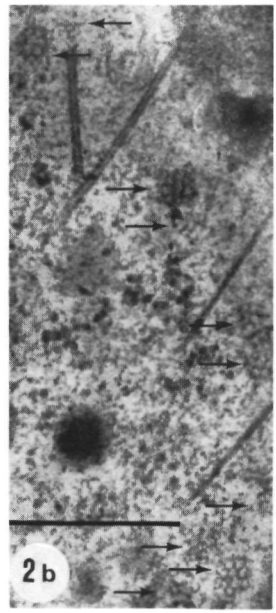
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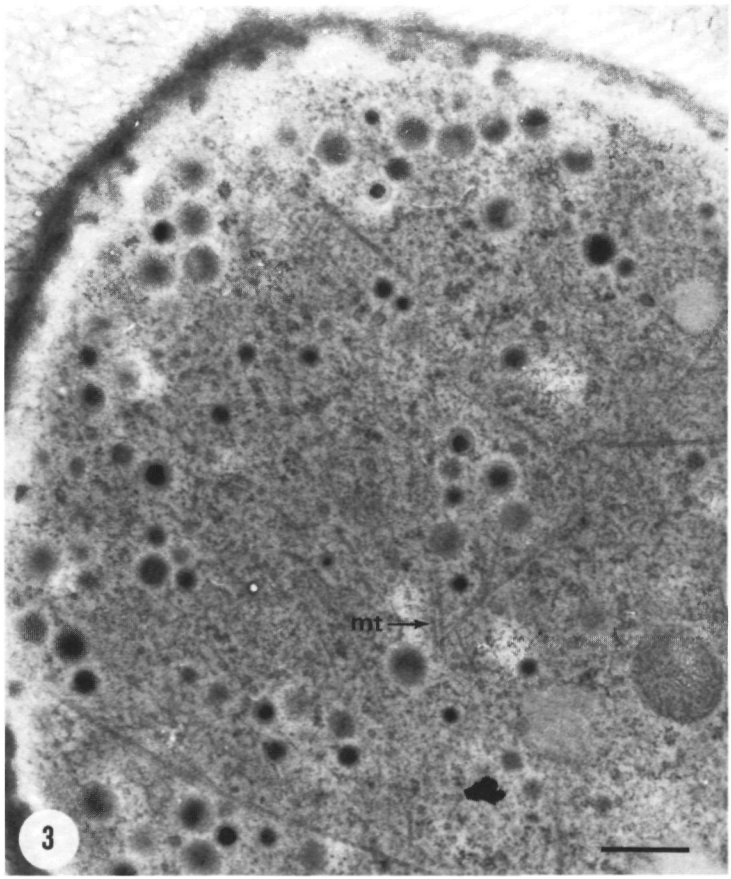
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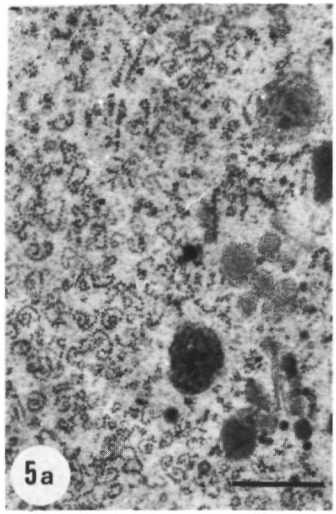
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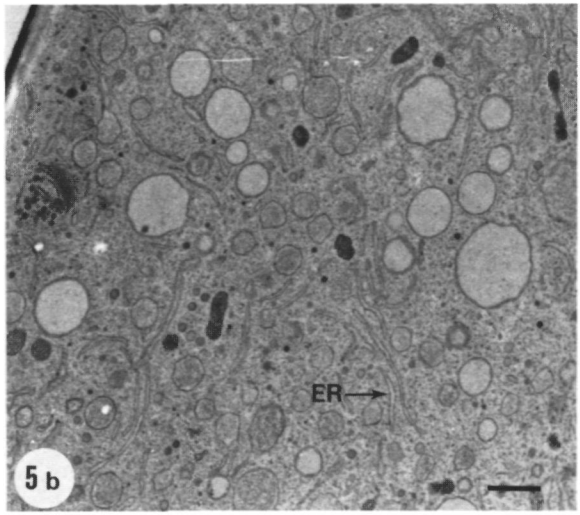
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Fig. 1: Longitudinal thin section of young root hair of Equisetum hyemale, showing the polar distribution of the cell (from the tip to 22 micrometers from the tip). The tip region is free from organelles other than secretory vesicles. Length of mitochondria and plastids increases from hair tip to hair base.
x 9000, Bar: 1000 nm.

Fig. 2a: Longitudinal thin section of root hair of Equisetum hyemale showing non-undulated plasma membrane profile appressed to the cell wall, leaving no periplasmic space.
x 45 500, Bar: 500 nm.

Fig. 2b: Tangential thin section of root hair of Equisetum hyemale, showing coated pits on the plasma membrane (arrows).
x 45 500, Bar: 500 nm.

Fig. 3: Tangential thin section of root hair tip of Equisetum hyemale, showing microtubules (mt) lining the plasma membrane in random orientations. The tip is filled with secretory vesicles.
x 22 000, Bar: 500 nm.

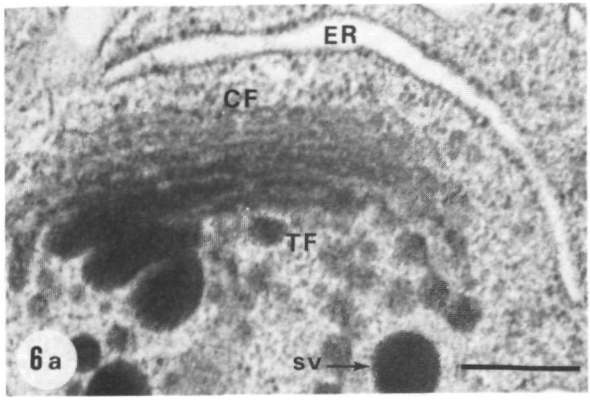
Fig. 4: Microtubule in cortical cytoplasm of Equisetum hyemale with adjacent secretory vesicles. Spacing between microtubules and vesicles is minimally 10 nm.
x 22 000, Bar: 500 nm.

Fig. 5a: Tangential thin section of subapical part of root hair of Equisetum hyemale, -subjacent to the cortical region with microtubules- showing polyribosomes. x 22 000, Bar:500 nm

Fig. 5b: Longitudinal thin section of root hair of Limnobium stoloniferum near the hair tip, showing cisternae of the endoplasmic reticulum (ER).
x 7500, Bar: 1000 nm.

Fig. 6a,b,c: Thin longitudinal sections of root hair of Limnobium stoloniferum showing dictyosomes in various positions with respect to each other and to the endoplasmic reticulum. Dictyosomes show structural gradation from cis- to trans-face: inter-cisternal width increases, intra-cisternal width decreases, cis-cisternae are lighter stained than trans-cisternae, size of attached vesicles increases from cis- to trans-face. ER: endoplasmic reticulim, CF: Cis-face of dictyosome, TF: Trans-face of dictyosome, sv: secretory vesicle, encircled: coated vesicle.
x 32 000, Bars: 500 nm.

