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

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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.

Keywords: Cell suspension cultures; Differentiation; Microtubules; Taxol; Xylogenesis.

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 (1980 a, b), 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.

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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, *Poterioochromonas* (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 in 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 minutes 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% $NaBH_4$ followed by PBS rinses. Rat monoclonal antibody to yeast tubulin (Cedarlane Lab., Hornby, Ont.) was layered on coverslips for 45 minutes, washed in PBS and followed by incubation for 45 minutes with fluorescein conjugated secondary antibody (rabbit anti-rat, Miles Lab., Rexdale, Ont.). Cells were briefly stained for DNA with Hoechst no. 33258 followed by a 3-minute 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.

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. 1 *a*), 2. Angled—alignment of microtubules intermediate between longitudinal and transverse (Fig. 1 *b*), 3. Transverse—aligned at right angles to the long dimension of the cell (Fig. 1 *c*), and 4. Random—no over-all orientation (Fig. 1 *d*).

The majority of newly cultured cells display longitudinally oriented microtubule arrays. On day 3 postculturing, 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. 2 *a*). 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. 2 *a*). Cultures were not sampled earlier than day 3 due to technical inability to stain the cells.

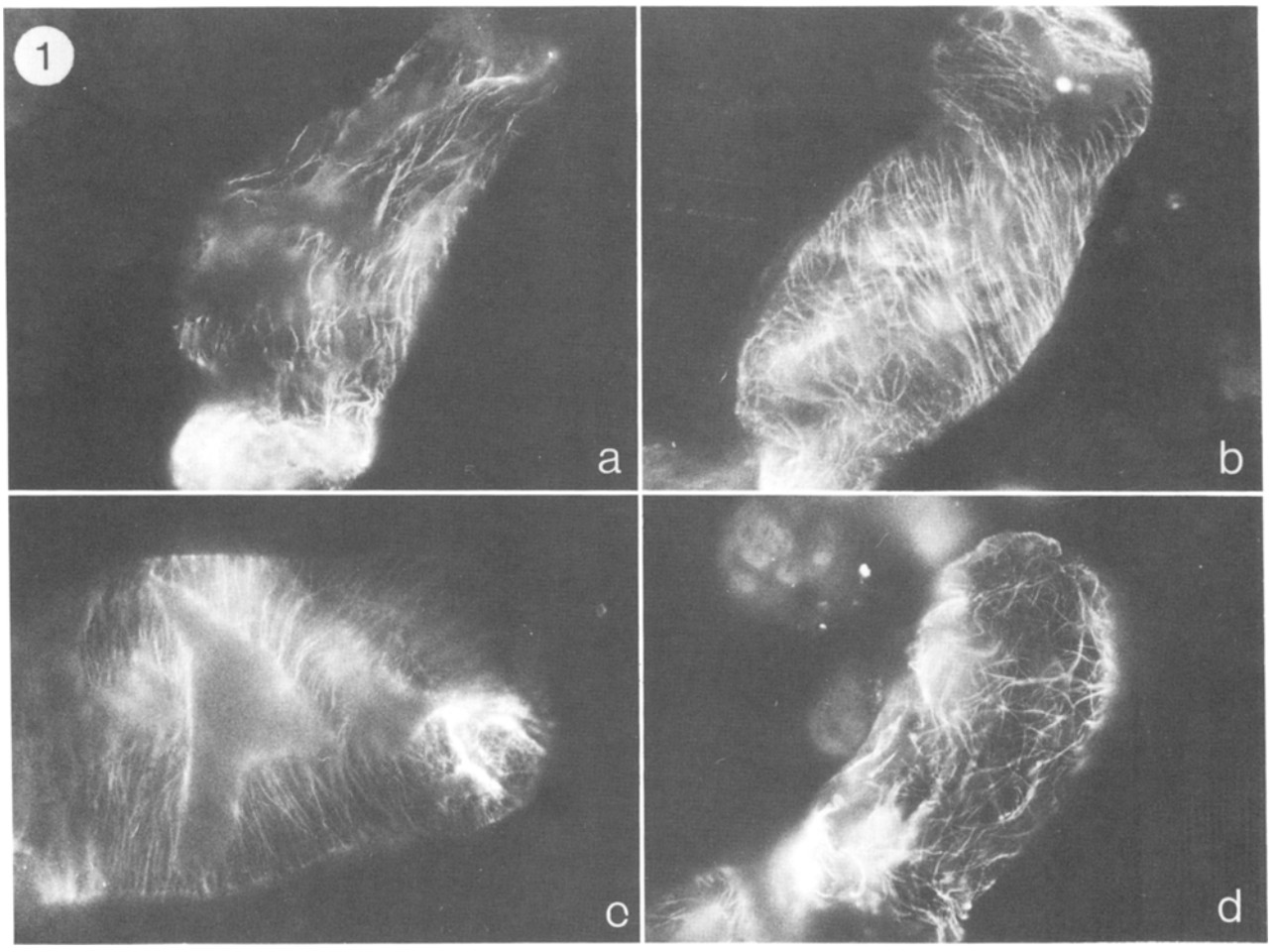

Fig. 1. Orientations of cortical microtubule arrays in cultured *Z. elegans*. *a* Longitudinal—microtubules parallel, or nearly parallel, to the long axis of the cell; *b* Angled—microtubules oriented between longitudinal and transverse; *c* Transverse—microtubules oriented approximately at right angles to the long axis of the cell; *d* Random—no over-all microtubule orientation. × 756

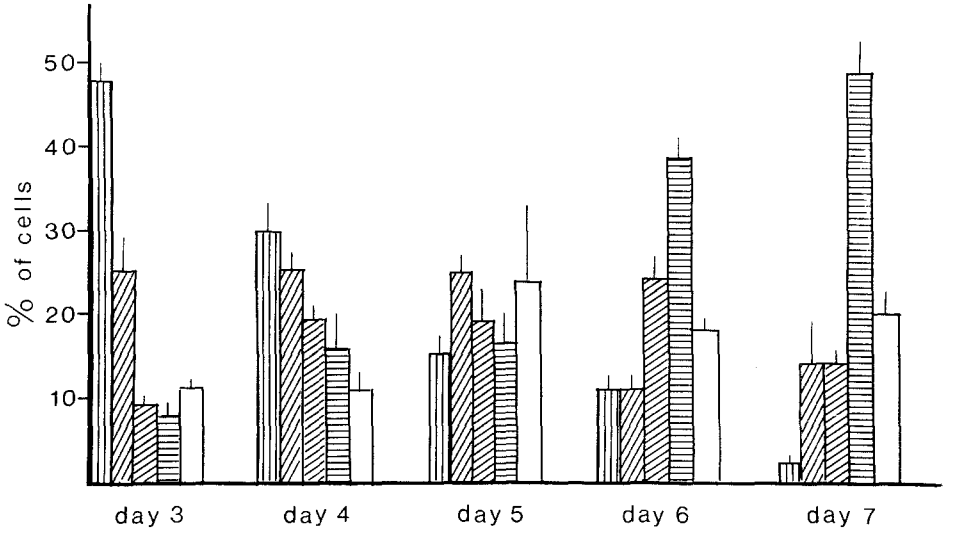

Fig. 2 *a*. Microtubule orientation changes with culture age. Cells sampled on days 3, 4, 5, 6, and 7 post culturing and fluorescently stained to show microtubule orientations. Lines in bar graph indicate orientation of microtubules with respect to the long axis of the cell (vertical lines—longitudinal microtubules; angled lines—microtubules increasingly angled toward the transverse; horizontal lines—transverse microtubules; and no lines—random microtubules). 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

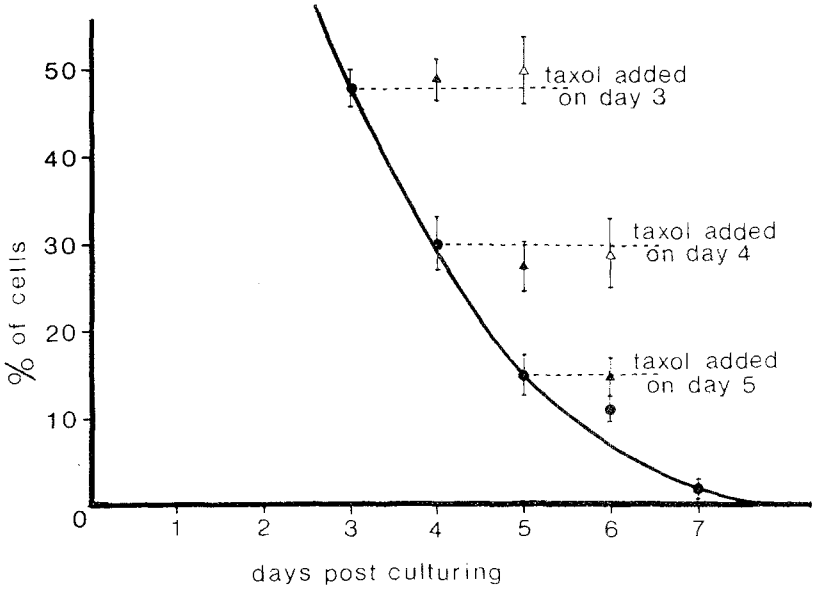

Fig. 2 *b*. Microtubules are stabilized in the longitudinal orientation for 48 hours in taxol. Control cultures (dots) sampled on days 3, 4, 5, 6, and 7. After sampling, taxol was added to cultures as noted. Taxol treated cultures were sampled at 24 (triangle) and 48 (open triangle) hours. Percent of 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

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. 2 *b* and 3 *a*). 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. 2 *b*). 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 hours (day 5) and 48 hours (day 6) samplings. Microtubules oriented transversely (Fig. 3 *b*) 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. 3 *b*) although this association is not pronounced.

