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Xylogenesis in Tissue Culture: An Immunofluorescent Study of the Changes in
Cortical Microtubule Arrays During Tracheary Element Differentiation

University — Université

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Degree for which thesis was presented — Grade pour lequel cette thèse fut présentée

M.Sc.

Year this degree conferred — Année d'obtention de ce grade

1985

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XYLOGENESIS IN TISSUE CULTURE

An Immunofluorescent Study of the Changes in Cortical
Microtubule Arrays During Tracheary Element Differentiation

by

Marcia M. Falconer, B.Sc.

A thesis submitted to the Faculty of Graduate Studies and
Research in partial fulfillment of the requirements for the
degree of Master of Science

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ABSTRACT

Immunofluorescent and Calcofluor/Hoechst staining of developing tracheary elements (TE) in Zinnia mesophyll cell cultures allows detection of three stages of differentiation:

1) Microtubules (mts) grouped into bands without evident secondary wall deposition; 2) Groups of mts subtending wall material only visible with Calcofluor/Hoechst staining; 3) Microtubule patterns reflected by Calcofluor/Hoechst staining, phase contrast and polarization optics.

In differentiating cultures, cortical mts reorient from longitudinal to transverse arrays as the culture ages. Taxol prevents this reorientation but does not inhibit TE formation. The orientation of secondary wall bands in TE which differentiate in taxol reflects the orientation of cortical microtubules at the time when they were stabilized by the addition of taxol.

These results allow the proposal of the following hypothesis: The role of mts in TE differentiation involves lateral association to form discrete bands typical of secondary wall patterns deposited during xylogenesis.

ACKNOWLEDGEMENTS

Special thanks and appreciation are due to Dr. Robert W. Seagull for suggesting this project, supporting it, providing stimulating discussions and advice and most of all - for being patient and considerate with a fledgling graduate student. Thank you.

A thank you is also due to Dr. George Setterfield for providing me with the initial opportunity to return to biology and for his continued interest in this project.

And finally, a special thank you to my husband, Dr. David Falconer, and our children, Ian and Leah, who supported me in this effort and who had to learn to cope with an absentee wife and mother.

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LIST OF ABBREVIATIONS

BA	6-benzylaminopurine
C/H	Calcofluor white and Hoechst #33258
DMSO	dimethylsulfoxide
EGTA	ethyleneglycol-N, N'-tetra-acetic acid
EM	electron microscope
FITC	fluorescein isothiocyanate
mfs	microfibrils
mts	microtubules
NAA	1-napthaleneacetic acid
PBS	phosphate buffered saline
PIPES	piperazine-N, N'-bis(2-ethane-sulfonic acid)
TE	tracheary element

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within 6 hours) and can encompass 50% or more of the total cell population (Fukuda and Komamine, 1980a, 1980b). Drug availability is uniform for all cells and penetration problems may be minimized by easy access to all cells. The influence of the whole plant, and to a large extent, the presence of neighboring cells has been removed. It was therefore the system of choice in which to study cytoskeletal involvement in differentiation of tracheary elements.

2) Xylem Differentiation - Protoxylem and Metaxylem

In the plant it is customary to distinguish between two types of xylem - the protoxylem and the metaxylem (O'Brien, 1981). The distinction is based upon the time when differentiation is completed in relation to the period of cell elongation.

Protoxylem matures before the cell has finished elongating (Esau, 1965). Secondary wall is deposited in annular rings or spiral bands with large areas of unlignified primary wall remaining between the bands (O'Brien, 1981). It is this area of primary wall which is capable of growth during cell elongation and can also expand after proteolysis. This allows the plant to maintain xylem vessel in functional condition throughout the periods of cell elongation (Esau, 1965).

The secondary wall of metaxylem cells is deposited after the period of elongation is completed (Esau, 1965). The large areas of primary wall, which allow for expansion in protoxylem cells, are no longer necessary - thus the secondary wall in metaxylem can be deposited

INTRODUCTION AND LITERATURE REVIEW

1) Introduction and Background

Tracheary elements (TEs) are the terminally differentiated cells which, upon lysis of the protoplast and selective removal of the cell end walls, will make up the mature xylem vessels in the plant (Esau, 1965). TE development is characterized by the deposition of secondary wall in various patterns. These patterns are visible with the light microscope and are birefringent under polarized light thus making them easily detectable markers of cell differentiation.

Due to this ease of observation, the study of developing xylem cells as an example of cellular differentiation goes back to the classical plant anatomists of the 19th century. Much of their work was descriptive and concerned locating areas of xylem differentiation in the root and shoot. However, one point raised by these investigators remains unsettled to date. As described by Sinnott and Bloch (1945), Crüger reported in 1855 that a pattern was formed in the cytoplasm of differentiating cells which predicts and precedes formation of the wall pattern. This was confirmed by Sinnott and Bloch (1945) and later by Hepler and Fosket (1971) among others. Ultrastructure analysis (Wooding and Northcote, 1964), (Cronshaw and Bouck, 1965), (Pickett-Heaps, 1967), (Maitre and De, 1971) has not shown anything which can be interpreted as the densely granular cytoplasm reported by Crüger in Sinnott and Bloch (1945). However, reports of accumulations of rough endoplasmic reticulum found between the wall thickenings (Pickett-Heaps, 1967), along with accumulations of dictyosomes and

vesicles (Maitre and De, 1971) and the grouping of microtubules which precede and accompany wall thickenings (Hepler and Newcomb, 1964), (Gunning, et.al., 1978) have all been confirmed. O'Brien (1981) has suggested that any or all of these organelles might produce enough of a phase change to give the appearance of bands in the cytoplasm. However, the existence of cytoplasmic prepatterning of wall bands remains an open question in the field of xylogenesis.

Many researchers have searched for the perfect system in which to study xylogenesis. Ideally this system consists of single cells which undergo synchronous differentiation into tracheary elements in a defined medium and under controlled environmental conditions. In the search for this perfect system, work has been done on roots and shoots, on wound vessel regeneration and on tissue culture.

All early studies of xylem were done by sectioning roots and shoots to provide an ordered sequence of events as one followed the increasingly more differentiated tissue through the elongation and maturation zones. Differentiation studies continue to make use of the whole root/shoot system with the elegant Azolla studies (Gunning, 1980) perhaps the best known. In the Azolla root, the planes of division (past, present and future) can be determined with geometrical precision allowing one to define the particular state of differentiation of each cell in the root. Drawbacks of this system include: differentiation is under the influence of conditions within the whole plant (Sachs, 1978) and cannot be experimentally manipulated with any precision; physical presence of neighboring cells may influence shape or other aspects of tracheary element differentiation; additionally, penetration of drug treatments may be a problem.

Wound regeneration of tracheary elements has been in use since the late 19th century (Sinnott and Bloch, 1945), (Hardham and McCully, 1982a, 1982b). This system is based on the wounding of vascular bundles causing previously unspecialized parenchyma cells to be reprogrammed into differentiating tracheary elements. With this system; TEs can be produced upon demand, however differentiation remains under the influence of the whole plant (Sachs, 1978) and drug penetrations and physical influences of neighboring cells continue to cause difficulties.

Tissue culture enables one to remove the influence of the whole plant. By manipulation of auxin and cytokinin combinations, TEs can be induced in callus explants from a wide variety of species (Kohlenbach et.al., 1982), (Dalessandro and Roberts, 1971), etc. However, these differentiation experiments in callus produced relatively low numbers of TEs. Callus tissue culture systems still have the problem of ensuring adequate and uniform drug penetration and, in callus growth, the cells are not free from at least the physical effects of neighboring cells.

A step toward achieving an ideal system in which isolated cells synchronously and totally differentiate into tracheary elements under controlled conditions was taken with the establishment of the Zinnia mesophyll culture system (Kohlenbach and Schmidt, 1975). Further characterized by Fukuda and Komamine (1980a, 1980b, 1981, 1983), this system uses single, isolated leaf mesophyll cells (or small groups of cells) which are suspended in defined medium and induced to differentiate into tracheary elements by addition of hormones. This differentiation is semi-synchronous, occurs rapidly (visible wall deposition is seen

within 6 hours) and can encompass 50% or more of the total cell population (Fukuda and Komamine, 1980a, 1980b). Drug availability is uniform for all cells and penetration problems may be minimized by easy access to all cells. The influence of the whole plant, and to a large extent, the presence of neighboring cells has been removed. It was therefore the system of choice in which to study cytoskeletal involvement in differentiation of tracheary elements.

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The secondary wall of metaxylem cells is deposited after the period of elongation is completed (Esau, 1965). The large areas of primary wall, which allow for expansion in protoxylem cells, are no longer necessary - thus the secondary wall in metaxylem can be deposited

in wide bands or in pitted patterns which provide strength for the xylem vessels at the expense of loss of extensibility (Esau, 1965).

Patterns of secondary wall deposition in TEs have been classified as annular, spiral, reticulate, scalariform and pitted (Cutter, 1978):

- Protoxylem consists of annular or spiral rings oriented at right angles to the long axis of the cell and is always elongated in shape (Sinnott and Bloch, 1945). Metaxylem cells are classified as having reticulate, scalariform or pitted patterns and have a tendency to be isodiametric in shape (Sinnott and Bloch, 1945).

One other distinction can be made between proto and metaxylem - that of microtubule susceptibility to colchicine as inferred by differences in wall patterns of treated TEs. Barlow (1969) has shown that proto and metaxylem in pea roots are differentially susceptible to colchicine. Protoxylem is unaffected by colchicine and cells differentiate to form secondary wall in typical, discrete annular rings or helices. Metaxylem, however, showed abnormal wall deposition in the presence of colchicine. Hepler and Fosket (1971) do not specifically address this point in their study on colchicine effects on differentiation but they do state that adding colchicine to wounded *Coleus* stems resulted in 87% of differentiating TEs showing abnormal secondary wall deposition while the remaining 13% had normal walls. While no mention is made of proto or metaxylem type cells, it is clear that colchicine affects wall deposition in some TEs and not in others.

3) Ontogeny of Wall Patterns

A very complete analysis of the ontogeny of proto and metaxylem was carried out on more than 1350 species by Bierhorst and Zamora (1965). They examined hundreds of thousands of tracheary elements with the light microscope in an attempt to determine how secondary wall patterns develop. Their results indicate that xylem secondary walls are formed by a two stage deposition.

The first stage of wall development is characterized by the deposition of secondary wall material in an annular, or more often, in a helical pattern which they term the first-order framework. This pattern is common to all tracheary elements with protoxylem ceasing to differentiate beyond this point while metaxylem continues on to the second stage of wall deposition.

In metaxylem, the second-order framework is deposited after completion of the first-order helical walls. Bierhorst and Zamora (1965) have suggested that all metaxylem cells deposit a second-order framework over the first-order one by: broadening of the helix and/or deposition of sheets of wall atop the first-order helix and/or formation of new, oblique thickenings between the rises of the helix. Criticism of this hypothesis (Roberts, 1976) rests on the relative paucity of intermediate states visible in the plant. This lack of observed intermediate wall patterns could, however, be explained if wall deposition is very rapid (as it is in the Zinnia system as shown by Fukuda and Komamine, 1980a).

To date there is no published analysis of TE wall pattern types which can be found to differentiate in tissue culture. Roberts (1976) states that TEs induced under experimental conditions nearly always

exhibit reticulate or scalariform wall patterns. He states that helical patterns are rare and there is no convincing evidence of annular thickenings forming in tissue culture. The Zinnia culture system had not been developed at the time when this was written. In this system annular and spiral TEs are common during the first burst of differentiation in the Zinnia system while reticulate, scalariform and pitted patterns dominate the later stages of differentiation.

4) Microtubule Involvement in TE Differentiation

Microtubules (mts) were first described in plant cells by Ledbetter and Porter (1963) who suggested they play a part in mitosis and in the orientation of cellulose microfibrils in the plant wall. In an effort to confirm the later role, Hepler and Newcomb (1964) looked at newly deposited cell walls in differentiating TEs of Coleus. They noted that in differentiating tracheary elements, groups of mts were localized beneath the plasma membrane in the cortical cytoplasm immediately adjacent to the developing bands of the secondary wall. These mts were associated with and ran roughly parallel to the particular wall bands observed with the electron microscope (EM). Microtubules were not found in the area between wall thickenings. This localization of mts in conjunction with the secondary wall band in tracheary elements has been confirmed by virtually all investigators who have looked for it.