Fig. 7: Freeze-fracture preparation of root hair of Equisetum hyemale, showing dictyosomes (arrows).
x 18 000, Bar: 500 nm.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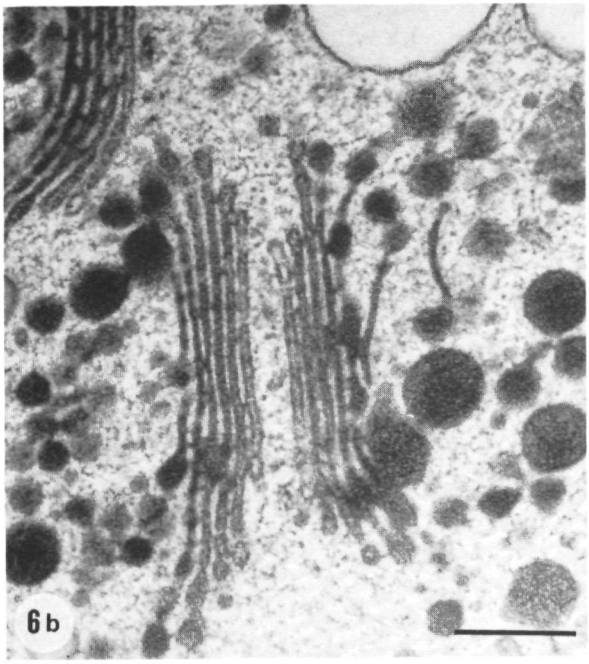
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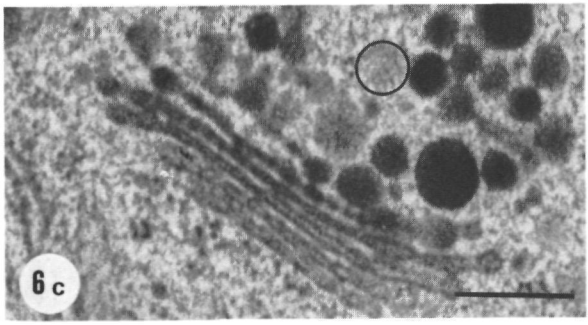
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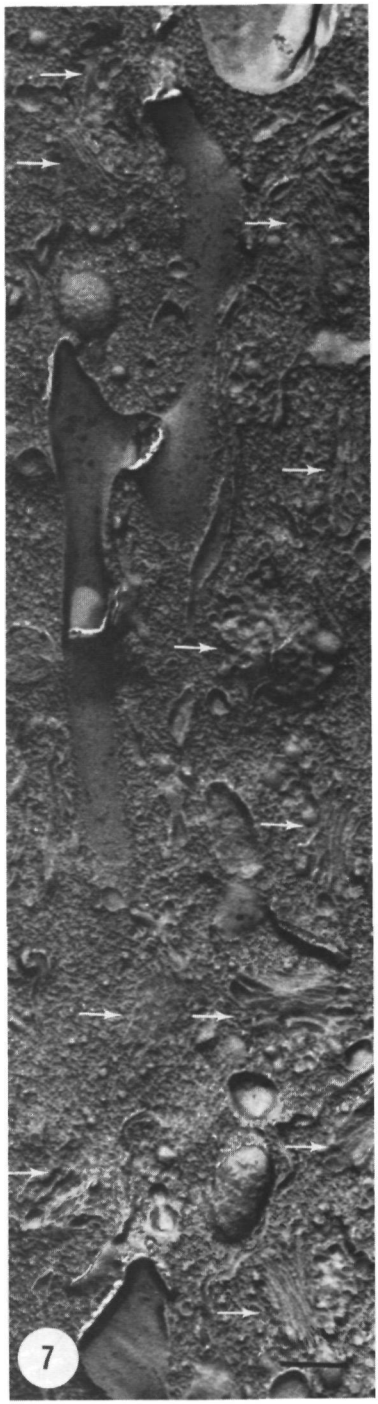
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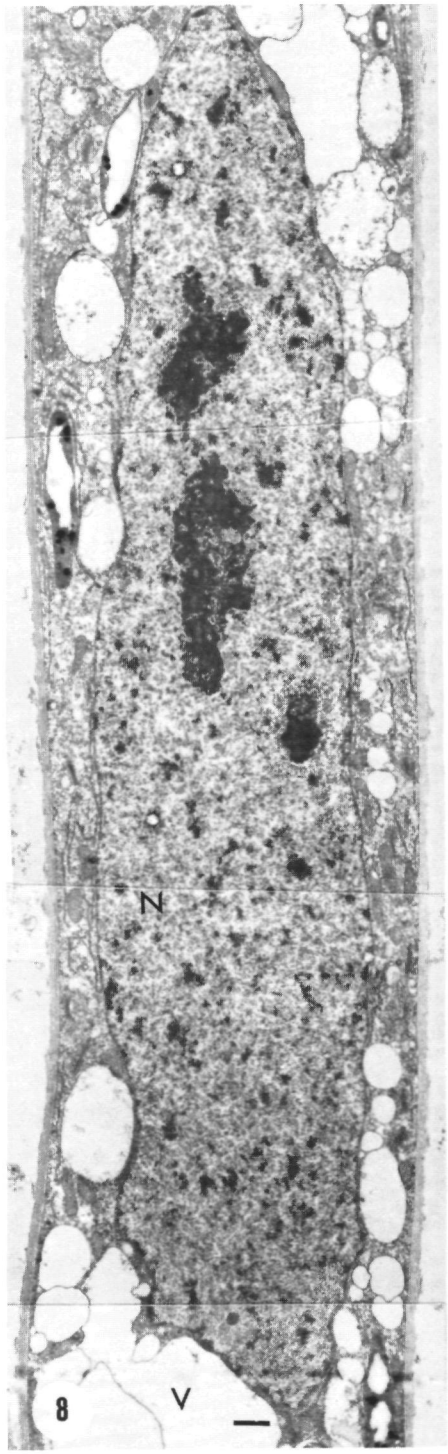
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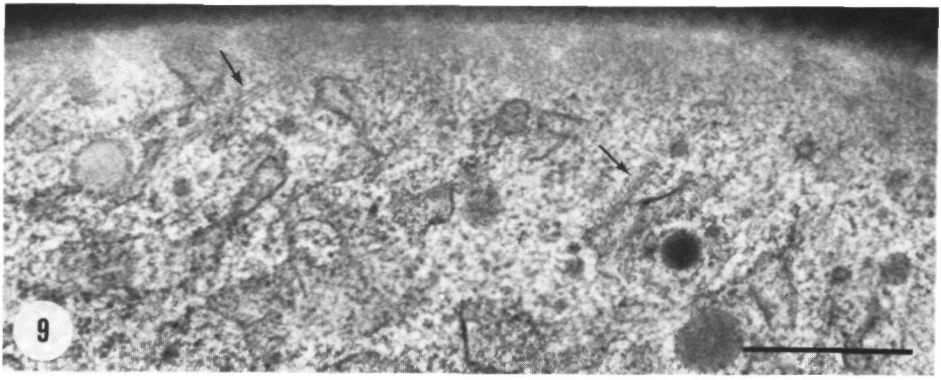
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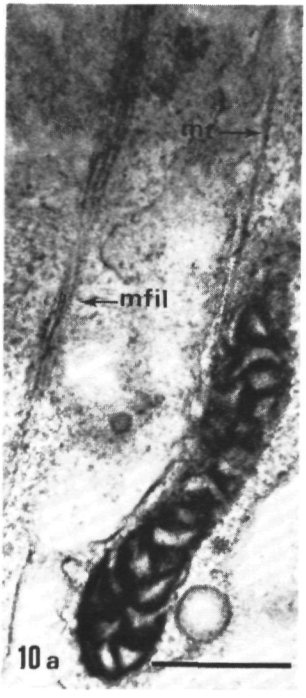
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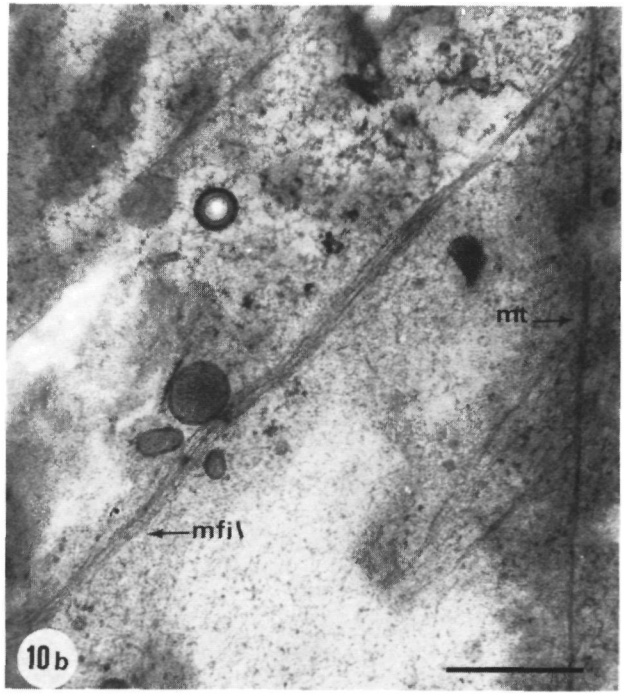
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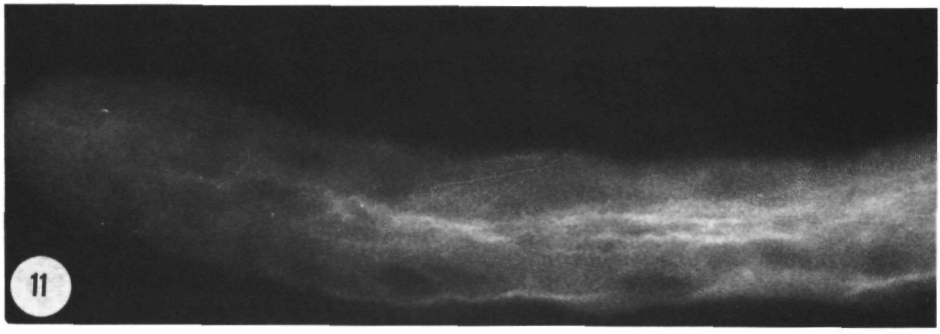
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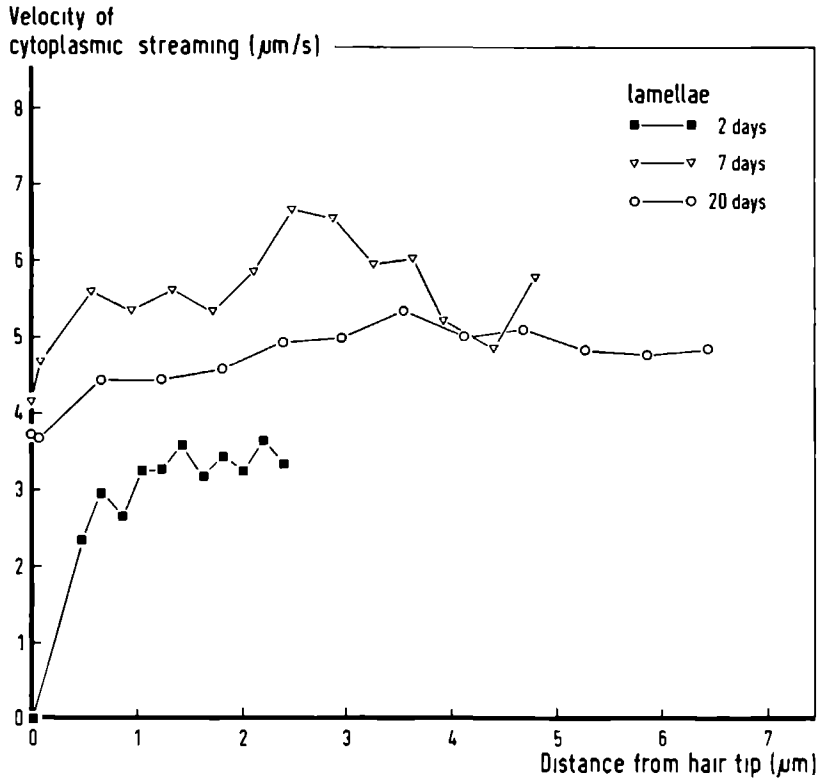
Fig. 8: Longitudinal thin section of basal part of long hair of Equisetum hyemale showing the nucleus. This hair was glutaraldehyde/osmium tetroxide fixed. For the basal region of long hairs chemical fixation gave better preservation than freeze-substitution. N: nucleus, V: central vacuole.
x 4 500, Bar: 1000 nm.