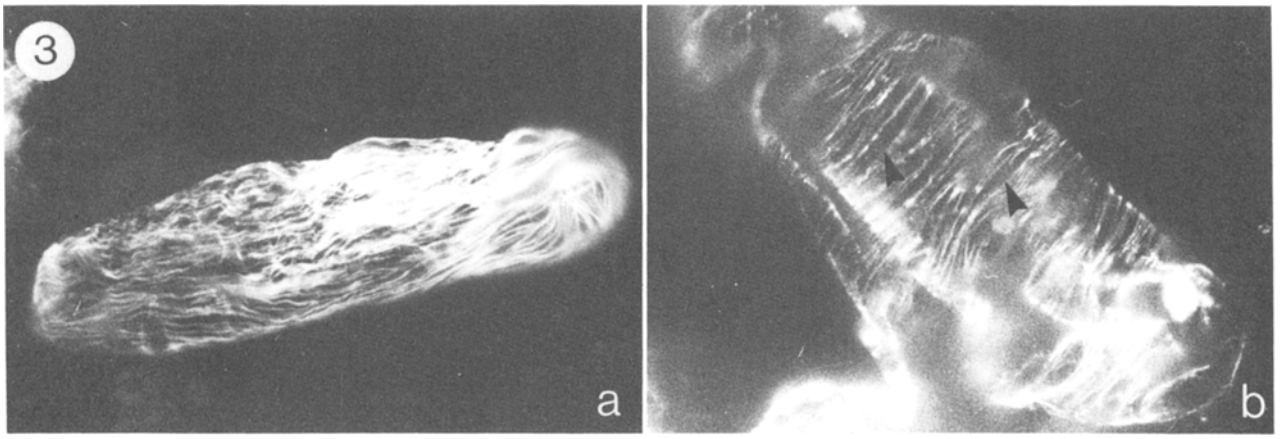

Fig. 3. Microtubule arrays stabilized by taxol. *a* Longitudinal microtubule array. Note increased numbers of microtubules in comparison with Figs. 1 *a* and *b*. Transverse microtubule array. Note spaces (arrowhead) apparently due to lateral association of microtubules. × 756

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. 3 *a* with Fig. 1 *a*). 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 postculturing. 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 white. 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 postculture. 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 Elements 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. 2 *a* and *b*). Tracheary elements which differentiate in these cultures will show wall bands parallel, or approximately parallel, to the long axis of the cell (Figs. 4 and 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. 2 *a*). Tracheary elements forming in these cultures still display predominantly longitudinal or