Hepler and Newcomb (1964) also reported that the distribution of microtubules in relation to the wall thickening varies with the stage of differentiation. In newly differentiating TEs, mts are evenly distributed over the entire wall thickening. At a later stage, after

more secondary wall has been deposited, there is a tendency to see mts clustered along the sides of the thickening with fewer mts located near the center of the wall band.

Brower and Hepler (1976) were the first to report that the earliest stages of mt involvement in differentiation could be seen in EM cross sections of tracheary elements. Groups of mts were spaced at intervals along the primary wall before any visible deposition of secondary wall could be detected. Similar early grouping of mts into bands without wall deposits was reported to occur during differentiation in Azolla root (Hardham and Gunning, 1978). Interestingly, Gunning also observed that at this early stage, mts might be banded on one side of a cell and evenly distributed as cortical mts on the opposite side. The mt bands in Azolla were found, by serial sectioning to be composed of short (3-5 μ m) mts arranged in overlapping arcs (Hardham and Gunning, 1978).

It has been suggested (Gunning, 1978) that these bands of short mts arise from microtubule organizing centers (MTOCs) (reviewed by Brown, et.al., 1982) located in specialized areas along the cell edges. These areas, typified by electron dense staining and small vesicles (Pickett-Heaps, 1967), have also been identified by Galatis (1980) in differentiating stomatal guard cells - a system which also involves spatially restricted wall deposition. Gunning (1978) proposes that localized activation of such MTOCs will result in creation of spatially restricted bands of mts such as those found in developing xylem. Results from this thesis allow proposal of an alternate hypothesis for the generation of mt groups in differentiating cells - that of lateral association of microtubules (to be discussed in a later section).

5) Microtubule/Microfibril Interaction in Tracheary Elements

The correlation of microtubule orientation with that of newly deposited microfibrils in the wall bands of TEs has made differentiation of TEs a test system for investigating control of wall deposition (Hepler and Newcomb, 1964), (Cronshaw, 1965), (Esau, et.al., 1966), (Robards and Kidwai, 1972), (Srivastava and Singh, 1972) and others. The mt inhibitor, colchicine (reviewed by Luduena, 1979) has often been used to facilitate these investigations.

Colchicine binds to tubulin subunits resulting in the formation of a "cap" on the plus end of mts. This cap prevents further addition of subunits and thus allows net depolymerization to become the dominant reaction (Luduena, 1979).

Pickett-Heaps (1967) applied colchicine to root tips and found a loss of organized wall pattern although actual deposition of secondary wall material was not inhibited. This loss was correlated with the disappearance of mts normally seen near the developing walls.

Hepler and Fosket (1971) applied colchicine to regenerating wound elements. If applied early, colchicine prevented division and subsequent differentiation. If applied later, TEs appeared but displayed aberrant wall deposition with the secondary wall being "smeared" over the primary wall. They concluded that mts are not actively involved in synthesis of wall material but are necessary for the deposition of wall material in an oriented manner.

6) Microfibril Deposition

Secondary wall is primarily made up of cellulose microfibrils (mfs) (Taiz, 1984). A correlation between mfs and mts has been shown not only in the case of tracheary element differentiation, as mentioned above, but also for the algae, Oocystis (Robinson and Quader, 1982), and Poterioochromonas (Schnepf, et. al., 1975) as well as for the root cells in Zea Mays L. (Mueller and Brown, 1982a, 1982b) and in cotton fibers (Yatsu and Jacks, 1981). In all of these cases a very close degree of parallelism between the orientation of the microtubules and that of the wall microfibrils has been demonstrated implying some sort of connection between the two.

This connection between microtubule and microfibril orientation is still hypothetical, however the advent of free-etching allowed Mueller et. al. (1976) to show micrographs of terminal complexes located at the end of freeze-etch impressions of microfibrils. More complex structures, termed rosettes, were also identified (Mueller and Brown, 1980a). It is believed that these varying structures are enzyme complexes involved in cellulose synthesis and/or deposition at the level of the plasma membrane (Montezinos, 1982). However, no direct proof of this has been accomplished to date.

It is assumed that these cellulose synthetases move in the plane of the membrane to deposit long microfibrils in the cell wall. Heath and Seagull (1982) have classified hypotheses to explain this motion as Direct or Indirect relationships of mfs, complexes and mts. Direct hypotheses propose links (of various types) between the mf and its

synthesizing complex and the orienting mts in the cortical cytoplasm. The complex is then moved along either by the mts sliding, by actin/microtubule motion or by dynein-like proteins which move the complex along stationary mts. Indirect hypotheses (Heath and Seagull, 1982) suggest that mt directed membrane flow moves the synthetic complexes. Herth (1980) suggests that mts serve to restrict and channel plasma membrane fluidity and thereby guide mf deposition in a manner consistent with the orientation of the mts. Experimental confirmation of any of these hypotheses has not been demonstrated.

7) Microfibril Orientation and Cell Wall Expansion

While research continues on how mts orient mf deposition, the pattern of that deposition has been a source of controversy. EM sectioning has shown that walls are made up of layers with varying orientations of mfs. Just what these mf orientations are and how they change during cell extension has produced two schools of thought:

- 1) The Multinet Growth Hypothesis first proposed by Roelofsen and Houwink in 1953 and 2) The Ordered Fibril Hypothesis proposed by Roland et.al., 1979.

The modified multinet growth hypothesis proposes that cellulose is deposited transversely or in a shallow helix at the plasma membrane. It is deposited under zero stress and zero strain but becomes passively reoriented toward the longitudinal axis as cell extension puts strain on the mf in the cell wall.

The ordered fibril hypothesis suggests that mfs are deposited in alternating transverse and longitudinal layers thus building up a plywood-like structure for the cell wall (Lloyd, 1984). Growth of the cell would occur by selective release of bonds and inter-fibril sliding. It may be that the differences between these two hypotheses are due to oblique sectioning at the EM level and subsequent interpretation (or misinterpretation) of these micrographs perhaps compounded by genuine differences in wall deposition due to different species (Sargent, 1978).

C Current research shows a tendency to view these two hypotheses as compatible variations due to a wide spectrum of mf deposition patterns. Species variation in wall organization and varying techniques may satisfactorily explain the origins of two separate hypotheses which may soon be combined into one.

The following two chapters are comprised of manuscripts published in the journal, Protoplasma, during 1985. Manuscript I details culture methods and indirect immunofluorescent staining procedures. It also explores the involvement of microtubules in the formation of the secondary wall of tracheary elements as seen from the cell-wide vantage point allowed with indirect immunofluorescence. Manuscript II uses the procedures outlined in the previous section to examine the reorientation of microtubule arrays in the period before and during tracheary element differentiation. The microtubule stabilizing agent, taxol, was used to demonstrate that although microtubule reorientation normally occurs before TE differentiation is seen, this reorientation is not necessary for deposition of the secondary wall. Together, the results of the two manuscripts allow the formulation of a working hypothesis regarding microtubule involvement in tracheary element differentiation.

7

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Manuscript 1.

Immunofluorescent and Calcofluor White Staining
of Developing Tracheary Elements in Zinnia elegans L. Suspension Cultures

Published in Protoplasma 125, 190-198 (1985).

SUMMARY

7

Xylogenesis has been studied in primary suspension cultures of Zinnia elegans L. The wall patterns produced in culture closely resemble those described for intact tissues (annular, spiral, reticulate, scalariform and pitted). Using fluorescence microscopy and immunocytochemical techniques we have followed both the changes in wall deposition and microtubule organization during xylogenesis. A combination of Calcofluor white and Hoechst #33258 has been used to detect secondary wall deposition before it can be observed using either phase contrast or polarization optics. The development of tracheary elements can be divided into three stages: 1. microtubules grouped into bands without secondary wall deposition evident; 2. groups of microtubules subtending wall material only visible using the combination of Calcofluor and Hoechst stains; 3. a complex microtubule pattern reflected by well developed wall thickenings detected using Calcofluor and Hoechst, phase contrast and polarization optics.

1. INTRODUCTION

One of the best examples of the relationship between microtubule disposition and microfibril orientation is found in the study of xylogenesis. During the formation of tracheary elements, secondary wall microfibrils are deposited in localized bands to form patterns described as annular, spiral, reticulate, scalariform or pitted (Esau, 1965). The wall thickenings in these patterns are highly birefringent indicating the microfibrils are oriented in coherent, parallel bundles. Electron microscopy has shown that the distribution of microtubules in tracheary elements is limited to the cortical cytoplasm directly adjacent to the wall thickenings (Hepler and Fosket, 1971). In the presence of microtubule inhibitors, secondary wall is not deposited in patterns consisting of discrete bands but rather is found smeared over a wide area of the primary wall (Brower and Hepler, 1976).

Thus far all studies of the role of microtubules during xylogenesis have been restricted to the use of electron microscopy on shoots and roots (Pickett-Heaps, 1967, Hardham and Gunning, 1978). While this method provides high resolution data regarding the relationship between microtubules and microfibrils, it does not provide a view of the overall microtubule organization nor does it provide data concerning changes in microtubule populations which occur during xylogenesis. In addition, the study of xylogenesis in intact roots and shoots has been hampered by the relatively low number of cells undergoing differentiation into tracheary elements at any one time.

We have attempted to circumvent these problems by studying xylogenesis in tissue culture using indirect immunofluorescence microscopy of microtubules. Using the Zinnia leaf mesophyll culture system, developed and characterized by Fukuda and Komamine (1980a, b), it is possible to produce many tracheary elements at various stages of development. Immunofluorescent techniques allow an analysis of overall microtubule patterns and pattern changes which occur during xylogenesis. In addition, we have used a combination of Calcofluor White and Hoechst #33258 fluorochromes to detect very early secondary wall deposition before it is visible with either phase contrast or polarizing microscopy.

In this preliminary paper we demonstrate that this culture system closely parallels the intact root and shoot with regard to the various changes in microtubule arrays associated with cell division, expansion and differentiation. In addition, we document the overall changes in microtubule organization and secondary wall deposition which occur during xylogenesis.

2. MATERIALS AND METHODS

2.1 Plant Material.

Seeds of Zinnia elegans L. cv. Canary Bird (Stokes Seed Ltd., St. Catharines, Ont.) were grown in potting soil in controlled environment chambers under 12-hour light (13 klux fluorescent) periods, at 23⁰ and 12-hour dark periods at 21⁰C.

2.2 Cell Cultures

Culture methods were adapted from Fukuda and Komamine (1980). The first true leaves were removed from seedlings 14 days after planting. Leaves were surface sterilized in 0.9% sodium hypochlorite for 15 minutes, rinsed 3 x with sterile deionized water. All subsequent operations were done under sterile conditions. Fourteen leaves were placed in 100 ml of medium (Fukuda and Komamine, 1980) containing 0.2 mg/L NAA (1-Napthaleneacetic acid) and 2 mg/L BA (6-Benzylaminopurine) in a small blender jar (Osterizer) and blended at low speed for 15 seconds. The resulting cell suspension was filtered through 125 μ m nylon mesh to give single cells and small clumps (2-10 cells) of leaf mesophyll cells. The cells were centrifuged at 50 x g for 2 minutes, resuspended in culture medium and centrifugation repeated. Cells were counted in a haemocytometer and volume adjusted to give 1×10^5 cells/ml. 4 ml of this suspension were plated in 60 x 15 mm disposable, plastic petrie dishes, sealed with Parafilm, covered with aluminum foil and incubated on a shaker with 14 strokes/min at 27°C for up to 3 weeks.

2.3 Immunofluorescent Staining of Microtubules

Indirect immunofluorescent staining procedures were adapted from Simmonds et.al (1983). Culture cells (5 to 12 days post-culturing) were layered on coverslips previously cleaned in hot chromic acid, rinsed in water and coated with poly-L-lysine (molecular weight 190 Kd) (Sigma Chemical Co., St. Louis, Mo.). After cells settled (about 15 minutes) coverslips were transferred to freshly made 4% paraformaldehyde (Polysciences, Ltd., Markham, Ont.) in MSB-1 (0.1 M Pipes,

13 mM EGTA, pH 6.9) for 1 hour. Cells were washed 3 x 3 minutes in MSB-1. Cells were treated with 0.1% ultrapure cellulase (s.a. 39 units/mg) (Worthington Biochemical Co., Mississauga, Ont.) in MSB-1, for 5 minutes followed by 2 x 3 minute washes in MSB-1. Cells were extracted 1 hour with 1% w/v Triton X-100 in MSB-2 (50 mM Imidazole, 50 mM KCl, 0.5 mM MgCl, 1 mM EGTA, 0.1 mM 2-mercaptoethanol, 4 M glycerol, pH 6.9) with 3 x 3 minute washes in MSB-2. Cells were fixed with 1% glutaraldehyde (JBEM Services, Dorval, Que.) in phosphate buffered saline (PBS) for 10 minutes. Free aldehydes were reduced by 3 successive 3 minute washes in 0.1% NaBH₄ in PBS. NaBH₄ was removed with 3 x 3 minute washes in PBS.