Fig. 9. Tangential thin section of cortical cytoplasm of Limnobium stoloniferum showing filaments lying along microtubules (arrows).
x 45 500, Bar: 500 nm.

Figs. 10a,b: Longitudinal thin sections of cortical cytoplasm of Equisetum hyemale 1 mm from the hair tip, showing microfilament bundles axially oriented (10a) and traversing the hair (10b). Note that in both preparations microtubules are axially oriented. mt: microtubule, mfil: microfilament bundle.
x 36 500, Bars: 500 nm.

Fig. 11: Young root hair of Equisetum hyemale stained with rhodamine-labelled phalloidin showing microfilament bundles in the hair tube and a fuzzy staining in the hair tip.
x 1500

Fig. 12: Graph showing velocity of cytoplasmic streaming in root hairs of Equisetum hyemale of different lengths.



*The cytoskeleton*

Cortical microtubules are observed in all cortical areas of the root hair and are oriented parallel to the long axis of the hair, the direction of growth. In the hair tip they lie in random orientations (Fig.1).

Two classes of filaments are observed. Single filaments lie parallel to the microtubules. Their diameter is ca. 6 nm (Fig. 9). Bundles of filaments are observed deeper in the cytoplasm (Fig. 10a and b). They are not observed in the tip region. Most filament bundles are in the direction of the long axis of the hair (Fig. 10a). However, bundles of filaments are occasionally seen in oblique directions (Fig. 10b).

By means of rhodamine-labelled phalloidin microfilament bundles are made visible in root hairs (Fig 11). Microfilament bundles are mainly longitudinally oriented, through the whole hair and do traverse the cytoplasm.

*Cytoplasmic streaming in Equisetum hyemale*

Large organelles, such as plastids, move singly, slowly and irregularly. Smaller granules (2.5 micrometers in diameter) move in rows over longer distances with a more regular velocity. They travel bidirectionally along specific cytoplasmic longitudinal tracks in the subapical and basal regions of the hair, occasionally stopping abruptly and starting in reversed direction. In the middle and basal region of the hair transvacuolar strands appear, where particles move from one side of the hair to the other. In 2 days old. 2.42 mm long hairs no streaming is measured in the tip region of the hair. From 0.3 mm of the hair tip onwards, streaming velocity increases to 3.5 micrometers/sec (Fig. 12). In longer growing (4mm long, 5 days old and 5mm long, 7 days old) and in full-grown (7mm long, 20 days old) hairs streaming reaches the tip. Streaming velocity in the full-grown hair is 6 to 7 micrometers/sec. (Fig.12)

The application of cytochalasin B ($5.10^{-3}$ mg/ml), an agent which destroys microfilaments, did stop streaming of most particles within 20 min. Very small particles, however, did not stop streaming.

DISCUSSION

Freeze-substituted biological specimens generally show improved ultrastructural preservation than chemically fixed material (Howard 1981, Hippe 1985, Tiwari et al. 1984). In freeze-substituted material membrane profiles are smooth. The root hair plasma membrane is appressed to the cell wall, leaving no periplasmic space as is often seen in chemically fixed material. The periplasmic space has been hypothesized to be the place where microfibrils self-assemble in a crystallization process like liquid crystals (Roland and Vian 1979).

*Secretory vesicles and Golgi apparatus*

Secretory vesicles are primarily located in the extreme tip of the root hair. They are suggested to bring wall matrix material in the cell wall (Sievers and Schnepf 1981). Pulse-chase experiments with [$^3$H] glucose confirmed the role of the Golgi apparatus in secretion of wall matrix polysaccharides in protoplasts (Griffing et al. 1986). For pollen tubes it has been shown that the rate of fusion of secretory vesicles with the plasma membrane is proportional to the rate of diffusion of calcium ion across the plasma membrane at the tip (Picton and Steer 1982).

In hyphae of *Saprolegnia*, Heath and coworkers (1985) have found a mixture of spherical and elongated secretory vesicles. In root hairs of *Equisetum hyemale* and *Limnobium stoloniferum* secretory vesicles are spherical and measure approx. 300 nm in diameter.

As in the hyphae the secretory vesicles have very intensely stained electron opaque contents; Together with the cell wall they are the most electron opaque structures of the hairs.

Dictyosomes in *Closterium* produce large trans-face flat vesicles during formation of a special cell wall layer (Noguchi and Ueda 1985) comparable to the spherical trans-face vesicles in *Equisetum* and *Limnobium* root hairs. Simultaneously hexagonal arrays of particle rosettes occur in the PF-face of the plasma membrane of *Closterium*. Noguchi and Ueda (1985) have hypothesized that hexagonal arrays of

particle rosettes are inserted in the membrane by the flat vesicles. *Equisetum hyemale* (Emons 1985) and *Limnobium stoloniferum* (Emons unpublished) have single particle rosettes in the PF-faces of the plasma membranes of the root hairs. Similarly these could be inserted in the plasma membrane by the secretory vesicles.

Secretory vesicles are smooth; they do not have the radial spokes of clathrin seen around coated vesicles.

Coated pits are seen in abundance on the plasma membrane of root hairs (Fig. 2b) and have been hypothesized to retrieve the excess membrane from the plasma membrane, which results from the incorporation of secretory vesicles (Emons and Traas 1986). Coated vesicles were only rarely seen near dictyosomes. In favourable sections coated pits were observed on the trans-face cisterna. They appear similar to the coated pits of the plasma membrane. They may function in membrane turn-over as has also been suggested by McFadden and Melkonian (1986) for the green flagellate *Scherffelia dubia*. A large polygonal vesicle as is seen in this flagellate is not present in the root hairs. In hormone secreting cells Orci and coworkers (1985) have detected a marked level of clathrin immunoreactivity at the trans-face of the Golgi apparatus only.

Dictyosome polarity in the root hair is typically expressed in terms of progressive differences in staining from one cisterna to the next within the stack. This structural gradient has been recorded for many plant and animal cells (Whaley and Dauwalder 1979, Shannon et al. 1982). In root hairs of *Equisetum hyemale* as in other cell types (Morré and Mollenhauer 1983, Morré and Ovtracht 1977) the polarity is from endoplasmic reticulum like at the cis-face to plasma membrane like at the trans-face. Evidence for the involvement of endoplasmic reticulum in the functioning of higher plant dictyosomes is controversial (Robinson 1980, review: Robinson 1985).

Although electron microscopy reveals the structural relationships of the cell cytoplasm and provides a basis for an assessment of the functional potential of the system, there is need to assess the pathway of molecules in the plant cell

by tracer studies. Recently, Kappler and coworkers (1986) showed in fractionated germinating tobacco pollen a rapid incorporation of [$^3$H]leucine into endoplasmic reticulum followed by a rapid chase out. The Golgi-apparatus fraction was labeled reaching a maximum 20 minutes post chase. Their results suggest membrane flow from endoplasmic reticulum to Golgi apparatus.

*Cytoplasmic streaming and wall texture*

It has been suggested that cytoplasmic streaming somehow influences microfibril orientation and therefore cell wall texture (Colvin 1980). The utmost 0.3 mm of the growing tip of *Equisetum hyemale* root hairs with little or no cytoplasmic streaming has a random cell wall texture (Emons and Wolters-Arts 1983); the tube of the young hair with cytoplasmic streaming up to 3.5 micrometers/sec has a helicoidal cell wall texture (Emons 1982, Emons and Wolters-Arts 1983); the full-grown hair with cytoplasmic streaming up to 7 micrometers/sec has a helical wall texture (Emons and Wolters-Arts 1983). One cannot, however, conclude that wall texture is directly related to cytoplasmic streaming, because root hairs of *Limnobium stoloniferum* (Pluymaekers 1982) and *Ceratopteris thalictroides* (Meekes 1985) both have helicoidal wall texture, but cytoplasmic streaming in *Limnobium* is fast and in *Ceratopteris* is barely visible.

*Cytoplasmic streaming and the cytoskeleton*

Cytoplasmic streaming is seen in the cytoplasm located between the plasma membrane and the vacuole membrane of root hairs. Microtubules are located in the utmost cortical region of the cytoplasm. Single filaments are sometimes observed in association with the microtubules (Fig. 9), as has also been shown by Tiwari and coworkers (1984). Microtubules with associated filaments occupy a sheath of cytoplasm of not more than 300 nm. Cytoplasmic streaming, however, is visible in the total compartment between plasma membrane and vacuole membrane and moves organelles as mitochondria and plastids. Also in *Nitella* and *Chara* internodal cells it has been found that microtubules are not in a position to be

part of a motive force generation system (review: Allen and Allen 1978).

The proximity of some secretory vesicles and microtubules might point to a transport function of these microtubules. However, only a minor number of vesicles is located near microtubules. By means of video-enhanced microscopy Allen and coworkers (1985) have shown transport of particles along microtubules. Using cytochalasin D, Picton and Steer (1981) showed that secretory vesicles in pollen tubes might be transported from the dictyosomes to the cell tip by a microfilament dependent process.

Bundles of filaments occur deeper in the cytoplasm (Fig. 10 a and b). Their morphology resembles the filaments seen in other higher plant cells (Tiwari et al. 1984), where they have been referred to as microfilaments. These bundles are probably the actin-microfilament bundles visualized with rhodamine-labelled phalloidin (Fig. 11). In thin sections filament bundles were not observed in the tip region of short, growing hairs of *Equisetum hyemale*. Rhodamine-labelled phalloidin staining produced a fuzzy staining in the tip and clear bundles in the tube of the hair. The bundles are mainly longitudinally oriented but do occasionally traverse the hair (Emons and Derksen 1986). Visible cytoplasmic streaming is also absent from the tip region of short, growing, hairs. Streaming is mainly in the longitudinal direction but does also traverse the hair occasionally. The data suggest that microfilament bundles play a role in cytoplasmic streaming as has been suggested before (Yamaguchi 1981, Pesacreta and Parthasarathy 1984, Parthasarathy 1985, Derksen et al. 1986). However, the number of microfilament bundles is not related to the velocity of cytoplasmic streaming. In *Ceratopteris* root hairs, which show a very slow cytoplasmic streaming, microfilament bundles occur in abundance (Derksen et al. in preparation). Cytoplasmic streaming in *Nitella* is caused by the interaction between subcortical filaments and an endoplasmic factor (Kamitsubo 1981).

Secretory vesicles do not need cytoplasmic streaming to reach the plasma membrane of the tip. In young hairs these vesicles are located in the part of the root hair where no visible streaming occurs.

ACKNOWLEDGEMENTS

I wish to thank Professor Dr. M.M.A. Sassen for his support, A.M.C. Wolters-Arts for technical assistence with thin-sectioning, Marluus Ruland for measuring cytoplasmic streaming in root hairs of Equisetum hyemale and Dr. J. Derksen for providing Fig. 11.

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CHAPTER 12

# A Mathematical Model for Helicoidal Cell Wall Deposition in Cells with Tip-Growth

# A MATHEMATICAL MODEL FOR HELICOIDAL CELL WALL DEPOSITION IN CELLS WITH TIP GROWTH

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extended version of a contribution presented at the 4th Cell Wall Meeting, Paris, 1986

## ABSTRACT

A mathematical model for the deposition of helicoidal wall texture is postulated. The model presupposes the insertion in the plasma membrane of microfibril inition complexes, which, once inserted are moved through the fluid plane of the membrane by the kinetic force of microfibril synthesis and leave microfibrils in their wake. The microfibrils are linked to wall matrix molecules. Microfibril orientation is ruled by the laws of geometry only.

KEY WORDS: helicoidal cell wall texture, mathematical model.

## INTRODUCTION

Spatial order can be used as a source of information about temporal order of events (Mendelson 1984). An example of spatial and temporal order in the biological world is the deposition of successive lamellae of the helicoidal cell wall of plant cells (Reis et al. 1985, Roland et al. 1983).

A helicoidal texture is defined as a stack of lamellae, each one fibril thick, in which the microfibril orientation is rotated with respect to the microfibril orientation of the previous lamella (Sargent 1978, Pluymaekers 1982, Emons 1982, Emons and Wolters-Arts 1983, Emons and van Maaren, submitted, Neville and Levy 1984, Neville 1985, 1986). The

helicoidal structure has not only been found in plant cells, but in several different biological materials (Bouligand 1972, Neville 1976).

Though microfibril orientation is generally believed to be determined by the orientation of the microtubules in the underlying cytoplasm, the nascent microfibrils of the helicoidal cell wall are not in parallel with the cortical microtubules (Emons 1982, Emons and Wolters-Arts 1983, Itoh and Brown 1984, Meekes 1985, Traas et al. 1985, Emons and Derksen 1986).

Because there is evidence that microfibrils in a helicoidal wall lengthen by tip growth (Emons 1985) it seems unlikely that the wall texture is formed like a liquid crystal, as has been proposed by Roland (1979) and Neville (1986).

A mathematical model can explain how helicoidal wall texture can be generated.

## THE CELL WALL TEXTURE OF THE ROOT HAIR OF EQUISETUM HYEMALE

To elucidate the organization of the cell wall texture, root hairs of stem cuttings of Equisetum hyemale have been studied by means of several methods: thin-sectioning, shadow-casting, dry-cleaving, freeze-fracturing, freeze-substitution (Emons 1982, Emons and Wolters-Arts 1983, Traas et al. 1985, Emons and Derksen 1986). Schematical representations of helicoidal wall texture in root hairs is shown in Figs. 1 and 2. In a growing root hair the number of lamellae and also the number of microfibrils within a lamella under deposition increases from hair tip to hair base (this thesis, chapter 5). Microfibrils grow from hair base to hair tip.

Particle rosettes and terminal complexes are the putative microfibril synthesizing structures (review: Brown 1985), and have been demonstrated in plasma membranes of root hairs of Equisetum hyemale (Emons 1985). The formation of microfibril bundles, and subsequently a lamella, by dispersed synthesizing complexes, is pictured in Fig. 3. Rosettes in the plasma membrane of Equisetum hyemale occur in large clusters. Reiss and coworkers (1984) have found areas in plasma membranes with and without particle rosettes in *Funaria* caulonema tip cells. The optical brightener Photine H.V.

revealed highly fluorescent bands and spots, which were supposed to be sites of cellulose synthesis in root hairs of *Peperomia* (Tampion et al. 1973). If particle rosettes are involved in cellulose microfibril formation, formation does not take place at the same time in the whole hair.

From the *Equisetum hyemale* root hair it is known that

1. the helicoidal wall is deposited against the inside of a cylinder: the randomly textured primary wall;
2. microfibrils have a constant diameter;
3. the number of microfibril lamellae increases from hair tip to base;
4. the number of microfibrils within a lamella increases from hair tip to base;
5. microfibrils within a lamella are not strictly parallel;
6. microfibrils are linked to wall matrix molecules;
7. rosettes in the plasma membrane occur in large clusters.

Assuming that rosettes and/or terminal globules are microfibril initiation points, it can be deduced that:

8. (from 3 and 4) a cluster of microfibril initiation points travels from hair base to hair tip;
9. (from 7) there are several clusters of microfibril initiation points, that pass along the hair;

It is further supposed that

10. microfibrils with the adherent matrix molecules pack in such a way that available surface is occupied completely, subjecting microfibril deposition to space-limiting conditions;
11. microfibrils have a given length.

## THE HELIX MODEL FOR HELICOIDAL WALL DEPOSITION

The geometrical relationships of the model can be demonstrated by wrapping string of constant width closely on a cylinder. Four variables define the system: 1) cylinder diameter, 2) string width, 3) pitch angle of the strings wrapped on the cylinder surface, 4) number of parallel strings on the cylinder surface.

The four parameters are related by the following equation: $\sin\varphi = N_{rel}$

in which $\varphi$ : pitch angle, $N_{rel} = N/N_{max}$
with N: number of strings, $N_{max}$: maximum number of strings, given by $N_{max} = D/d$
with D: cylinder diameter, d:string width.

$$\text{sine of pitch angle} = \frac{\text{number of strings x width of string}}{\text{cylinder diameter x } \pi}$$

This relationship holds for strings of any diameter, as long as all strings have the same diameter, for strings of any length, as long as all strings are longer than the cylinders' circumference and for strings of any composition. The relationship in which cylinder diameter and string width are constant and therefore pitch angle varies as a function of string number is given in Fig. 4.

Given a cylinder of constant diameter and strings of constant width, the orientation of a single string is nearly perpendicular to cylinder length, and its pitch angle is defined by the width of the string itself. However, when a second string of similar diameter is inserted in parallel, the pitch angle of the two-string array changes as shown in Fig. 4. Addition of new strings will cause constant shift of pitch angle until all strings lie parallel to the long axis of the cylinder, at which point the pitch angle of the string is 90°. The product of string number times string diameter will then equal cylinder circumference. No new strings can be added without increasing cylinder diameter.

Strings may be inserted at a single location on the cylinder. This is the simplest situation. But also a series of independent string insertion locations, may be present on a given length of cylinder. The helical wrapping may be right or left handed, but once initiated all strings pack parallel to the first string in the same helix hand.

Mendelson (1982) developed the helix model of cell growth and regulation for the helical growth of Bacillus subtilis.

Fig. 1.: Schematical representation of the cell wall texture of an Equisetum hyemale root hair: randomly oriented microfibrils in the outer layer of the whole hair and helicoidal texture consisting of superimposed lamellae with progressive rotation of microfibril orientation.

Fig. 2.: Schematical representation of helicoidal cell wall texture in section and in surface view.