Coverslips with cells were layered with 100 μ l of diluted (1:300 with PBS) rat monoclonal antibody to yeast tubulin (Cedarlane Lab., Hornby, Ont.) and incubated at room temperature for 45 minutes. Following 3 x 3 minute washes in PBS, 100 μ l of diluted (1:30 in PBS) FITC labeled goat antibody to rat IgG (Miles Lab., Rexdale, Ont.) was layered onto the cells. After 45- minute incubation at room temperature and 3 x 3 minute washes in PBS, cells were treated with Hoechst #33258 stain (1 μ g/ml in PBS) for 1 minute. Cells were then treated with 0.01% Calcofluor white (Ciba, Geigy, Greensboro, N.C.) (also known as Tinopal LPW) in PBS for 3 minutes, washed 3 x 5 minutes in PBS to remove excess stain and then coverslips were mounted in glycerol/PBS (1:1) containing 0.1% phenylene diamine. All cells were examined using a Zeiss photomicroscope fitted with epifluorescence optics. Photos were taken on Ilford XP 1-400 ASA film.

For immunofluorescent, Hoechst and Calcofluor control procedures and results, see Appendix II. For filter combinations, see Appendix II.

3. RESULTS

3.1 Isolation and Culture of Cells

While generally following the cell culture methods of Fukuda and Komamine (1980), we found the isolation of leaf mesophyll cells by mortar and pestle was completely unsuccessful, resulting in no viable cells. However, separation of cells by blending leaves in culture medium consistently yields 75% (or higher) live cells capable of establishing continuous cultures.

3.2 Xylogenesis

Differentiation in this culture system parallels that described for intact roots and shoots with the production of annular, spiral, reticulate, scalariform and pitted tracheary elements (Fig. 1). Differentiation occurs in two bursts, the first at 4 to 6 days after culturing. At this time only 1% to 3% of the total cells will form tracheary elements with annular, spiral and reticulate patterns predominating. The second burst occurs 10 to 13 days post-culturing when up to 30% of the total cell population differentiates. These tracheary elements form largely thickly banded reticulate and scalariform patterns.

We find that replicate plates, made from the same suspension of mesophyll cells vary both in the percentage of cells that become tracheary elements and in the timing of initial differentiation. Differentiation of 5% of the total cells in one plate versus 30% in another replicate plate is not uncommon. Similarly, replicate plates

can vary between day 4 and day 6 post-culturing in appearance of the first tracheary elements. We are currently investigating the possible causes of this variation. Regardless of differences in degree and/or rate of differentiation, all plates will show some tracheary element development and can be used in a descriptive study of xylogenesis.

3.3 Immunofluorescent Staining of Cell Cultures

The microtubule arrays seen in this culture system closely parallel those described for intact plants (Wick et.al., 1981). All phases of the cell cycle can be identified (Fig. 2) with microtubules displayed as preprophase bands, spindles or phragmoplasts. During interphase most cells have highly ordered cortical microtubules typical of cells that are expanding in a preferred direction (Fig. 2d). However about 25% of the total population exhibits random microtubule organization (Fig. 2e). These cells tend to be isodiametric in shape.

Plant cells grown in suspension culture are free from the growth confining effects of neighboring cells and thus tend to have irregular shapes. This can make it difficult to find large areas of cell wall (and adjacent cortical cytoplasm) which are in focus in a single viewing plane. As a result, microtubule patterns can appear discontinuous when they are actually going out of the plane of focus. Optical sectioning shows that the microtubules continue in an unbroken fashion into the next focal level (Fig. 3).

3.4 Calcofluor/Hoechst Staining of Tracheary Elements

When Z. elegans cultures are stained with C/H, the primary walls are faintly fluorescent while the secondary walls exhibit much stronger fluorescence (Fig. 4b). In a well developed tracheary element, the fluorescent secondary wall pattern is easily detected and identical to the wall pattern seen in phase contrast microscopy (compare Figs. 4b and c). Such a cell has birefringent secondary wall bands when viewed with polarized light (Fig. 4d).

In a newly developing tracheary element, C/H staining provides the first evidence of secondary wall deposition. Almost no wall thickenings are evident when the cells are viewed using phase contrast (Fig. 5c). Similarly, birefringence patterns in polarized light are very weak at this time (Fig. 5d). C/H staining however reveals a well developed wall pattern (Fig. 5b). The C/H positive wall pattern closely mimics the microtubule organization in the cell (Fig. 5a).

3.5 Microtubule Involvement in Xylogenesis

Xylogenesis can be classified into progressive stages according to the overall microtubule patterns and accompanying secondary wall deposition and birefringence.

In the first stage, bands of microtubules are evident, however not all microtubules are associated with these bands (Figs. 6 and 7). The mt patterns may predict the development of an annular/spiral wall pattern (Fig. 6a) or a reticulate/scalariform wall pattern (Fig. 7a). Bands of microtubules may appear more evident on one side

of the cell than the other (Fig. 7). At this stage secondary wall deposition is not evident with combined Calcofluor and Hoechst (C/H) staining (Figs. 6b and 7c) or phase contrast microscopy (Fig. 6c).

In the second stage, microtubule are found in distinct bands typical of those seen in well developed tracheary elements (Fig. 5). C/H staining confirms that secondary wall material has been deposited in the same pattern as the microtubule array (compare Figs. 5a and 5b) but at this stage almost no secondary wall thickenings are visible using phase contrast microscopy (Fig. 5c). The cell outwardly appears to be undifferentiated. Polarizing microscopy shows very little birefringence (Fig. 5d). In a proportion of cells at this stage, the banding pattern revealed by C/H staining appears to be associated with the plasmalemma as seen in very shrunken cells (Fig. 8). In such cells, C/H staining shows an unbanded cell wall while the typical tracheary element pattern of bands is found on the cell membrane.

In the third stage, the secondary wall is well developed and visible using phase contrast microscopy (Fig. 4c) as well as exhibiting typical birefringence patterns in polarized light (Fig. 4d). The C/H staining pattern is identical to the wall pattern in phase (compare Figs. 4b and 4c) and is matched by the distribution of microtubule bands in the adjacent cortical cytoplasm (Fig. 4a).

Very fine microtubule strands can sometimes be seen joining the large groups of microtubules. These fine strands seem to presage new wall development since such connecting strands may show: (a) no visible accompanying wall deposition (Fig. 5), (b) early wall deposition

visible only with C/H staining (Fig. 5) or, (c) a faint wall visible both with C/H staining and in phase contrast microscopy (Fig. 4).

4. DISCUSSION

4.1 The Culture System

The differentiation of tracheary elements involves changes in the organization of microtubules as well as synthesis and localized deposition of secondary wall material. Until now, study of the relationship between microtubule organization and wall deposition in tracheary elements has been limited to electron microscopy, a method which is labor intensive and provides information on only small portions of the cell. Developmental studies of microtubule involvement in the differentiation of tracheary elements therefore, were difficult to carry out. By applying immunofluorescent techniques to a culture which produces large numbers of tracheary elements within a short time, it is possible to observe varying stages of differentiation and to formulate a step-wise theory of microtubule involvement.

The microtubule patterns associated with the cell cycle of Zinnia elegans cell cultures are virtually identical to the microtubule arrays reported for whole plants (Wick, et.al., 1981). Similarly, all patterns of tracheary element secondary wall thickenings found in plants (i.e. annular, spiral, reticulate, scalariform and pitted) are also found in this culture. One may assume, then, that the microtubule involvement seen in tracheary element differentiation in this culture system is similar to events that occur in the whole plant.

4.2 Xylogenesis

Lai and Srivastava (1976) report that the first stage of differentiation is shown by development of lobed nuclei. We also see lobed nuclei however they appear to be the normal morphology for this system and therefore cannot be used as an early indicator of xylogenesis.

4.2.1 Stage 1

The grouping of some of the cortical microtubules into bands is the first indication of xylogenesis in this system. While the bands are fairly prominent, other microtubules, not associated with the bands, are still visible (Figs. 6 and 7). No secondary wall deposition is detected in association with these early microtubule bands. The bands or groups of microtubules are only visible within the time frame when xylogenesis may be expected to occur (i.e. about days 4-6 and 10-13) in this cell culture system.

4.2.2 Stage 2

In the second stage of xylogenesis microtubules are organized into well defined bands (Figs. 5 and 8). The microtubule pattern is reflected by the deposition of C/H positive staining wall material. Secondary cell wall deposition is not yet visible using phase contrast or polarization optics. During what we believe to be the early stages of wall deposition the C/H staining material seems to be more tightly associated with the plasma membrane than with the cell wall (Fig. 8). This may be a transient condition however, since at later stages the

C/H staining remains with the cell wall after cell shrinkage (unpublished observation). We cannot speculate as to the chemical nature of the C/H positive wall material since Calcofluor binds a number of wall components (Hughes and McCully, 1975)(Wood, 1980) and the staining mechanism of Hoechst for TE walls is unknown.

The deposition of C/H positive staining material seems to occur simultaneously over the cell surface since we have not detected partial patterns of wall deposition with C/H stains.

4.2.3 Stage 3

At this stage wall thickenings can be detected with phase contrast or polarized light microscopy (Figs. 4c and 4d). The fact that some wall thickenings become visible in phase contrast before others (Fig. 5c), indicates that the rates of wall deposition may vary between bands in the cell. This point is further emphasized by the apparent differences in the level of birefringence between wall thickenings in the same cell (Fig. 5d). Further wall deposition results in the development of wall bands with strong birefringence, highly visible in phase contrast microscopy (Fig. 4d).

4.3 Microtubule Organization During Xylogenesis

We have been able to examine overall cellular organization of microtubules using immunofluorescence techniques. When cells develop reticulate and scalariform wall thickenings their microtubules are organized in a pattern which exactly predicts the wall deposition (Figs. 4, 5 and 7). We do not yet know how the cell establishes a microtubule pattern with the various orientations necessary to produce

such a wall pattern. Observations made thus far (Figs. 6 and 7) indicate to us that perhaps a reorganization of the microtubule network via some form of microtubule aggregation may be occurring. We are currently testing this hypothesis.

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Fig. 1. Patterns of wall thickening found in cultured Z. elegans.

a) Annular, b) Spiral, c) Reticulate, d) Scleriform, e) Pitted.

Scale bar = 10.0 μm .

Fig. 2. Microtubule organization during the cell cycle of cultured Z. elegans. a) Preprophase band of microtubules. Note dispersed microtubules (arrow) at the periphery of the band. b) Late anaphase spindle, b') Hoechst #33258 staining of chromosomes, c) Phragmoplast, c') Hoechst #33258 staining of daughter nuclei, d) Ordered microtubule arrays in an elongating cell, e) Randomly organized microtubules in an isodiametric cell. Scale bar = 10.0 μm .

Fig. 3. Two focal planes (a,b) through a culture cell in the early stages of xylogenesis. Strands of cortical microtubules can be followed from one plant to the next (black arrows) thus indicating a continuous network. Microtubules show early evidence of grouping into bands (white arrowheads). Scale bar = 10.0 μ m.

5

Fig. 4. A tracheary element with well developed wall thickenings. a) Microtubule staining pattern. b) C/H staining of the pattern of wall thickening. c) Cell viewed using phase contrast microscopy. d) Birefringence pattern of wall thickenings using polarization optics. The arrangement of microtubules is precisely mimicked by the C/H staining pattern of the secondary wall (compare a and b). The pattern of wall thickening is assessed earlier and easier with C/H staining compared to either phase, or polarization optics (compare b with c and d).

Scale bar = 10.0 μm .

Fig. 5 A cell in the early stages of xylogenesis. a) Cortical microtubules are organized into discrete bands (arrowhead). Bands vary in thickness. Lower left portion of the cell contains organized microtubules which are outside the plane of focus. b) C/H staining reveals a reticulate pattern of secondary wall deposition with wall bands of varying thickness. A prominent nucleus (arrowhead) is also visible. c) Phase contrast microscopy reveals only one wall thickening (arrowhead) which is located in a position consistent with the C/H and microtubule staining (compare c with a and b). d) Polarization optics reveal a band of weak positive birefringence (arrow) in the region of the cell where a wall thickening is identified (compare d with b and c). Fine microtubule strands (long arrow) do not yet have C/H staining associated with them (compare a and b).