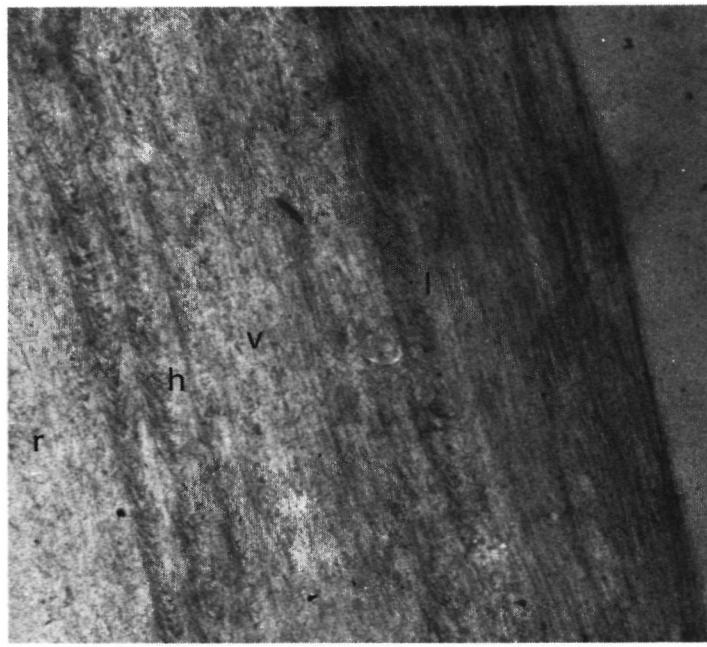
Fig. 3.: Schematical representation showing the formation of microfibril bundles and the resulting lamella by dispersed synthesizing complexes.

Fig. 4.: Relationship between helix pitch angle and number of parallel strings for helical wrapping of strings on a cylinder under space limiting conditions.

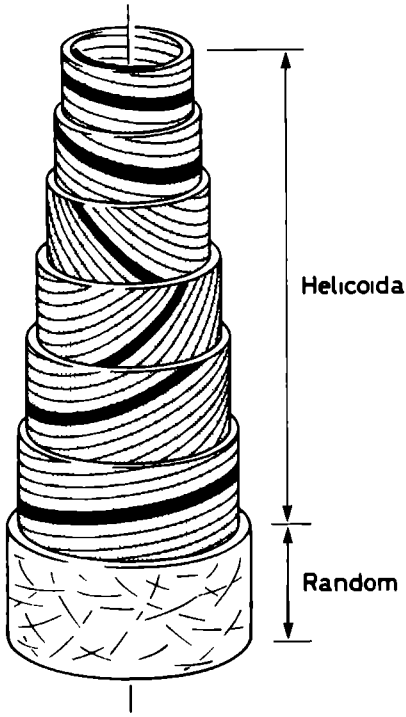
Fig. 5.: Schematical drawing showing microfibril orientation in developmental stages of microfibril deposition according to the "helix model". The hair is cut open lengthwise, revealing the complete circumference of the hair in a plane. For explanation see text.

Figs. 6a,b.: Schematical representation showing part of the helix described by a single microfibril while encircling the hair. Fig.6a.: Pitch angle is constant. Fig. 6b.: Pitch angle changes constantly.

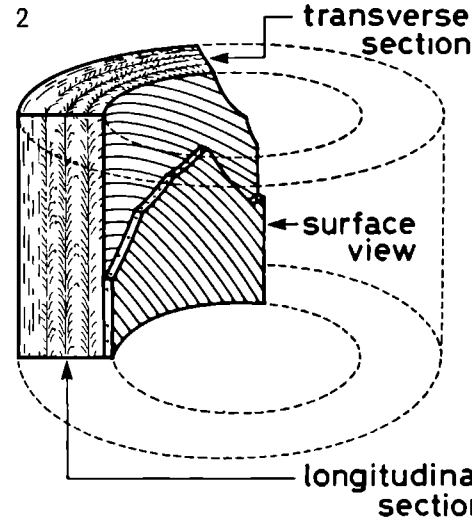
Fig. 7.: Longitudinal thin section of the cell wall of a full-grown root hair of Equisetum hyemale, showing the outer layer with randomly oriented microfibrils (r), three arcs of the helicoidal layer (h), a transition layer having microfibrils in various orientations except the transverse orientation (v), and the longitudinally inclined helical layer (l).



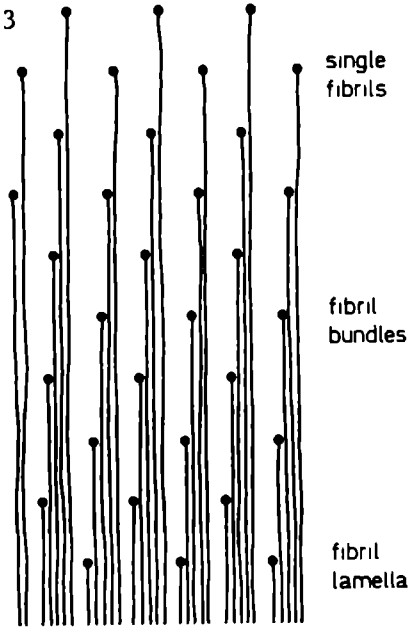
1


Helicoidal
Random

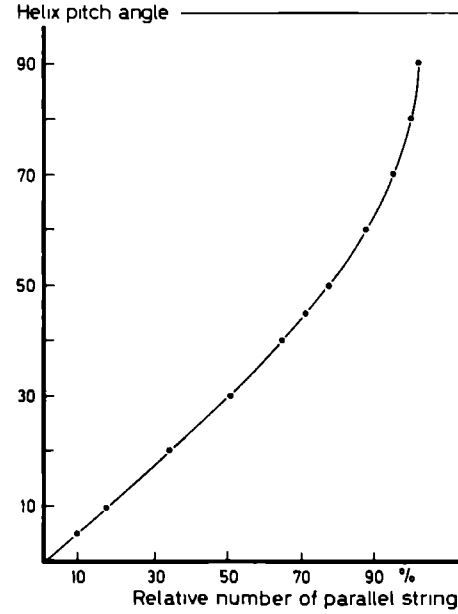
2


transverse section
surface view
longitudinal section

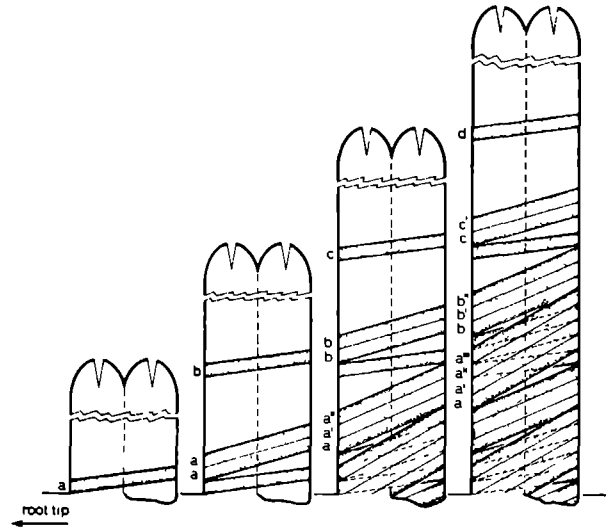
3


single fibrils
fibril bundles
fibril lamella

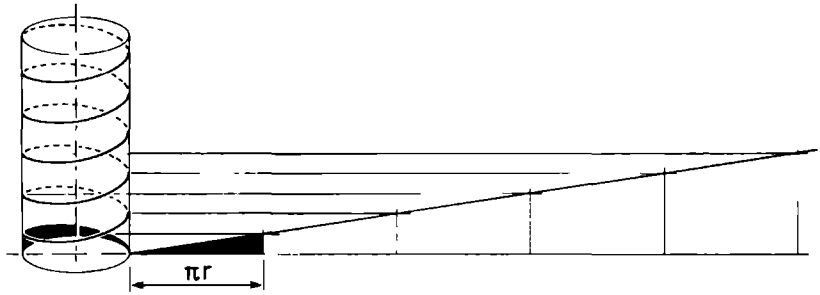
4


Helix pitch angle
90
70
50
30
10
10
30
50
70
90 %
Relative number of parallel strings

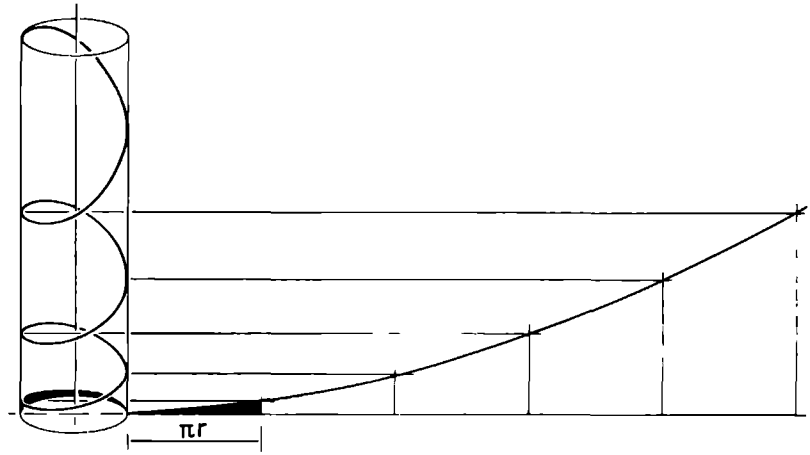
5




6




6b



The relationship in which cylinder diameter and string width are constant and therefore pitch angle varies as a function of string number represents the geometrical basis for secondary wall deposition in root hairs (Fig. 4). It must be noted, however, that in root hairs "string-insertion" occurs at the inside of the "cylinder".

A field of microfibril initiation points, probably particle rosettes and terminal globules, moves in the plasma membrane of the root hair. At the distal end of the field new microfibril initiation points are constantly added; at the proximal end they disintegrate after having completed the formation of a microfibril. Thus "string insertion" occurs via a mobile "insertion site", which moves in one direction at the inside of a "cylinder".

At (a) (Fig. 5) the first initiation point is brought into the membrane and circles the inner surface of the hair leaving a microfibril in its wake. According to the laws of geometry the orientation will be nearly transverse but will depend on microfibril width including adherent matrix molecules. Some time later two new microfibril initiation points are inserted: (b) and (a'). (b) lies above (a) and (a') lies at the same level as (a) is at this stage. Microfibril (b) will circle the inner surface of the hair in the same direction as (a) did before. Microfibrils (a,a'), however, will have a pitch angle larger than microfibril (a) had before. The same interval of time later three microfibrils will be inserted: (c), lying above (b), being single and travelling nearly transverse; (b'), lying at the level of (b), forming a two-string pattern on the hair with a pitch angle larger than (c), but similar to the two-string pattern (a,a') had before; (a'') joining (a,a'), forming a three- string pattern, with pitch angle larger than (a,a').

Up to this point there is little variation in orientation of microfibrils. Microfibrils do not cross and they form a lamella composed of "parallel" microfibrils deviating around a mean orientation. For the sake of simplicity, not every single microfibril is drawn in Fig. 5.: (a) contains x microfibrils, (a,a') contains 2x microfibrils, etc.

At a certain time starting at the base of the hair, new microfibrils cannot be added to the existing lamella and will have to cross the already apposed microfibrils. In Fig. 5 the array (a,a',a'',a''') crosses already apposed microfibrils. This is the onset of a new lamella. The orientation of the microfibrils in this array is still determined by the number of microfibrils in the array. While towards the tip of the hair the process of depositing a lamella with near transverse microfibrils is going on, a second lamella is started at the base with microfibrils in oblique orientation and is apposed against the earlier lamella with near transverse microfibrils.

The whole process is repeated until microfibrils in the last lamella under deposition lie more or less axially, which occurs first at the base of the hair. Towards the hair tip a lamella with microfibrils in oblique direction is under deposition and near the hair tip a lamella with transverse microfibrils is still being formed. Microfibrils in every lamella deviate around a mean orientation. A single microfibril describes a helix of continually changing pitch (Fig. 6), but the mean microfibril orientation in a lamella remains contstant (cf.this thesis, chapter 5). An individual microfibril takes part in several successive lamellae (Fig. 5) in time and its orientation is according to the curve of Fig. 4, an arc sine. A lamella is made up of microfibrils (in Fig. 5: a,a',a'', b,b',b'', c,c',c'', etc.) all describing the same part of this arc sine. The angle between lamellae is determined by the proportion of the curve, that microfibrils can describe without crossing. This proportion is determined by the parameters of the equation and by the distance between the insertion sites of (a) and (b).

In Equisetum hyemale root hairs the lamella directly following the axially oriented lamella is oblique, but in the reversed helix-hand. If microfibril initiation points have a limited time span and move in clusters, their number decreases if no new microfibril initiation points are inserted in the membrane. The orientation of individual microfibrils changes in a process which is the reverse of what has been described above. Pitch angle will decrease from 90° to 0°.

The last initiation point will move transverse to the cylinder axis. One helicoidal arc has been completed.

A new cycle may be started.

## DEDUCTIONS FROM THE MODEL

$$\text{sine of pitch angle} = \frac{\text{number of strings x width of string}}{\text{cylinder diameter x } \pi}$$

1. Cell dimension (D) determines microfibril orientation.

If cylinder diameter decreases while number of strings and width of strings are constant, helix angle increases: the microfibril angle will be transverse over a shorter stretch of hair. A still narrower cylinder will have helical or axial wall texture. Indeed the cell wall texture of the young Equisetum hyemale root hair, which has a larger diameter, is helicoidal, whereas the wall texture of the full-grown hair, which, due to extensive wall deposition, has a much smaller diameter, is helical (Fig. 7).

2. Size, shape and density of microfibril initiation field (N) determines microfibril orientation.

If number of strings increases, while cylinder diameter and width of strings are constant, the axial orientation is attained sooner. If the field of microfibril initiation points has a wide front, transverse microfibril orientation does not occur. The insertion of microfibril initiation points is a cell regulated process.

3. Cellulose-linked matrix polysaccarides determine microfibril orientation

Width of cellulose microfibrils of higher plant cell walls is constant (3 to 4 nm in thin sections, 6 to 10 nm in shadowed preparations (Emons and Derksen 1986, Preston 1974), but microfibrils are linked to other molecules in the cell wall (Albersheim 1976, Hayashi and Maclachlan 1984). Therefore, the matrix molecules highly determine string width and therefore orientation.

On the basis of results indicating a correlation between a local concentration in glucuronoxylan and the helicoidal

transition zone in Linden wood, Vian and coworkers (1986) concluded that glucuronoxylans, which constitute the major hemicellulosic component of the wall matrix, act in the formation of helicoidal assembly of cellulose.

## THE EFFECT OF COLCHICINE ON CELL WALL TEXTURE

The application of colchicine on plant cells does not have a uniform effect on cell wall texture. After colchicine treatment the orientation of microfibrils in a cell wall may be "random" (Schnepf et al. 1975), "in swirls" (Hepler and Fosket 1971), helicoidal (Quader 1978), unidirectional (Quader 1978), or undisturbed (Itoh 1976).
Colchicine depolymerizes microtubules; microtubules determine cell shape (Dustin 1984). In the model, cell shape determines cell wall texture.
Colchicine depolymerizes microtubules; microtubules function in exocytosis of matrix material containing vesicles (Dustin 1984). In the model the wall matrix determines cell wall texture by determining the intermicrofibrillar space.

The model predicts that if the diameter of a root hair with axial wall texture increases, while the number of microfibril initiation points and width of microfibrils with adherent matrix molecules remains constant, the helix-pitch angle will change and a helicoidal-like wall with microfibrils in varying orientations will be formed. But, if the diameter of a root hair with helicoidal wall texture increases, the helicoidal wall texture will be preserved. A high increase of hair diameter in a short hair will lead to transverse layers only.

Colchicine was applied to roots of Equisetum hyemale and Raphanus sativus. (this thesis, chapter 7). The treatment depolymerized microtubules and widened root hairs. The cell wall texture in Equisetum hyemale remained helicoidal, as before treatment. The newly formed wall texture of Raphanus sativus root hairs, however, contained lamellae with microfibrils in oblique and even transverse orientations.

ACKNOWLEDGEMENT

I am indebted to Dr. J. Molenaar, Mathematical Consulting Services University of Nijmegen, for mathematical advice.

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# SUMMARY

# SUMMARY

The cell wall of higher plants constists of a configuration of crystalline microfibrils embedded in a matrix of proteins and non-cellulosic polysaccharides. The configuration of microfibrils constitutes the texture of the cell wall. This thesis reports on an investigation of cell wall texture and cytoplasmic structures, that are thought to contribute to microfibril deposition.

As a prototype of a higher plant cell the root hair was chosen as object of study because of its accessibility to chemical and physical fixation and the possibility to follow cell growth in vivo. The wall texture, the microfibril deposition and the cytoplasmic structures supposed to contribute to wall deposition as cortical microtubules, microfilaments, the inner faces of the plasma membrane, the cytoplasmic side of the plasma membrane with coated pits and the Golgi-derived wall vesicles were studied.

Most root hairs of aquatic plants have a helicoidal wall texture. But this wall texture is found also in root hairs of some terrestrial plants. With the exception of the grasses wall texture coincides with a special type of trichome, the root hair initiating cell (chapter 1). The wall textures found in root hairs of the European species of the genus *Equisetum* lends support to the division of this genus in the two subgenera *Equisetum* and *Hippochaete* (chapter 2).

The cell wall texture of young root hairs of *Equisetum hyemale* is helicoidal. A helicoidal wall consists of a stack of microfibril lamellae. Microfibril orientation in successive lamellae is rotated with respect to the orientation in the previous lamella. In the cell wall of the young *Equisetum hyemale* root hair density of microfibrils in a lamella is 5 to 7 per micrometer, angle between fibril orientations in adjacent lamellae is 30° to 40°, and rotation mode of the helicoid is counter clockwise (chapters 4 and 5). In the tip-growing root hair, microfibril lamellae are stacked vertically but also lie next to one another horizontally.

Thus, along the hair tube microfibril lamellae with microfibrils in subsequently differing orientations lie adjacent to the plasma membrane (chapters 4 and 5). The cortical microtubules, however, align in the axial direction of the hair only, and thus do not coorientate with the nascent microfibrils (chapters 3, 4 and 5). Nor do microtubules and microfibrils coorientate in the trichoblastic part of the cell during root hair initiation and growth (chapter 6). The observation, based on thin-sectioning of chemically fixed material, that nascent microfibrils are not parallel to the cortical microtubules was verified by means of freeze-substitution (chapter 5). The observations contradict the currently held hypothesis that the orientation of microtubules determines microfibril orientation.

In the expanding root hair tip, microtubules show a random configuration. In the transition area of hemisphere and tube they are gradually more axially aligned, and in the hair tube they align axially (chapter 5). It is hypothesized that microtubules in plant cells play a role in morphogenesis.

The application of colchicine causes depolymerisation of most of the microtubules of root hairs of *Equisetum hyemale* and *Raphanus sativus*. The wall texture deposited during colchicine treatment remains helicoidal. In *Raphanus sativus*, however, a root hair with axial microfibril alignment, colchicine, in a concentration that depolymerized microtubules, effects the formation of a wall texture containing lamellae with oblique and even transverse microfibril orientations (a helicoidal-like cell wall texture) (chapter 7). The data are in keeping with the hypothesis drawn from ultrastructural data found in chapters 3, 4, 5 and 6.