Scale bar = 10.0 μm

2

2. MATERIALS AND METHODS

2.1 Preparation of cell cultures. Leaf mesophyll cells were isolated from 14 day old leaves of Zinnia elegans L. cv. Canary Bird and cultured in medium (Fukuda and Komamine, 1980a) containing 0.2 mg/L NAA (1-Napthaleneacetic acid) and 2 mg/L BA (6-Benzylaminopurine) as previously described (Falconer and Seagull, 1985).

2.2 Taxol treatment of cultures. Taxol (National Cancer Institute, Bethesda, Md.) was dissolved into DMSO (Dimethylsulfoxide) at 10 mM concentration and stored at -80°C , 4 μL of stock taxol were added to cultures containing 4 ml of medium for a final dilution of 1×10^{-5} M. Taxol was added to 3 day, 4 day and 5 day old cultures. For fluorescent staining, 0.2 mL was removed from each culture before addition of taxol and again at 24 hour intervals until differentiation occurred. Taxol was not added at the time of subsequent samplings of a culture. DMSO controls were treated in the same manner as taxol. DMSO at 1×10^{-5} M final concentration had no effect on cell number, differentiation or type of tracheary element formed. All DMSO treated cultures were identical with untreated controls and therefore are not shown in micrographs or bar graphs.

2.3 Cell counts. For each sample, 0.2 mL was removed from a culture and layered on a coverslip. Cell counts were done by scanning the coverslips and counting fluorescently stained cells. A minimum of 500 cells was counted for each sample. Counts of cell types are expressed

Manuscript 2.

Xylogenesis in Tissue Culture: Taxol Effects on Microtubule
Reorientation and Lateral Association in
Differentiating Cells

Published in Protoplasma (1985).

SUMMARY

In Zinnia elegans tissue cultures, cortical microtubules reorient from longitudinal to transverse arrays as the culture age increases and before differentiation of tracheary elements is visible. The orientation of microtubules, in the period just before visible differentiation, determines the direction of the secondary wall bands in forming tracheary elements. Taxol, applied early in culture, stabilizes the microtubules of most cells in the longitudinal direction. Tracheary elements differentiating in these taxol treated cultures show secondary wall bands parallel to the long axis of the cell while those differentiating in control cultures always have wall bands transverse to the long axis of the cell.

It is proposed that, in untreated Zinnia cultures, microtubules are reoriented by a gradual shift from longitudinal to transverse and this reorientation normally occurs before differentiation becomes visible. Once initiated, tracheary element differentiation involves lateral association of microtubules to form the discrete bands typical of secondary wall patterns.

1. INTRODUCTION

Xylogenesis has been intensively studied yet relatively little is known about the precise involvement of cytoskeletal elements in tracheary element differentiation. Microtubule reorganization has been shown to precede secondary wall deposition (Brower and Hepler, 1976), (Hardham and Gunning, 1978) while groups of microtubules are associated with the deposition of secondary wall material (Hepler and Newcomb, 1964), (Pickett-Heaps, 1967), (Robards and Kidwai, 1972). Recently microtubules have been implicated in the transformation of annular/spiral wall patterns into scalariform or pitted patterns through continuing wall deposition (Falconer and Seagull, 1985). However, there is little evidence to indicate how wall patterns are determined and what role microtubules may play in this.

In the Zinnia mesophyll culture system, first developed by Kohlenbach and Schmidt (1975), and later characterized by Fukuda and Komamine (1980a, 1980b), one can follow the direct differentiation of mesophyll cells into tracheary elements. Using this system, it is possible to monitor cytoskeletal involvement before and during the appearance of secondary walls. We have previously demonstrated (Falconer and Seagull, 1985) that the various microtubule arrays associated with interphase, mitosis and cytokinesis in this system are identical with those of intact plants (Wick, et al., 1981) and can therefore assume that events which occur during the differentiation of tracheary elements are also similar to those occurring in intact plants.

The first microscopically visible sign of secondary wall formation involves the reorganization of microtubules from cortical arrays into distinct groups (Brower and Hepler, 1976), (Falconer and Seagull, 1985). In this paper we demonstrate the orientation of these cortical microtubule arrays changes before tracheary element differentiation occurs. To investigate if this reorientation is necessary to differentiation, we treat Zinnia cultures with the microtubule stabilizing drug, taxol.

In vitro, taxol has been shown to stabilize polymerized microtubules (Schiff and Horwitz, 1981), to shift the tubulin equilibrium in favor of polymerization (Schiff, et.al., 1979), and to cause lateral association of microtubules to form bundles (Turner and Margolis, 1984). Taxol has similar effects in vivo, stabilizing existing microtubules in HeLa cells (Schiff and Horwitz, 1980) and in the alga, *Poteroiochromonas* (Herth, 1983), polymerizing new microtubules (DeBrabander et.al., 1981) and inducing microtubules to laterally associate (Bajer et.al., 1982).

Results obtained in these experiments allow us to suggest that in Zinnia cultures:

- (a) Cortical microtubule arrays reorient from longitudinal to transverse with respect to the long dimension of the cell. (b) Taxol stabilizes the orientation of these cortical microtubules. (c) Stabilization of microtubules does not prevent xylogenesis. (d) While cortical microtubules normally shift orientation from longitudinal to transverse before xylogenesis occurs, this shift is not essential for differentiation. (e) The pattern of the secondary wall bands is determined by microtubule orientation and may be the result of lateral association of these cortical microtubules.

2. MATERIALS AND METHODS

2.1 Preparation of cell cultures. Leaf mesophyll cells were isolated from 14 day old leaves of Zinnia elegans L. cv. Canary Bird and cultured in medium (Fukuda and Komamine, 1980a) containing 0.2 mg/L NAA (1-Napthaleneacetic acid) and 2 mg/L BA (6-Benzylaminopurine) as previously described (Falconer and Seagull, 1985).

2.2 Taxol treatment of cultures. Taxol (National Cancer Institute, Bethesda, Md.) was dissolved into DMSO (Dimethylsulfoxide) at 10 mM concentration and stored at -80°C . 4 μL of stock taxol were added to cultures containing 4 ml of medium for a final dilution of 1×10^{-5} M. Taxol was added to 3 day, 4 day and 5 day old cultures. For fluorescent staining, 0.2 mL was removed from each culture before addition of taxol and again at 24 hour intervals until differentiation occurred. Taxol was not added at the time of subsequent samplings of a culture. DMSO controls were treated in the same manner as taxol. DMSO at 1×10^{-5} M final concentration had no effect on cell number, differentiation or type of tracheary element formed. All DMSO treated cultures were identical with untreated controls and therefore are not shown in micrographs or bar graphs.

2.3 Cell counts. For each sample, 0.2 mL was removed from a culture and layered on a coverslip. Cell counts were done by scanning the coverslips and counting fluorescently stained cells. A minimum of 500 cells was counted for each sample. Counts of cell types are expressed

as a percentage of the total number of cells counted in that sample (usually 500 cells). When the total number of cells/slide was needed, 8 random fields were counted. The number of fields/coverslip was determined and the appropriate calculations done to obtain number of cells/slide. Mean and standard deviation are based on counts of at least 500 cells.

2.4 Immunofluorescent staining of cells. Indirect immunofluorescent staining was adapted from Simmonds et.al. (1983) and previously described (Falconer and Seagull, 1985). Briefly, cell samples were layered onto coverslips and fixed for 1 hour in 3% paraformaldehyde made-up in 0.1 M Pipes buffer, pH 6.9. After rinsing in the same buffer, coverslips were incubated for 5 min. in 0.1% w/v ultrapure cellulase (Worthington Biochemical, Mississauga, Ont.) made up in buffer. Cells were then extracted in 4 M glycerol containing 1% Triton for 1 hour, fixed with 1% glutaraldehyde in PBS and subsequently washed, first in 0.1% NaBH₄ followed by PBS rinses. Rat monoclonal antibody to yeast tubulin (Cedarlane Lab., Hornby, Ont.) was layered on coverslips for 45 min., washed in PBS and followed by incubation for 45 min. with fluorescein conjugated secondary antibody (rabbit anti-rat, Miles Lab., Rexdale, Ont.). Cells were briefly stained for DNA with Hoechst #33258 followed by a 3 min. stain with 0.01% Calcofluor White (Ciba Geigy, Greensboro, N.C.) to stain cell walls. Coverslips were rinsed in PBS and mounted in glycerol/PBS (1:1) containing phenylene diamine.

For immunofluorescent, Hoechst and Calcofluor control procedures and results, see Appendix II. For filter combinations, see Appendix II.

All cells were examined using a Zeiss photomicroscope fitted with epifluorescence optics. Photos were taken with Ilford XP1-400 ASA film.

3. RESULTS

3.1 Microtubule orientation in cultured cells. In cells from Zinnia elegans cultures, cortical microtubules can be found with the following orientations: 1) Longitudinal, or nearly longitudinal - i.e., parallel to the long axis of the cell (Fig. 1a), 2) Angled - alignment of microtubules intermediate between longitudinal and transverse (Fig. 1b), 3) Transverse - aligned at right angles to the long dimension of the cell (Fig. 1c) and 4) Random - no over-all orientation (Fig. 1d).

The majority of newly cultured cells display longitudinally oriented microtubule arrays. On day 3 post culturing, 48% of elongated cells show longitudinal orientation; 33% show angled and only 7% show transverse microtubule orientation (the remainder are randomly arranged or in mitosis) (Fig. 2a). As the culture ages, the prevailing orientation changes from longitudinal to transverse. Thus by day 7, the percentages are reversed with 48% of cells now showing transverse microtubule orientation, 30% angled and only 2% with longitudinal arrays (again, the rest are random or in mitosis) (Fig. 2a). Cultures were not sampled earlier than day 3 due to technical inability to stain the cells.

3.2 Microtubule orientation in taxol treated cultures. The addition of 1×10^{-5} M taxol inhibits reorientation of cortical microtubules for

up to 48 hours. In the sample from a 3 day old control culture, 48% of columnar cells display longitudinal microtubule arrays (Figs. 2b, 3a). When this same culture is treated with taxol and sampled after 24 and 48 hours, about 50% of the cells continue to show microtubules parallel to the long axis (Fig. 2b). Similarly the sample from a 4 day old control culture shows 29% of cells have longitudinally oriented microtubules. When taxol is added at day 4, this figure remains constant for 24 hour (day 5) and 48 hour (day 6) samplings. Microtubules oriented transversely (Fig. 3b) remain stable in this configuration for up to 48 hours as well (data not shown). In some cells with transverse orientation, lateral association of microtubules into bands can be detected (Fig. 3b) although this association is not pronounced.

3.3 Microtubule polymerization in taxol treated cultures. We did not attempt to quantify the apparent increase in microtubule numbers resulting from taxol treatment. However, many cells appear to have more cortical microtubules than comparable cells in control cultures (compare Fig. 3a with Fig. 1a). Taxol treated cells also show increased intensity in fluorescence which may be associated with increased polymerization of tubulin. This brilliant fluorescence remains for at least 4 months while the staining of control cells fades almost to invisibility in the same time period (data not shown).

3.4 Tracheary element differentiation in cultured cells. In our cultures tracheary element differentiation routinely occurs by day 6 post-culturing. We have previously shown (Falconer and Seagull, 1985)

that tracheary element differentiation can be divided into 3 stages:

- 1) Microtubules grouped into bands without secondary wall evident.
- 2) Groups of microtubules subtending wall material which is visible only when stained with Calcofluor/Hoëchst.
- 3) A complex microtubule pattern reflected by well developed wall thickenings detected using Calcofluor, phase contrast and polarization optics.

In these experiments, all control cultures display Stage 3 differentiation by 6 days post-culture. There was no evidence of any secondary wall deposition prior to day 6 in any control culture.

Secondary wall patterns in tracheary elements, whether annular, spiral or reticulate, are always oriented primarily transverse to the long axis of the cell. We have never seen a tracheary element, in a control culture, which displays wall bands parallel to the longest dimension of the cell.

3.5 Tracheary element differentiation in taxol treated cultures.

3.5.1 Orientations of microtubules and wall thickenings. The secondary wall patterns of tracheary elements formed in the presence of taxol are related to the microtubule orientation of the cell at the time of addition of taxol. When taxol is added to a 3 day old culture, the majority of cells have microtubule arrays stabilized in the longitudinal direction (see Figs. 2a, 2b). Tracheary elements which differentiate in these cultures will show wall bands parallel, or approximately parallel, to the long axis of the cell (Figs. 4, 5).

When taxol is added to a 4 day old culture, the shift to transverse microtubule orientation is well underway with many cells

showing angled microtubule orientations (Fig. 2a). Tracheary elements forming in these cultures still display predominantly longitudinal or sharply angled wall bands although some tracheary elements may contain combinations of longitudinal and transverse orientations within the same cell (Fig. 6).

Addition of taxol to a 5 day old culture (which has more cells containing transverse microtubule arrays, see Fig. 2a) results in the formation of increased numbers of tracheary elements with transverse wall patterns (Fig. 7). These tracheary elements often are indistinguishable in appearance from those forming in control cultures.

When a tracheary element differentiates in taxol, the wall bands often display "waves" or "swirls" which are particularly noticeable in longitudinally oriented patterns (Fig. 5). These sub-patterns are characteristic of taxol treated cultures and are not found in control tracheary elements. Transverse wall patterns which develop in taxol (and are most like the wall bands found in control cultures) may show slight "waves" or "swirls" in which the wall band doubles back on itself, but these are not common (data not shown). All tracheary elements formed in taxol, irregardless of wall band pattern or orientation, follow stage 1, stage 2 and stage 3 sequential differentiation (data not shown).

Cell counts were done to ascertain the correlation between microtubule orientation and subsequent secondary wall orientation.

Distinguishing between longitudinal and angled wall bands is complicated since individual cells may contain bands with both orientations (Fig. 6).

In addition, bands demonstrated waved or swirled configurations (Fig. 5). Consequently it was decided to use only two wall band orientation categories: 1) "Longitudinal" defined as cells having bands oriented at angles less than 45° off the long axis; 2) "Transverse" defined as cells having some or all bands oriented at angles between 45° and 90° off the long axis. If cortical microtubule arrays are categorized as either "Longitudinal" or "Transverse" (using the same definitions as for wall bands), then a very close correlation is evident between the microtubule orientation at the time of taxol addition and the orientation of tracheary element wall patterns which subsequently differentiate in this culture (Fig. 8).

3.5.2 Taxol alters kinetics of xylogenesis. Taxol has two additional effects on tracheary element differentiation: 1) Taxol treated cultures differentiate at least 24 hours earlier than control cultures. Stage 2 differentiation (walls visible only with Calcofluor/ Hoechst) is found in taxol treated cultures on day 5. No tracheary elements, in any stage of differentiation, are visible in control cultures until day 6. 2) A greater percentage of cells differentiate into tracheary elements in a 6 day old taxol treated culture in comparison to similar age control cultures. While the absolute numbers of tracheary elements varied between experiments, within each experiment taxol treated cultures contain about 3 times more tracheary elements than controls.

4. DISCUSSION

Columnar cells change the orientation of their cortical microtubules from longitudinal to transverse during the first few days of culture and before differentiation into tracheary elements occurs. Two questions arise from this observation: 1) How does this shift in microtubule orientation occur? and 2) Is this reorientation necessary for tracheary element differentiation in the Zinnia culture system? To address these questions, cultures were treated with the microtubule stabilizing drug, taxol.

In vivo, taxol treatments stabilize polymerized microtubules, promote polymerization of new microtubules and cause lateral association of microtubules into bundles (DeBrabander et.al., 1982), (Herth, 1983), (Bajer et.al., 1982), (Schiff and Horwitz, 1980). We find the same range of taxol effects occurring in the Zinnia culture system. In taxol, cortical microtubules are stabilized and prevented from reorienting for up to 48 hours. Taxol induces polymerization as indicated by an apparent increase in numbers of microtubules as well as increased fluorescence. Lateral association of microtubules can be seen in taxol treated cultures.

4.1 Microtubule reorientation before xylogenesis. The change in microtubule orientation takes place over a period of several days and involves gradual displacement from longitudinal to transverse (Fig. 2a). This reorientation may be the result of either depolymerization followed by repolymerization of microtubules in a new direction, or by a

shifting of the cortical microtubule array from longitudinal to transverse. Taxol evidence, which indicates that the orientation of microtubule arrays is stabilized for up to 48 hours, does not conclusively support one mechanism over the other.

Reorientation by depolymerization/repolymerization is consistent with the taxol results but requires that the cell must expend energy to form intermediate, angled arrays which function for only a short time and are soon replaced. A shifting of the microtubule array from longitudinal to transverse through a series of angled stages seems, to us, to be the simplest explanation. Taxol stabilization of a shifting array can be ascribed to the ability of taxol to promote polymerization of microtubules and to cause lateral associations between them. By polymerizing long microtubules which laterally associate to form a stable, grid-like configuration, microtubule arrays may be physically constrained in a particular orientation.

4.2 The development of tracheary elements in taxol. Tracheary elements can differentiate in the presence of taxol. The orientation of the secondary wall bands mirrors the microtubule orientation at the time of taxol addition. If taxol is added on day 3, a majority of tracheary elements show main wall bands parallel, or approximately parallel, to the long axis of the cell. If taxol is added on day 5, when many cells have transverse microtubule arrays, a similar proportion of tracheary elements show transverse wall bands. Clearly, reorientation of microtubules is not essential for tracheary element differentiation.

While reorientation is not a prerequisite for differentiation, nonetheless, it always occurs in untreated cells which subsequently differentiate into tracheary elements. If this were not the case, some TEs with longitudinal wall patterns should be seen since there are cells in control cultures which exhibit longitudinal microtubule arrays at the time of differentiation.

We have preliminary evidence to suggest that cultures which are slow to reorient microtubules, or do so in low proportions, show low percentages of tracheary element differentiation. Cultures which reorient microtubule arrays quickly and completely have high rates of tracheary element production.

In addition to stabilizing microtubule arrays and thereby affecting subsequent secondary wall orientation, taxol also stimulates cultures to differentiate earlier and to produce more tracheary elements.

4.3 Lateral association of microtubules during differentiation. One can postulate two mechanisms by which cortical microtubules are formed into the discrete bands which precede wall deposition during xylogenesis. Dispersed arrays of cortical microtubules may laterally aggregate directly into discrete bands possibly due to microtubule interactions with a MAP-like protein with minimal involvement of depolymerization, repolymerization. Alternatively, the development of distinct groups of microtubules can occur by localized activation of specialized regions (MTOCs) in the edges of the cell, with depolymerization, repolymerization playing a major role (Gunning et al., 1978). We believe this paper provides evidence to support the first hypothesis invoking lateral association to form groups of microtubules.

The development of tracheary elements in taxol indicates that microtubule arrays can change from dispersed arrays to distinct groups with minimal turnover (depolymerization/repolymerization). If lateral association occurs during differentiation, it may be enhanced by the ability of taxol to induce similar lateral association (Herth, 1983), (Turner and Margolis, 1984). This would be consistent with the observation that earlier development and increased proportions of tracheary elements are found in the presence of taxol.

Activation of specialized sites implies depolymerization of microtubules to form an inter-band area free of microtubules with repolymerization of new microtubules into discrete groups. Taxol treatment should impede this depolymerization and consequently the mt-free inter-band areas seen in tracheary elements would not form. Considering that wall deposition mirrors mt position, tracheary elements differentiating in taxol treated cultures would not have these mt-free areas and therefore should not show distinct bands of secondary wall. As shown in Figs. 4, 5, 6, and 7, tracheary elements differentiating in taxol do form well-defined wall bands. This evidence again points to lateral association as the best explanation for microtubule grouping in the early stages of xylogenesis.

Although this work does not indicate what mechanism is behind the lateral association of microtubules, we would like to suggest that a microtubule associated protein could fulfill this role.

In vitro work demonstrates that a MAP-like protein found in crude brain extract mediates taxol induced bundling of microtubules (Turner and Margolis, 1984). Although no specific MAP has been shown to be

associated with tracheary element differentiation, Fukuda and Komamine (1983) have demonstrated that the synthesis of two polypeptides is shut off while two other polypeptides are newly synthesized in the period preceeding morphologically detectable differentiation in the Zinnia system. These, or other proteins, related to differentiation may be involved in causing the lateral association.

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Fig. 1: Orientations of cortical microtubule arrays in cultured *Z. elegans*. a) Longitudinal - microtubules parallel, or nearly parallel, to the long axis of the cell; b) Angled-microtubules oriented approximately at right angles to the long axis of the cell; d) Random - no over-all microtubule orientation. Magnification, 756x.

Fig. 3: Microtubule arrays stabilized by taxol. a) Longitudinal microtubule array. Note increased numbers of microtubules in comparison with Fig. 1a. b) Transverse microtubule array. Note spaces (arrowhead) apparently due to lateral association of microtubules. Magnification, 756x.

Fig. 2a: Microtubule orientation changes with culture age. Cells sampled on days 3, 4, 5, 6 and 7 post culturing and fluorescently stained to show microtubule orientations. Lines in bar graph indicate orientation of microtubules with respect to the long axis of the cell (vertical lines - longitudinal mts), (angled lines - mts increasingly angled toward the transverse), (horizontal lines - transverse mts) and (no lines - random mts). Note the decreasing longitudinal orientation and the increasing transverse microtubule orientation relative to culture age. Random cells include those with random microtubule arrays plus cells in mitosis.

Fig. 2b: Microtubules are stabilized in the longitudinal orientation for 48 hours in taxol. Control cultures (dots) sampled on days 3, 4, 5, 6 and 7. After sampling, taxol was added to cultures as noted. Taxol treated cultures were sampled at 24 (triangle) and 48 (open triangle) hours. Percent of cells exhibiting longitudinal microtubule arrays was determined for all samples based on 500 cells/sample. Note that arrays remained stable in microtubule orientation for up to 48 hours.

Fig. 2a

Fig. 2b

Fig. 4: Longitudinal secondary wall pattern of tracheary element which differentiated in taxol. a) Groups of microtubules (arrow) are oriented parallel to long axis of cell. b) Calcofluor/Hoechst staining of secondary wall. Note similarity to microtubule pattern. c) Phase contrast micrograph showing secondary wall pattern. Note similarity to microtubule and Calcofluor patterns. Magnification, 756x.

Fig. 5: Calcofluor/Hoechst staining of longitudinal wall patterns in tracheary elements which differentiated in taxol treated cultures. a) Longitudinal wall bands showing "waves" (curved arrow). b) Two daughter cells, one remaining undifferentiated (note Hoechst stained nucleus, arrowhead), the other differentiated into a tracheary element displaying longitudinal secondary wall bands. Note "swirls" in which wall band doubles back upon itself (long arrow). Also note the continuation of wall bands in the wall between the two cells (arrow). c) Longitudinal wall patterns may have "waves" and/or "swirls" or can be virtually straight as shown in this tracheary element. Magnification, 756x.

Fig. 6: Two focal planes of a tracheary element which differentiated in taxol and shows longitudinal, angled and transverse patterns within the same cell. a) Microtubules in upper focal plane - note change in orientation. Dispersed microtubules (arrowhead) in upper left portion of cell laterally associate to form distinct groups (arrow). b) Calcofluor/Hoechst staining of secondary cell wall in upper focal plane. c) Microtubules in lower focal plane - note change in orientation and grouping of cortical microtubules from dispersed to distinct bands. d) Calcofluor/Hoechst staining of wall in lower focal plane. e) Phase contrast of same cell. Secondary wall pattern is just becoming visible; as cell ages and more wall material is deposited this cell wall will be as visible as those in Fig. 4c. Magnification, 756x.

Fig. 7: Transverse secondary wall pattern of tracheary element which differentiated in taxol. a) Microtubules are primarily oriented transverse to the long axis of the cell. b) Calcofluor/Hoechst staining of secondary wall. c) Phase contrast of same cell showing secondary wall pattern. This tracheary element is indistinguishable from those which differentiate in control cultures. Magnification 756x.

Fig. 8: Comparison of microtubule orientation and the secondary wall orientation of tracheary elements in taxol treated cultures. Microtubule orientations were stabilized in longitudinal (vertical lines) or transverse (horizontal lines) arrays by the addition of taxol on days 3, 4 and 5. Longitudinal tracheary elements (L) and transverse tracheary elements (T) differentiating in these cultures were assayed on day 6. Note the very close correlation between the percent of cells with a particular microtubule orientation and the percent of tracheary elements with the same orientation which differentiated in that culture. The percent of cells with a given microtubule orientation is based on a count of 500 cells. The percent of tracheary elements shown is based on the total number of longitudinal and transverse tracheary elements on one slide.