The plasma membrane lying adjacent to the microfibrils of the helicoidal wall of *Equisetum hyemale* root hairs contains the putative microfibril synthesizing complexes: terminal globules in the extraplasmic (EF) face and particle rosettes in the protoplasmic (PF) face. Their presence indicates that in a helicoidal wall the microfibrils lengthen by tip-growth (chapter 8). This contradicts the hypothesis that helicoidal walls form like liquid crystals in a self-assembly process.

The complexes disappear after loss of turgor of the cells, even by fixation of the cells in glutaraldehyde, which explains why they have often not been found in freeze-fractured plasma membranes of plant cells.

Particle patterns in plasma membranes of root hairs are compared with patterns in pollen tubes and other cells. It is hypothesized that particle rosettes are configurations of protein in the membrane, allowing microfibril precursors to pass the membrane (chapter 9). In this view, terminal globules of the EF-face of the membrane might be the microfibril synthesizing enzymes. Isolation and biochemical characterization of terminal complexes, i.e. particle rosettes and terminal globules, is needed to understand the process of microfibril formation.

The cytoplasmic side of growing root hairs bears many coated pits, in the expanding tip as well as in the non-expanding tube, whereas full-grown hairs have a much smaller number of pits (chapter 10). From the ultrastructural data obtained and from additional data from the literature it is hypothesized that coated pits in plant cells function in endocytosis.

Golgi-derived wall vesicles are incorporated in the plasma membrane of the tip of growing hairs. They release their contents against the cell wall. The excess membrane is retrieved from the plasma membrane by coated pits. There is a clear structural polarity of the dictyosome from cis-face to trans-face: in membrane staining and in inter- and intra-cisternal width of the dictyosome and the dictyosome-derived vesicles (chapter 11). Coated pits near dictyosomes might function in turn-over of excess membrane of dictyosome stacks.

Cytoplasmic streaming is absent from the apical part of the growing root hair. During hair growth, velocity of streaming increases from 3.5 micrometers per second in the subapical part of young hairs to 7 micrometers per second in the whole tube of full-grown hairs. The pattern of streaming is related to the pattern of microfilaments (chapter 11).

A mathematical model for helicoidal wall deposition is proposed (chapter 12). In this model mobile microfibril initiation complexes are inserted in the plasma membrane.

The complexes move in the fluid plane of the membrane, leaving microfibrils in their wake. Microfibril orientation is forced upon the depositing microfibrils by the laws of geometry only. A single microfibril describes a helix of continually changing pitch on the cell cylinder, an arc sine. The orientation of the microfibrils depends on i) the density of microfibril initiation complexes in the plasma membrane, ii) the amount and composition of matrix polysaccharides linked to the cellulose microfibrils and iii) cell dimension.

# SAMENVATTING

# CELWANDVORMING IN WORTELHAREN
## De rol van microtubuli en plasma membraan in de afzetting van microfibrillen.

## SAMENVATTING

De celwand van hogere planten bestaat uit een configuratie van kristallijne cellulose microfibrillen, ingebed in een matrix van andere polysacchariden en eiwitten. Deze configuratie van microfibrillen noemt men de textuur van de celwand. Van wortelharen van hogere planten zijn deze textuur en de cytoplasmatische structuren, die mogelijk bijdragen aan de totstandkoming ervan onderzocht.

De wortelharen van de meeste onderzochte waterplanten hebben een helicoidale celwandtextuur (H.1). Anders dan voorheen aangenomen is, komen er onder de landplanten echter ook soorten voor met een uitsluitend helicoidale textuur in de celwand van de wortelharen. Met uitzondering van de grassen lijkt er een verband te bestaan tussen een bepaald type trichoblast, de wortelhaarvormende cel, en de textuur van de celwand (H.1).

De verschillen in de textuur van de celwand in wortelharen van het genus *Equisetum* is in overeenstemming met de opsplitsing van dit genus in twee subgenera (H.2).

*Equisetum hyemale*, schaafstroo, heeft in de jonge wortelharen een helicoidale celwand. Een helicoidale wand is opgebouwd uit lamellen van fibrillen. Een lamel is een fibril dik en fibrillen in een lamel lopen ongeveer parallel. De richting van fibrillen in opvolgende lamellen maakt een constante hoek met de richting in de vorige lamel. Voor jonge wortelharen van Equisetum hyemale is de dichtheid van microfibrillen 5 tot 7 per micrometer, de hoek tussen de fibrillen in opvolgende lagen 30 tot 40 graden, en de richtingsverandering van microfibrillen in opvolgende lagen tegen de wijzers van de klok in (H.4 en 5).

De corticale microtubuli in jonge wortelharen van *Equisetum hyemale* lopen min of meer in de lengterichting van de cel (H.3, 4, en 5), dus niet evenwijdig aan de microfibril-

len, zoals voorkomt in de meeste onderzochte plantecellen. In de top van groeiende wortelharen vertonen microtubuli en microfibrillen beide een 'random' patroon. Daarom is de hypothese opgesteld, dat microtubuli weliswaar niet rechtstreeks microfibrillen richten, maar wel een morfogenetische factor zijn, evenals de microfibrillen (H.5 en 6). Tijdens de uitgroei van de wortelhaar zijn ook in de trichoblast microtubuli en microfibrillen niet parallel (H.6).

Behandeling van wortels met colchicine veroorzaakt depolymerizatie van de meeste microtubuli in wortelharen van *Equisetum hyemale* en *Raphanus sativus*, radijs, en een vergroting van de diameter van de wortelhaar. De celwand afgezet na colchicine behandeling is in *Equisetum* helicoidaal, evenals in onbehandelde haren. In *Raphanus sativus*, een haar met van nature axiale microfibrillen, worden door de colchicine behandeling microfibrillen in verschillende richtingen afgezet; dit lijkt meer op een helicoidale celwandtextuur (H.7). Deze data zijn in overeenstemming met de hypothese, dat microtubuli een rol spelen bij de vormbepaling van een cel (H.5 en 6), dat microtubuli niet nodig zijn om een helicoidale wand te vormen (H.3, 4, 5 en 6) en dat de diameter van de cel mede de textuur van de celwand bepaalt (H.12).

De plasma membraan van groeiende wortelharen van *Equisetum hyemale* bevat in het PF-vlak de rozetten en in het EF-vlak de terminale complexen, waarvan wordt aangenomen dat zij samen het microfibril-synthetiserende-complex van de hogere planten vormen (H.8). De aanwezigheid van deze complexen verwijst ernaar, dat microfibrillen ook in helicoidale wanden groeien aan de top en niet, zoals wel is verondersteld, crystalliseren als "liquid crystals" in de periplasmatische ruimte.

Het verband dat gelegd kan worden tussen de hexagonale patronen en de rozetten, het kleinste hexagonale patroon, kan een aanwijzing zijn dat de rozetten niet de microfibril synthetiserende enzymen zelf zijn, maar plaatsen in de membraan waar microfibril precursors de membraan passeren (H.9). Volgens deze hypothese zijn de terminale complexen in het EF-vlak de microfibril synthetiserende enzymen. De isolatie en

biochemische karakterisering van het microfibril synthetiserende complex zal een belangrijke stap zijn in het celwandonderzoek.

Op de cytoplasmatische kant van groeiende wortelharen komen vele 'coated pits' voor, zowel in de expanderende top als in de niet-expanderende buis, terwijl volgroeide wortelharen slechts weinig 'coated pits' hebben (H.10).

De dictyosomen vertonen een duidelijke polariteit van cis- naar trans-cisternae (H.11). De blaasjes aan de transcisternae hebben dezelfde structuur en inhoud als de blaasjes, die langs de plasma membraan en met name in de groeiende wortelhaartop voorkomen. De blaasjes brengen waarschijnlijk matrix moleculen in de wand en enzymen voor de cellulose synthese in de membraan. Het surplus aan membraan dat ingebracht wordt, wordt via de 'coated pits' weer in de cel gebracht (H.10). 'Coated-pits' bevinden zich ook aan de trans-cisternae van de dictyosomen (H.11).

In het apicale deel van groeiende wortelharen komt geen lichtmicroscopisch zichtbare cytoplasma stroming voor. De snelheid van stroming neemt toe van 3 micrometer/sec in jonge wortelharen tot 7 micrometer/sec in volgroeide haren (H.11). Het patroon van cytoplasma stroming is gecorreleerd aan het patroon van microfilamenten. Deze microfilamenten bestaan waarschijnlijk uit F-actine (H.6 en 11). Een rol van cytoplasma stroming in het orienteren van de microfibrillen zoals wel is verondersteld, kon niet aangetoond worden.

Een mathematisch model voor helicoidale wandvorming wordt gepresenteerd (H.12): microfibril-initierende-complexen bewegen in de membraan en vormen microfibrillen. De richting van de microfibrillen wordt uitsluitend bepaald door geometrische wetten. Een individuele microfibril beschrijft een helix met een steeds veranderende stijghoek, een boogsinus. Lamellen worden gevormd door niet overkruisende microfibrillen, die eenzelfde deel van deze boogsinus beschrijven. De orientatie van de microfibrillen is afhankelijk van i) de dichtheid van de microfibril-initierende-complexen in de plasma membraan, ii) de hoeveelheid en aard van matrix moleculen, die tegelijk met de microfibrillen door de cel afgezet worden, iii) de celdiameter.

# CURRICULUM VITAE

# CURRICULUM VITAE

Anna, Maria, Catharina Emons is op 15 mei 1942 geboren te Milsbeek. In 1961 behaalde zij het diploma gymnasium B aan het lyceum Mater Dei te Nijmegen.

Van 1961 tot 1962 werkte zij als analiste op het zoölogisch laboratorium van de Universiteit Nijmegen bij drs. A.M.Th. Beenakkers aan het onderzoek "Vetzuuroxidatie in de vliegspieren van Locusta migratoria" en behaalde het diploma Leerling-analist, zoölogische richting.