Fig. 8

DISCUSSION

1) Zinnia mesophyll tissue culture system.

Historically, scientists have searched for the ideal system in which to study xylogenesis: a system in which cells synchronously and totally differentiate under controlled conditions. The Zinnia leaf mesophyll tissue culture system appears to be the closest approximation to this ideal. Fukuda and Komamine (1980a,b) established the conditions under which cells from young Zinnia leaves are grown in defined medium (see Appendix) and under defined conditions until differentiation into tracheary elements occurs. Using this tissue culture system in combination with staining techniques developed in this project, it is possible to monitor the changes in microtubule arrays which can be detected as the cells go from leaf mesophyll to cultured cell to differentiated tracheary element.

The morphology of tracheary elements differentiating in Zinnia culture is very similar to that of xylem cells found in plants.

Annular rings, spirals, reticulate, scalariform and pitted patterns formed by secondary wall deposition are common to both tissue

culture and plant tracheary elements. Similarly, indirect immunofluorescent staining of the microtubule arrays present during the cell cycle (interphase cortical microtubule, preprophase bands, spindles and phragmoplasts) shows that the microtubule organization of Zinnia tissue culture cells is virtually identical with that seen in cells from intact plants (Falconer and Seagull, 1985), (Wick, et.al., 1981). This similarity in morphology and in

microtubule organization allows direct comparison of changes in microtubule arrays which occur during differentiation in cultured cells with those changes occurring during xylogenesis in plants.

2) The alterations in microtubule arrays during culture.

The alterations in microtubule patterns can be divided into two phases: those which occur as leaf cells adapt to culture conditions and those which occur during tracheary element differentiation.

Accommodation to tissue culture can be seen by examining cells in the first week after culturing. Approximately 50% of columnar shaped cells in three day old cultures exhibit microtubules aligned with the long axis of the cell. At the same time less than 8% of elongated cells have transverse microtubules. During the next few days of growth in culture, the predominant microtubule alignment goes through a complete shift of 90° until by seven days post culturing, 50% of elongated cells now have microtubule arrays transverse to the long axis of the cell. (See Fig. 2a, page 61).

Reorientation of microtubule arrays from longitudinal to transverse normally occurs before overt differentiation into tracheary elements becomes visible. Tracheary elements with secondary walls deposited in patterns that run parallel to the long axis of the cell have never been seen in untreated cultures. If this shift in microtubule orientation did not normally occur

before differentiation, one would predict that at least a few tracheary elements with longitudinal wall patterns should be found. There are cells which exhibit longitudinal microtubule orientation present in the culture at the time of tracheary element differentiation.

3) Changes in microtubule array related to tracheary element formation.

The second phase of microtubule array alteration is found during the differentiation of tracheary elements. One aim of this project was to establish a technique to simultaneously monitor any changes in microtubule arrays plus the deposition of secondary wall material. This was accomplished by use of indirect immunofluorescent staining of microtubules combined with staining of wall material with C/H stain and the use of phase contrast and polarizing microscopy. The stages of differentiation which can be observed using this technique form a single, continuous process. It is, however, convenient to divide this process into distinct stages with the understanding that abrupt divisions between one stage and the next do not occur.

Before visible differentiation is seen, cortical microtubules are uniformly dispersed across the cell surface. Electron microscopy (Hepler and Newcomb, 1964) (Gunning, et.al., 1978) demonstrates that clustering of microtubules into groups with no apparent wall deposition is the first sign of microtubule changes which occur during tracheary element differentiation. The indirect immunofluorescent technique and Calcofluor / Hoechst staining technique extend this observation to include cell-wide grouping of cortical microtubules.

a) Stage One - microtubule changes during tracheary element differentiation.

Stage One is defined as the first changes to occur in the microtubule array during differentiation of tracheary elements. Microtubules are loosely grouped into transverse bands or into a net-like pattern reminiscent of the wall pattern seen in reticulate tracheary elements. Not all microtubules are included in these groups, a few "stray" microtubules can be seen. No secondary wall material has been deposited at this time as judged by lack of C/H staining. (See Fig. 6, 7 page 40).

b) Stage Two - Mt changes during TE differentiation.

Stage Two differentiation consists of microtubules now tightly grouped into patterns typical of tracheary element walls. Secondary wall has been deposited in the same patterns that exhibited by the microtubule groups. However, this wall material is not visible with phase contrast optics nor is it birefringent. (See Fig. 5, page 39).

Very early in this period, C/H stain can be seen to be closely associated with the membrane (See Fig. 8, page 40). In a collapsed cell C/H staining is visible on the cell membrane and forms a pattern typical of an annular tracheary element wall. No such pattern is visible with C/H staining on the cell wall itself.

Micrographs of cells exhibiting membrane-associated staining are almost exact duplicates of the very precise drawings done by Dipple in 1855 as reproduced in Sinnott and Bloch (1945). These pen and ink illustrations show what is termed "pre-attened cytoplasm" (Sinnott and Bloch, 1945). Such pre-patterned cells

have been noted by others including Hepler and Fosket (1971) but no satisfactory ultra-structural explanation has been given. Based on observations of cultured Zinnia cells, it is possible to suggest that Dipple described a very transient period in which wall material may be closely associated with the membrane thus producing a phase change which can be detected by careful observation. Dipple also observed that the pre-patterned cytoplasm showed active streaming. Perhaps the streaming may be involved in bringing or directing some wall materials to the desired location. Holt and Milligan (1974) showed that Calcofluor can bind to proteins when subject to irradiation. It is unlikely that this occurred in these experiments as Calcofluor was applied for 3 minutes without the presence of UV irradiation or sunlight and then the cells were washed 3 times for 5 minutes each time to remove unbound Calcofluor. Nevertheless, if the stain seen in Fig 8b (page 40) is a stain of proteins in spite of the above precautions, such a protein would still seem to be specifically involved in secondary wall deposition as judged by the pattern in which it is seen to occur.

c) Stage Three- Microtubule changes during TE differentiation.

A third stage can be defined in the lateration of mt arrays during TE formation. Mts are found in distinct groups and are mirrored by the position of the secondary wall in a complex pattern typical of TEs. This wall is easily visible in phase contrast and is highly birefringent under polarized light.

4) Reorganization of microtubule arrays.

During growth and differentiation in tissue culture, the mts can be reorganized many times. The first alteration is seen as the cells accomodate to culture when the mt array is shifted in orientation from longitudinal to transverse.

Reorganization can also be seen in dividing cells as microtubules are rearranged to form preprophase bands, spindles and phragmoplasts. The beginning of tracheary element differentiation signals another reorganization when the microtubules are formed into distinct groups which precede and predict complicated patterns of wall deposition. Finally, microtubules may be reorganized or assembled between existing secondary walls to guide the deposition of wall material during continuing wall production.

5) Experiments with taxol.

In an effort to understand the mechanism behind microtubule orientation and behind the changes seen during differentiation, the microtubule stabilizing drug, taxol, was applied to Zinnia cultures. In vitro and in vivo, taxol stabilizes existing microtubules, causes increased polymerization of microtubules and induces lateral aggregation of microtubule arrays (DeBrabander, et.al., 1982) (Herth, 1983), (Bajer et.al., 1982). All of these effects were also seen in the Zinnia tissue culture system (Falconer and Seagull, 1985).

a) Taxol stabilization of microtubule arrays.

Reorientation of the microtubule array, which occurs during the first few days following culturing, is completely prevented for up to 48 hours by the addition of taxol. Unfortunately, application of taxol did not clarify the mechanism by which this reorientation can occur. One effect of taxol is to prevent depolymerization of microtubules. If the shift in microtubule arrays is caused by depolymerization of one array and repolymerization of a subsequent

array in a new orientation, then addition of taxol would prevent microtubule reorientation. Thus depolymerization, repolymerization of multiple microtubule arrays may be the mechanism behind the shift in microtubule orientation from longitudinal to transverse. However, this method would be costly in terms of energy needed to form multiple arrays each of which would function for only a short time before being replaced.

An alternate explanation of the mechanism involved in reorienting microtubule arrays suggests that shifting of preformed arrays may be taking place. Taxol can also inhibit this mechanism if the remaining two properties of taxol are invoked: i.e. the ability of taxol to induce polymerization of microtubules and the ability of taxol to cause lateral aggregation between them (Schiff, et.al., 1979) and (Turner and Margolis, 1984). If many (and perhaps long) microtubules are laterally associated together, they could form a stable, grid-like configuration which may be constrained from reorienting simply by the physical presence of a rigid cell wall.

Further experiments using microtubule inhibitors such as colchicine may help to elucidate the mechanism behind the microtubule shifts in orientation.

b) Differentiation of tracheary elements in taxol treated cultures.

The application of taxol to differentiating cultures did not prevent the formation of tracheary elements. Tracheary elements can differentiate and do so 24 hours earlier and in larger numbers than

in the untreated control cultures. When taxol is used to stabilize the cortical microtubule orientation parallel to the long axis of cells in three day old cultures, tracheary elements differentiating in this culture will form secondary wall bands also parallel to the long axis of the cell. These longitudinal wall patterns are never seen in untreated cultures. (Figs.4,5,8, pages 62 and 65).

When taxol stabilizes microtubule arrays as they are in the process of reorienting from longitudinal to transverse, tracheary elements forming in these cultures will show a complex, angled wall pattern. Often three different orientations of wall can be seen within the same cell: longitudinal, angled and transverse (Fig. 6, page 63).

It can be shown that the percentage of longitudinal and transverse wall patterns in tracheary elements is identical with the percentage of cells displaying these microtubule orientations at the time taxol is applied (Fig. 8, page 65). Apparently, the orientation of the microtubule array just before visible differentiation occurs determines the wall pattern in differentiating tracheary elements.

As noted earlier, taxol treated cultures produce tracheary elements earlier and in greater amounts than do untreated control cultures. This effect may be related to the ability of taxol to cause lateral association of microtubules. The grouping of dispersed cortical microtubule arrays into discrete bundles is the first visible change seen during tracheary element differentiation.

Taxol may act to enhance this effect thereby allowing the culture to produce earlier and more tracheary elements.

6) Conclusion.

These observations allow the formulation of a working hypothesis: The orientation of cortical microtubules, in the period before visible differentiation can be detected, determines the secondary wall pattern in tracheary element formation. Lateral association between microtubules may be the mechanism behind this event.

This project has opened many new areas of research in the study of tracheary element differentiation. The mechanism behind the shift in orientation of microtubule arrays remains to be elucidated. The hypothesis proposing clustering of microtubules via lateral association needs to be tested. More evidence should be gathered to support the argument that the orientation of microtubules before differentiation determines the wall pattern seen in tracheary elements. Research into the possibility of MAP or actin microfilament involvement in differentiation of tracheary elements may also be profitable. In conclusion, the Zinnia tissue culture system, observed with the techniques developed in this project and guided by the hypothesis which arose from work in this project, should prove very useful in understanding microtubule involvement in xylem differentiation.

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APPENDIX I

Table 1 Composition of Culture Medium

Macroelements	mg/L	Microelements	mg/L
KNO_3	2020	$\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$	25
NH_4Cl	54	H_3BO_3	10
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	247	$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	10
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	147	$\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$	0.25
KH_2PO_4	68	$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	0.025
		Na_2EDTA	37
		$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	28
Organic Growth Factors	mg/L	Growth Substances	mg/L
Glycine	2	NAA	0.2
myo-Inositol	100	BA	2.0
Nicotinic acid	5		
Pyridoxine HCl	0.5	Sucrose	10 g/l
Thiamin	0.05		
Folic acid	0.05	Mannitol	36.4 g/l
Biotin	0.05	pH 5.5	

Table 2: Bershadsky Buffer

(Weights given make 500 ml of a 2x stock)

	gm/500 ml	mM
Imidazol	3.4	50
KCl	3.73	50
MgCl ₂ ·6H ₂ O	0.1015	0.5
EGTA	0.38	1
EDTA	0.037	0.1
Glycerol	368.0	4 M

Adjust pH to 6.9, May be stored at 4°C for extended periods

For use dilute 1:1 with H₂O, add 7μl 2-mercapto-ethanol per 100ml of 1x concentrated Bershadsky Buffer just before using.