In 1962 begon zij met de studie biologie aan de Universiteit Nijmegen. In 1963 en 1964 was zij studentassistent bij de afdeling zoölogie en assisteerde als vrijwilliger Dr. H.C.J. Oomen en Dr. J.F.M. Geelen bij bronnenonderzoek. In 1965 was zij studentassistent en daarna tot 1969 candidaatsassistent bij de afdeling botanie. In oktober 1966 legde zij het candidaatsexamen af. In 1968 deed zij een onderzoek naar de geschiedenis van de alpenfloristiek aan het Conservatoire Botanique in Genève. Op 1 april 1969 behaalde zij het doctoraalexamen, met de aantekening "cum laude". Het doctoraalexamen omvatte het hoofdvak Plantenfysiologie[1], bewerkt onder leiding van Prof. Dr. H.F. Linskens en het bijvak Submicroscopische Morfologie[2], bewerkt onder leiding van Dr. M.M.A. Sassen. Als tweede bijvak bestudeerde zij de Natuurfilosofie bij Prof. Dr. A.G.M. van Melsen.

Van 1969 tot 1973 gaf zij biologielessen en daarnaast van 1971 tot 1973 lessen in de gezondheidszorg aan het St. Stanislas college te Delft. Van 1974 tot 1976 gaf zij biologielessen aan de Rijksscholengemeenschap te Meppel en van 1978 tot 1980 aan het Rhedens Lyceum te Velp.

Van 1980 tot 1986 was zij werkzaam als wetenschappelijk medewerker aan de afdeling Submicroscopische Morfologie van de Universiteit Nijmegen voor het in dit proefschrift beschreven onderzoek naar de celwandvorming in wortelharen. Tevens leverde zij een bijdrage aan het onderwijs: eerste jaars practicum botanie, derdejaars cursus electronenmicroscopie, stagebegeleiding van doctoraalstudenten. Zij nam deel aan celbiologische congressen in Göttingen, Fribourg, Norwicn, Cambridge en Parijs. Zij bezocht het John Innes Institute, Cell Biology Department, te Norwich en het Laboratoire de Biologie Végétale, Cytology Expérimentale, Ecole Normale Supérieure te Parijs.

Vanaf 1 september 1986 heeft zij een tijdelijke aanstelling als wetenschappelijk medewerker aan de afdeling Plantencytologie en -morfologie van de Landbouwhogeschool te Wageningen.

Van 1982 tot 1984 was zij secretaris en van 1984 tot 1986 voorzitter van de Commissie voor de Plantenmorfologie, -anatomie en -cytologie van de Koninklijke Nederlandse Botanische Vereniging. Vanaf 1986 is zij vice-voorzitter van het bestuur van de Koninklijke Nederlandse Botanische Vereniging en hoofdredacteur van het tijdschrift Acta Botanica Neerlandica. In 1985 heeft zij binnen de Nederlandse Vereniging voor Celbiologie de werkgroep Plantecelbiologie opgericht, waarvan zij momenteel werkgroepcoördinator is.

Zij is gehuwd en heeft drie kinderen.

[1]G.W.M. Barendse, A.S. Rodrigues Pereira, P.A. Berkers, F.M. Driessen, A.M.C. Emons, H.F. Linskens: Growth hormones in pollen, styles and ovaries of Petunia hybrida and of Lilium species, 1970, Acta Bot. Neerl. 19, 175-186.

[2]M.M.A. Sassen, A.M.C. Emons, A. Lamers, F. Wanka: Cell wall formation in Chlorella pyrenoidosa: a freeze-etching study, 1970, Cytobiologie 1, 373-382.

STELLINGEN

behorende bij het proefschrift

# CELL WALL FORMATION IN ROOT HAIRS

## Role of plasma membrane and cytoskeleton in microfibril deposition

ANNE MIE C. EMONS

I

Bij de helicale celwandtextuur van wortelharen valt de as van de mathematische figuur samen met de as van de wortelhaar, terwijl bij de helicoïdale textuur de as van de mathematische figuur loodrecht staat op de as van de wortelhaar.

II

Aangezien glucuronoxylanen een rol lijken te spelen in de helicoïdale assemblage van cellulose (Vian et al.), verdient het aanbeveling om te onderzoeken of er verschillen in matrixmateriaal zijn tussen wortelharen met verschilende celwandtexturen.

Vian, B., Reis, D., Mosiniak, M., Roland, J.C., Protoplasma 131, 185-190, 1986

III

Albersheims interpretatie van het boogjespatroon, dat zichtbaar is in doorsneden van helicoïdale celwanden, als cellulose microfibrillen met aangehechte polysacchariden, past goed in zijn concept van de celwand, maar is onjuist.

Albersheim, P., Scientific American 232 (4) 81-95, 1975

IV

De diameter van microfibrillen in de celwand van de alg Boergesenia forbesii is niet 300 nm (Brown 1985), maar 30 nm.

Brown, R.M., Jr., J.Cell Sci., suppl. 2, 13-32, 1985

## V

In het boek "Cell Components" is te weining aandacht besteed aan het Golgi-apparaat, het cytoskelet en de coated pits.

Linskens, H.F., Jackson, J.F., eds.: Cell Components, Modern Methods of Plant Analysis, New Series Volume 1, 1985, Springer-Verlag, Berlin Heidelberg New York Tokyo

## VI

Protofilamenten van ongekleurde microtubuli, zichtbaar gemaakt door middel van cryo-electronenmicroscopie, vertonen een rechtshandige draaiing rond de as van de microtubulus.

Mandelkow, E.M., Mandelkow, E., J. Mol. Biol. 181, 123-135, 1985

## VII

Het feit, dat bij een gekruiste helicale celwandtextuur in wortelharen van de broccoli een axiaal patroon van microtubuli voorkomt en bij eenzelfde textuur in wortelharen van de ui de microtubuli in verschillende richtingen liggen, (Lloyd en Wells 1985), ondergraaft de stelling van Lloyd en Wells (1985), dat helicale patronen van microtubuli in wortelharen overeenkomen met de richting van de jongste microfibrillen van de celwand.

Lloyd, C.W., Wells, B., J. Cell Sci. 75, 225-238, 1985

## VIII

Op grond van de door Heuser en Kirchhausen gerapporteerde data over clathrine-assemblages moet het door Crowther en Pearse opgestelde schema voor de assemblage van triskelions tot polygonale clathrine-structuren herzien worden.

Heuser, J., Kirchhausen, T., J. Ulstrastr. Res. 92, 1-27, 1985
Crowther, R.A., Pearse, B.M.F., J. Cell Biol. 91, 790-797, 1981

## IX

De gist Saccharomyces cerevisiae heeft geen clathrine nodig voor celgroei en eiwitsecretie.

Payne, G.S., Schekman, R., Science 230, 1009-1014,1985

X

Myosine is aanwezig in hogere planten en vertoont tijdens de cytokinese eenzelfde verdeling als actine.

Parke, J., Miller, C., Anderton, B.H., Eur. J. Cell Biol. 41, 9-13, 1986

XI

Tabaksexplantaat, gebruikt door Tran Than Van en medewerkers, is een te complex systeem om te onderzoeken welke rol oligosaccharinen uit de celwand spelen in de cel.

Tran Than Van, K., Toubart, P., Cousson, A., Darvill, A.G., Collin, D.J., Chelf, P., Albersheim. P., Nature 314, 615-617, 1985

XII

In de cuticula van de Drosophila-pop gaan verschillen in helicoïdale gelaagdheid samen met verschillen in eiwitsamenstelling.

Wolfgang, W.J., Firstrom, D., Firstrom, J.W., J. Cell Biol. 102, 306-311, 1986

XIII

Hadleys gebruik van de term "lamella" is niet conform het gebruik in de literatuur over helicoïdale wanden van plantecellen.

Hadley, N.F., Scientific American 255 (1) 98-106, 1986

XIV

Vermindering van het aantal niveaus van voortgezet onderwijs leidt tot een lager gemiddeld niveau van de leerlingen.

John H.A.L. de Jong, Studies in Educational Evaluation 12 (3), 1986, in press.

XV

In het Disciplineplan Biologie 1986 is het onderzoek van cytoskelet, celmembraan, celwandvorming en celwandafbraak van plantecellen ten onrechte ingedeeld bij plantenmorfologie.

## XVI

De verwerking in tandpasta van Equisetum hyemale (schaafstroo), een plant met een sterk siliciumhoudende stengel, wijst erop dat men zijn tanden dient te onderhouden als gereedschap.

## XVII

De vrouwelijke natuurwetenschapper lijkt gevangen tussen twee tegengestelde idealen. De westerse cultuur van de negentiende eeuw accepteerde voor vrouwen slechts zachtheid, gevoeligheid en niet-competitieve activiteiten, terwijl de zich in dezelfde tijd ontwikkelende natuurwetenschap werd gezien als nuchter, zakelijk en competitief. Een synthese van deze idealen is mogelijk.

## XVIII

Tweede-kans-onderwijs impliceert vernederingen voor degene die dit onderwijs geniet.

## XIX

De bewering van Rousseau: "A tout âge l'étude de la nature émousse le goût des amusements frivoles, prévient le tumulte des passions, et porte à l'âme une nourriture qui lui profite en la remplissant du plus digne objet de ses contemplations" geldt voor de biologie als zuivere wetenschap en niet voor de biotechnologie.

Rousseau, J.J., "La Botanique", lettre première, 1771