Table 3: Phosphate Buffered Saline (PBS) Solution

(Weights given make 1 liter of 10x stock)

	gm/L
NaCl	76.5
Na ₂ HPO ₄ anhydrous	7.25
K ₂ HPO ₄ anhydrous	2.12

Adjust pH to 6.9 after diluting to 1x concentration

APPENDIX II

A. Microscopy

- 1) Examination of cells for birefringence was done using a Zeiss Photomicroscope II equipped with polarization optics.
- 2) Bright field and phase contrast observations were carried out using a Zeiss Photomicroscope III equipped with bright field, phase contrast and epifluorescence optics.
- 3) Epifluorescence observations were also carried out using the Zeiss Photomicroscope III which was equipped with an HBO-50W lamp and a DC transformer. Objectives used for epifluorescence work were: Plan-Neofluar 16x/0.5 oil, Plan-Neofluar 40x/0.9 oil, and Plan-Neofluar 63x/1.25 oil

B. Filter Systems

- 1) Filter System I - For observations using fluorescein isothiocyanate (FITC) as a fluorochrome. Filter System I consists of a BP 450-490 exciter filter, a FT 510 chromatic beam splitter, an LP 520 barrier filter and an additional KP 560 barrier filter (for a band pass of 520-560 nm wavelengths).
- 2) Filter System II - For observations using Calcofluor White and Hoechst #33258 fluorochromes. A G 365 exciter filter, an FT 395 chromatic beam splitter and an LP 420 barrier filter combined with an additional KP 560 barrier filter were used (for a band pass of 420-560 nm wavelengths).

C. Photography

At this point it is necessary to detail how the accompanying micrographs were exposed and printed so that reference can be made to them. Note that for each Figure (numbers 1 through 14) there are 5 illustrations: (a), (b), (c), (d) and (e). In all cases these illustrations represent the following:

- (a) TE photographed with bright field optics using an automatic exposure meter and printed on #3 Ilford paper with enlarger setting f/11 for two seconds unless otherwise noted in the figure legend.
- (b) TE photographed using Filter System II (i.e. Calcofluor/Hoechst channel) with an automatic exposure meter to ensure that even faint fluorescence would show up on the micrograph. Printed on #5 Ilford paper (the paper routinely used to print micrographs in which microtubules are to be the main subject) at enlarger setting f/8 for two seconds unless otherwise noted.
- (c) TE photographed using Filter System I (i.e. FITC channel) with an automatic exposure meter to ensure that any faint fluorescence would show on the micrograph. Printed on #5 Ilford paper at f/8 for two seconds unless otherwise noted.
- (d) TE photographed using Filter System I (FITC channel) with a 3 second exposure. (This exposure time was determined to be sufficient to allow FITC indirect staining of mts to be easily visible on the ensuing micrograph.) Printed on #5 Ilford paper at a constant enlarger setting of f/8 for 2 seconds. This illustration allows comparison of autofluorescence,

undesired non-specific staining with the secondary antibody (2^0 Ab) or "spill-over" fluorescence from the Calcofluor and/or Hoechst stains which might appear in the FITC channel. Thus all illustrations (d) are comparable between Figures 1 through 14. Because the other illustrations (a,b,c and e) were made using an automatic exposure meter, the actual intensity of any visible fluorescence may vary greatly while the exposure meter may cause this intensity variation to be greatly minimized. Any comparison between Figures should keep this fact in mind.

- (e) TE photographed using Filter System I with the narrow band barrier filter removed. Thus all wavelengths longer than 520 nm can now be photographed. Exposures were made using the automatic exposure meter and micrographs were printed on #5 Ilford paper at the enlarger f stop and time specified in the figure legend. This filter combination was not used during the normal course of observation but is included here to show that the fluorescence of lignin (yellow) and that of chlorophyll (red) is blocked by the presence of the 560 nm barrier filter and therefore is not a problem (compare 3e and 3d). Note that the presence or absence of lignin and/or chlorophyll and/or plastids was not of concern in this project. A small amount of fluorescence in the green-yellow (narrow band FITC) range can be detected at the same location as the yellow lignin and red chloroplasts but this fluorescence is not brilliant, (compare 5d and 5e, 6d and 6e) and does not interfere with observations made in the normal manner in this project.

D. Fluorochromes

- 1) Calcofluor - Calcofluor White is an optical brightener which has been shown to bind to chitin, cellulose and other β -linked polysaccharide polymers (Maeda and Ishida 1967). It has also been shown to bind to callose and other wall components (Hughes and McCully, 1975). Its use in these experiments is merely to locate cell walls, not to identify the composition of these wall. It was empirically determined that, using Filter system II, Calcofluor stains the primary wall (1^0 wall) blue and the secondary wall (2^0 wall) a much more intense blue fluorescence thereby allowing easy observance of the 2^0 wall pattern.

Fischer, et. al. (1985) report that Calcofluor stains the nuclei and cytoplasm of fixed cells. In these preparations the nuclear staining may be present (although not with brilliant fluorescence - figure not shown) or the nuclear staining may be absent (See Fig. 14b, a cell which has an intact protoplast but no nuclear staining was observed). I cannot explain this observation at this time. The cytoplasm has been largely extracted in these preparations (Simmonds, et. al. 1985) and thus cytoplasmic fluorescence is difficult to detect considering that the walls also provide a brilliant source of fluorescence.

Holt and Milligan (1974) have shown that proteins when irradiated with sunlight or UV light in the presence of a large excess of Calcofluor, can be covalently bound to Calcofluor.

This does not present a problem in these experiments because at the time when the cells are being stained in a large excess of Calcofluor, no sunlight or UV irradiation is present. After staining, the cells are rinsed 3 times for 5 minutes each time with PBS to remove unbound Calcofluor before mounting and viewing. It is unlikely that sufficient molar excess of Calcofluor remains to cause the binding reaction to go forward.

Hughes and McCully (1975) state that when Calcofluor is excited by wavelengths between 325 and 500 nm and viewed with a barrier filter which transmits all wavelengths above 530 nm, a greenish-yellow fluorescence is observed. This indicates that Calcofluor staining of the 2⁰ wall should be visible with the FITC Filter System I as well as with the C/H Filter System II due to "spill-over" of fluorescent wavelengths which can be accommodated not only by the desired Filter System II but also by Filter System I (i.e. in the 520-560 nm range). This is, however, not a problem. Both unstained Fig. 3c and Calcofluor stained Fig. 5c, show the presence of 2⁰ wall fluorescence. In Fig. 3c this fluorescence must be due to autofluorescence of the 2⁰ wall. In Fig. 5c it can be a combination of autofluorescence and Calcofluor "spill-over". However, by comparing 3d and 5d, it can be seen that the intensity of such staining is low and will not interfere with mt observations (see mt staining in Fig. 13 d) which are far more brilliant. Additionally, both autofluorescence and Calcofluor and Hoechst staining patterns cover the 2⁰ wall with an opaque fluorescence (see Figs. 3b, 3c, 5b, 5c, 7b, 7c). Microtubule staining is clearly fibrillar in nature (compare the above Figures with Fig. 13c) and the difference is easily discernable.

- 2) Hoechst #33258 - Technical information from the Hoechst manufacturer indicates that Hoechst #33258 staining is specific for DNA (brochure from Calbiochem-Behring, no date). Hoechst #33258 fluoresces with a very pale, blue/white color when excited and viewed with Filter System II.

Filion et. al. (1976) used Hoechst #33258 to stain root tips and they report the stain to be specific for DNA.

LaLoue, et. al. (1980) report staining both plant tissue culture cells and sections of plant tissue with Hoechst #33258. They state that a bright fluorescence of nuclei and chromosomes was seen while "cytoplasm and cell walls do not fluoresce".

When Hoechst #33258 was added to the indirect immunofluorescent procedure outlined in Manuscript I, a control was done in which the dye was added to fixed and extracted cells. The culture used for this control had differentiated to a minimal extent and (apparently) no TEs were included in the stained sample. Hoechst #33258 was seen to stain only nuclei and chromosomes as indicated in the references above (Fig. not shown). However Hoechst staining of xylem in hand sections of plant tissue has been reported (personal communication Dr. M.E. McCully). Consequently the Hoechst control was done again using a culture with a high rate of TE differentiation. It was observed that Hoechst #33258 did, indeed, stain the 2⁰ TE walls (no other cell walls showed staining) as well as the expected staining of nuclei and chromosomes. This staining of the 2⁰ walls is virtually identical to that shown by Calcofluor (compare Fig. 7 with Figs. 5 and 6) with the

exception that Calcofluor also stains the 1^0 walls and the nuclear staining shown by Hoechst is much more brilliant than the nuclear staining which may be seen with Calcofluor.

This finding does not, however, alter the interpretation of the results in this work. Both Calcofluor and Hoechst stain the 2^0 walls of Stage Two TE differentiation (see Manuscript I for definition of Stage Two) (compare Figs. 12 and 14). It is not possible to ascertain which stain binds to the newly forming 2^0 walls first or whether both stain the new walls at the same period of development. When combined together, as shown in Fig. 5 of Manuscript I, the C/H combination also stains 2^0 walls of Stage Two differentiation. Again, it is not possible to determine the relative timing of this staining reaction. Stage I TE differentiation (defined in Manuscript I) can be demonstrated to occur in cultures stained with either Calcofluor or Hoechst or a combination of both (Fig. not shown).

3) Fluorescein isothiocyanate (FITC) - According to information provided by Zeiss (Zeiss, no date given), fluorescein isothiocyanate excited by wavelengths between 450 and 490 nm will fluoresce yellowgreen when viewed with wavelengths between 500 and 550 nm. It is therefore the fluorochrome of choice when observing with Filter System I.

In these experiments, FITC has been conjugated with the 2^0 Ab (secondary anti-body), a rabbit-anti-rat IgG. The primary anti-body (1^0 Ab) was raised in rats using purified yeast tubulin as the antigen. Using the method of monoclonal antibody production

(Milstein, 1980), a 1⁰ Ab is produced which is specific to tubulin and therefore binds to microtubules. Free tubulin in the cytoplasm is largely removed during extraction and therefore does not cause a significant amount of background fluorescence in the cells after application of the 2⁰ Ab.

The specificity of the 2⁰ Ab binding is determined by using a control in which fixed and extracted cells are incubated with only the 2⁰ Ab (Figs. 8 and 9). While a certain amount of non-specific binding of 2⁰ Ab to the secondary walls of mature TEs has been seen, this does not present a problem in interpreting the results for several reasons.

- (1) Most non-specific binding of 2⁰ Ab to TE 2⁰ walls occurs with older cultures (i.e. during the second burst of differentiation between days 10 and 13 post culturing. These culture ages were not under study for this project. Cultures 6 days old or younger, which were used in these experiments, show only minimal, if any non-specific 2⁰ Ab binding and this only to mature TEs (see Figs. 8c and 8d , 9c and 9d). Fig. 8d clearly demonstrates that the intensity of any non-specific 2⁰ Ab binding is clearly much less than the intensity of the indirect immunofluorescent staining of mts and will not cause a problem in interpretation.
- (2) Part of the fluorescence seen in the 2⁰ walls in Fig. 8c must be ascribed to autofluorescence of the mature TE 2⁰ walls (see Fig. 3c). Yet even in combination, the

autofluorescence and non-specific 2⁰ Ab binding shown in Fig. 8c are not intense enough to show serious interference with mt patterns in 8d or 10d.

- (3) Finally, non-specific binding of the 2⁰ Ab, when it does occur, binds with an opaque and uniform intensity across the 2⁰ wall. It is easily distinguishable from the fibrillar staining shown by microtubules (see Fig. 10c).

E. Figure Legends

It is necessary to make some general statements about the Figures at this point. The results of each particular control are shown as they apply to mature tracheary elements and to immature tracheary elements. In this study, the primary objects of observation were cells in the process of undergoing TE differentiation, i.e. undifferentiated cells through Stage One, Stage Two and up to Stage Three differentiation with particular respect to the changes in microtubule arrays during this period. Thus the results of the various controls are particularly interesting with regard to the immature TE.

For the purposes of these illustrations only, a mature TE is defined as one in which the 2⁰ wall pattern is visible with bright field or phase contrast optics. A mature TE may be one that has undergone autolysis and has no remaining protoplast or it may be a TE with a visible 2⁰ wall which had an intact

protoplast at the time of fixation. Obviously in controls which require the presence of an intact protoplast (i.e. those demonstrating mt staining), cells which have undergone autolysis will not be illustrated. However, where only the staining patterns of 2⁰ walls are under observation, the mature TE may be one without an intact protoplast. In these cases, information regarding the staining of cytoplasm, nuclei, etc. can be obtained by inspecting micrographs of the immature TE control. Obviously all immature TEs contain intact protoplasts.

In a number of TEs with intact protoplasts, when observed with Filter System I without the presence of the narrowband filter (i.e. observing all wavelengths above 520 nm), red autofluorescence may be seen as discrete dots (see Fig. 7b, small arrow), as more generalized fluorescence (see Fig. 12e, arrow) or as a combination of discrete dots and generalized fluorescence (see Fig. 6e, arrow). The discrete dots are presumably chloroplasts; the generalized red autofluorescence may be chlorophyll which has dispersed in the cytoplasm. The discrete plastid and dispersed chlorophyll fluorescence is almost entirely filtered out by the presence of the narrow band filter (which creates a band pass of 520-560nm). The small component of this fluorescence which is transmitted between 520 and 560 nm (see Fig. 6c) is very dim as shown by its absence in the 3 second exposure (Fig. 6d).

Fig. 1. Live cell. Mature TE. (a) Bright field. (b) Filter System II (FS II), i.e. Calcofluor/Hoechst channel. Autofluorescence of 2⁰ wall is clearly seen. (c) Filter System I₂ (FS I), i.e. FITC channel. Autofluorescence of 2⁰ wall is very dim, with automatic exposure meter, exposure time was about 11 seconds. (d) No 3 second exposure because fluorescence in (c) was so dim. (e) FS I without additional 560 nm barrier filter. 2⁰ walls show yellow fluorescence of lignin. Small dots within TE are red chloroplast fluorescence. Printed at f/8 for 2 seconds. Magnification 480x.

Fig. 2. Live cell. Immature TE. (a) Bright field. Note the just barely visible rings of the newly forming 2⁰ wall (arrowheads). (b) FS II (C/H channel). No 2⁰ wall autofluorescence is detectable. (c) Very faint fluorescence except for bright dots in cortical cytoplasm. (d) Exposure time in (c) was so long that no 3 second exposure was taken. (e) The bright dots in (c) fluoresce red when viewed without the 560 nm barrier filter. This chlorophyll fluorescence is excluded from FS I but some component which fluoresces in the 520-560 nm range can be seen. Printed at f/8 for 2 seconds. Mag. 480x.

Fig. 3. Fixed and extracted cell. Mature TE (lysed). (a) Bright field. (b) FS II, note autofluorescence of 2⁰ walls in mature TE. (c) FS I, 2⁰ walls display some autofluorescence but not enough to show up on a 3 second exposure shown in (d). (e) 2⁰ walls display yellow autofluorescence of lignin. Printed at f/8 for 4 seconds. Magnification 480x.

e

Fig. 4. Fixed and extracted. Immature TE. (a) Bright field. Note the very small thickenings indicating the appearance of 2^0 walls (arrowheads). (b) Faint autofluorescence of what may be 2^0 wall material and of cytoplasm. Nuclear autofluorescence is visible. (c) Faint autofluorescence of what may be 2^0 walls and cytoplasm. No nuclear autofluorescence. (d) No fluorescence appears in the 3 second exposure. (e) Autofluorescence similar to that in (c). Printed at f/5.6 for 4 seconds. Magnification 480x.

Fig. 5. Fixed, extracted, plus Calcofluor White. Mature TE. (a) Bright field. (b) This TE is partially collapsed and therefore the brilliant Calcofluor staining of the 2^0 walls reflects the combined pattern of both upper and lower focal planes of the TE. (c) Autofluorescence of this lysed, mature TE can be seen but is not brilliant enough to show on a 3 second exposure (d). (e) Autofluorescence of 2^0 walls and chloroplasts and dispersed chlorophyll (arrow) in the adjacent cell is seen. Printed at f/5.6 for 3 seconds. Magnification 480x.

Fig. 6. Fixed, extracted, plus Calcofluor White. Immature TE. (a) Bright field. Note the very faint wall thickenings (arrowheads) indicating newly forming 2^0 walls. (b) Calcofluor staining of 2^0 wall pattern. Note absence of nuclear staining. (c) No autofluorescence of 2^0 walls is visible. (d) No visible fluorescence in a 3 second exposure. (e) Red autofluorescence of chlorophyll is present as discrete dots and dispersed chlorophyll (arrow). No fluorescence of the 2^0 wall is seen. Printed at f/5.6 for 3 seconds. Magnification 480x.

Fig. 7. Fixed and extracted plus Hoechst. Mature and immature TEs.

(a) Bright field. Mature TE with visible 2⁰ wall bands. Immature TE (arrow) with no visible wall bands. (b) FS II. Hoechst staining of mature TE shows no nucleus (TE has autolysed, bright spot is nucleus of adjacent, undifferentiated cell) but brilliant staining of 2⁰ wall. Staining of 2⁰ wall pattern in immature TE is also visible although less intense. Note nuclear stain in immature TE (large arrow).

Small arrow points to fluorescence of plastid DNA. (c) Fluorescence of 2⁰ wall in mature TE may be due to autofluorescence or to Hoechst "spill-over" of fluorescence but it is not an intense fluorescence as shown by lack of detection in the 3 second exposure (d). In (c) also note the lack of 2⁰ wall autofluorescence in the immature TE. (e) Yellow lignin and red chlorophyll autofluorescence are visible, no autofluorescence of 2⁰ walls in immature TE can be seen.

Printed at f/5.6 for 2 seconds. Magnification 480x.

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Fig. 8. Fixed and extracted plus 2⁰ Ab only. Mature TE. (a) Bright field. (b) FS II. Fluorescence may be due to autofluorescence or more likely to a combination of autofluorescence and "spill-over" of non-specific FITC stain. (c) FS I. This micrograph was printed at f/5.6 for 8 seconds to arrive at a black background. Therefore the 2⁰ wall stain by non-specific binding of the 2⁰ Ab is moderately intense. However, the 3 second exposure of (d) shows only a faint outline of the 2⁰ walls, thus relative to indirect mt staining with FITC, the non-specific 2⁰ Ab stain is fairly faint and does not cause problems in interpretation of results. Note also that the staining and/or autofluorescence in (b) and (c) do not resemble the fibrillar stain pattern shown by indirectly labelled mts. (e) The yellow autofluorescence of lignin is easily detected. Printed at f/5.6 for 4 seconds. Magnification 480x.

Fig. 9. Fixed, extracted plus 2⁰ Ab only. Immature TE. (a) Bright field. Note the very faint outlines of the 2⁰ walls (arrowheads). The protoplast is visibly shrunken in this TE. (b) FS II. Very faint autofluorescence of 2⁰ walls is visible (arrow). (c) Due to very low level of fluorescence with FS I, the exposure meter allowed a very long exposure time (about 12 seconds) to detect the very faint background fluorescence of 2⁰ Ab. Since the 2⁰ walls of the immature TE did not stain with the 2⁰ Ab, they appear black (arrow). No 2⁰ wall fluorescence appears in either (d) or (e). Printed at f/5.6 for 3 seconds. Magnification 480x.

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Fig. 10. Fixed, extracted, plus 1⁰ and 2⁰ Abs. Mature and Immature TEs in the same illustration. (a) Bright field. Note 2⁰ walls of mature TE. Immature TEs have no visible 2⁰ walls (arrows) but are located by their microtubule pattern in (c). (b) FS II. Auto-fluorescence and/or non-specific but low level 2⁰ Ab binding are seen with the Mature TE. The immature TEs show faint but detectable autofluorescence of the newly forming 2⁰ walls. Printed at f 8 for 4 seconds. (c) FS I. Note the mt staining pattern in the immature TEs. The mature TE has undergone autolysis and consequently no mts are visible. Note that only very faint autofluorescence or non-specific 2⁰ Ab is visible with the mature TE. Clearly it does not interfere with mt observation. The 3 second exposure of (d) shows the intensity of the indirect immunofluorescent staining of the mt population with regard to any non-specific or autofluorescent wall image that may appear. (e) The 2⁰ wall of the mature TE shows yellow fluorescence of lignin. In the immature TEs in this illustration, no chlorophyll fluorescence was seen. However, as it is removed by the presence of the 560 nm barrier filter, its presence is immaterial with regard to the current project. Printed at f/5.6 for 3 seconds. Magnification 480x.

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Fig. 11. Fixed, extracted, 1⁰ and 2⁰ Abs plus Hoechst #33258.

Mature TE. (a) Bright field. Printed at f/8 for 3 seconds.

(b) FS II. Hoechst staining of the 2⁰ wall is quite brilliant.

The nucleus is in a portion of the cell not shown. Note that

the Hoechst staining is opaque and covers the 2⁰ wall surface.

It is not fibrillar in nature. (c) FS I. Mt staining is clearly

seen. In mature TEs the mt pattern is located predominantly at

the edges of the 2⁰ walls (this will be investigated in a later

project on continuing wall synthesis). However, some mts can be

seen away from the wall edges (arrow). The fibrillar nature of

this staining is evident and quite distinct from that of auto-

fluorescence, Calcofluor or Hoechst staining of the 2⁰ wall.

(d) 3 second exposure shows a somewhat dim pattern of mt staining.

No 2⁰ wall autofluorescence or other staining is apparent however.

(e) No yellow lignin autofluorescence was seen in this mature TE.

One may assume that lignin deposition had not yet occurred in any

significant amount. Printed at f/8 for 2 seconds. (Fig. 11b was

printed at f/5.6 for 2 seconds. Fig. 11c was printed at f/5.6 for

2 seconds.) All magnifications 480x.

Fig. 12. Fixed, extracted, 1⁰ and 2⁰ Abs plus Hoechst #33258.

Immature TE. (a) Bright field. Note 2⁰ wall band just barely visible (arrowhead). (b) FS II. Hoechst staining of the newly deposited 2⁰ wall is quite visible. Note nuclear staining as well (arrow).

Printed at f/5.6 for 2 seconds. (c) Although the protoplast has shrunk, the mt staining pattern is clearly visible using FS I. Note the obvious fibrillar nature (arrow) of the staining. No 2⁰ wall fluorescence is evident. In the 3 second exposure shown in (d), only the mt pattern is visible. Thus in the developing TE, autofluorescence, non-specific binding of 2⁰ Ab or "spill-over" fluorescence from Calcofluor or Hoechst staining does not pose a problem in interpretation. In (e) red autofluorescence of dispersed chlorophyll is present in an adjacent cell (arrow).

The mt staining is easily detected, but no 2⁰ wall fluorescence is present. Printed at f/5.6 for 4 seconds. (Fig. 12c was printed at f/5.6 for 4 seconds also). All magnifications 480x.

Fig. 13. Fixed, extracted, 1⁰ plus 2⁰ Abs plus Calcofluor White. Mature TE with protoplast intact. (a) Bright field. Printed at f/11 for 3 seconds. Although the 2⁰ walls are not as thick as in many mature TEs, they are clearly visible (arrowheads). (b) FS II. Calcofluor stain of the 2⁰ wall is readily seen and brilliant. Nuclear staining is not seen (nucleus may be in portion of the cell not included in the micrograph). Printed at f/5.6 for 5 seconds. (c) Although fixation in this example is not optimal, the mts are obviously fibrillar in nature (arrow). Autofluorescence, non-specific staining of the wall by 2⁰ Ab, and/or "spill-over" of Calcofluor staining into FS I is clearly not a problem in interpreting mt organization and, in fact, is not detectable. the 3-second exposure of (d) also shows only mt patterns. (e) No yellow autofluorescence of lignin could be detected at this time. Printed at f/5.6 for 5 seconds. All magnifications 480x.

Fig. 14. Fixed, extracted, 1⁰ and 2⁰ Abs plus Calcofluor White. Immature TE. (a) Bright field. No 2⁰ walls can be seen (arrow). (b) FS II. Calcofluor shows the newly developing 2⁰ wall pattern. No nuclear fluorescence can be detected. Printed at f/5.6 for 6 seconds. (c) The mt staining pattern clearly shows a fibrillar nature (arrow). No 2⁰ wall fluorescence can be detected in (c) or in (d). (e) Only the indirect immunofluorescent staining of mts is detectable in this micrograph. The protoplast is somewhat shrunken in this TE. Note the lack of any wall staining in (c) (curved arrow). (e) Was printed at f/5.6 for 4 seconds. All magnifications 480x.

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