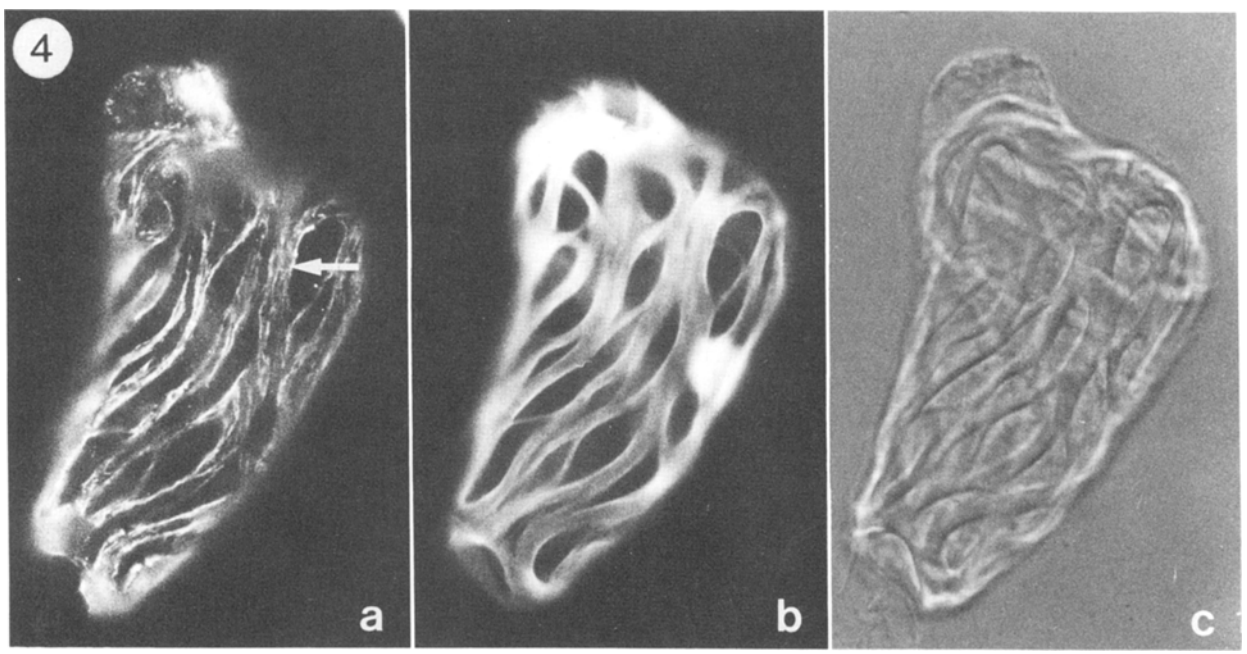

Fig. 4. Longitudinal secondary wall pattern of tracheary element which differentiated in taxol. *a* Groups of microtubules (arrow) are oriented parallel to long axis of cell. *b* Calcofluor white staining of secondary wall. Note similarity to microtubule pattern. *c* Phase contrast micrograph showing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

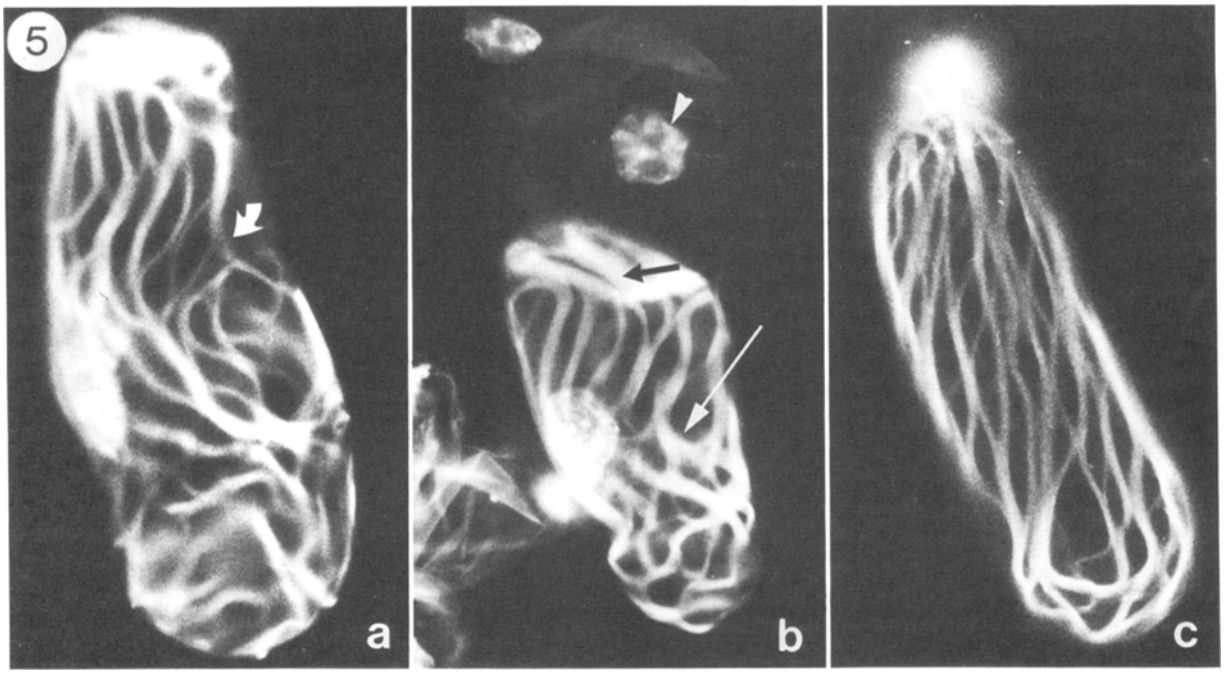

Fig. 5. Calcofluor white staining of longitudinal wall patterns in tracheary elements which differentiated in taxol treated cultures. *a* Longitudinal wall bands showing "waves" (curved arrow). *b* Two daughter cells, one remaining undifferentiated (note Hoechst stained nucleus, arrowhead), the other 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. × 756

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. 2 *a*) 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 subpatterns 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 staining) is found in taxol treated cultures on day 5. No tracheary elements, in any stage of differentiation, are

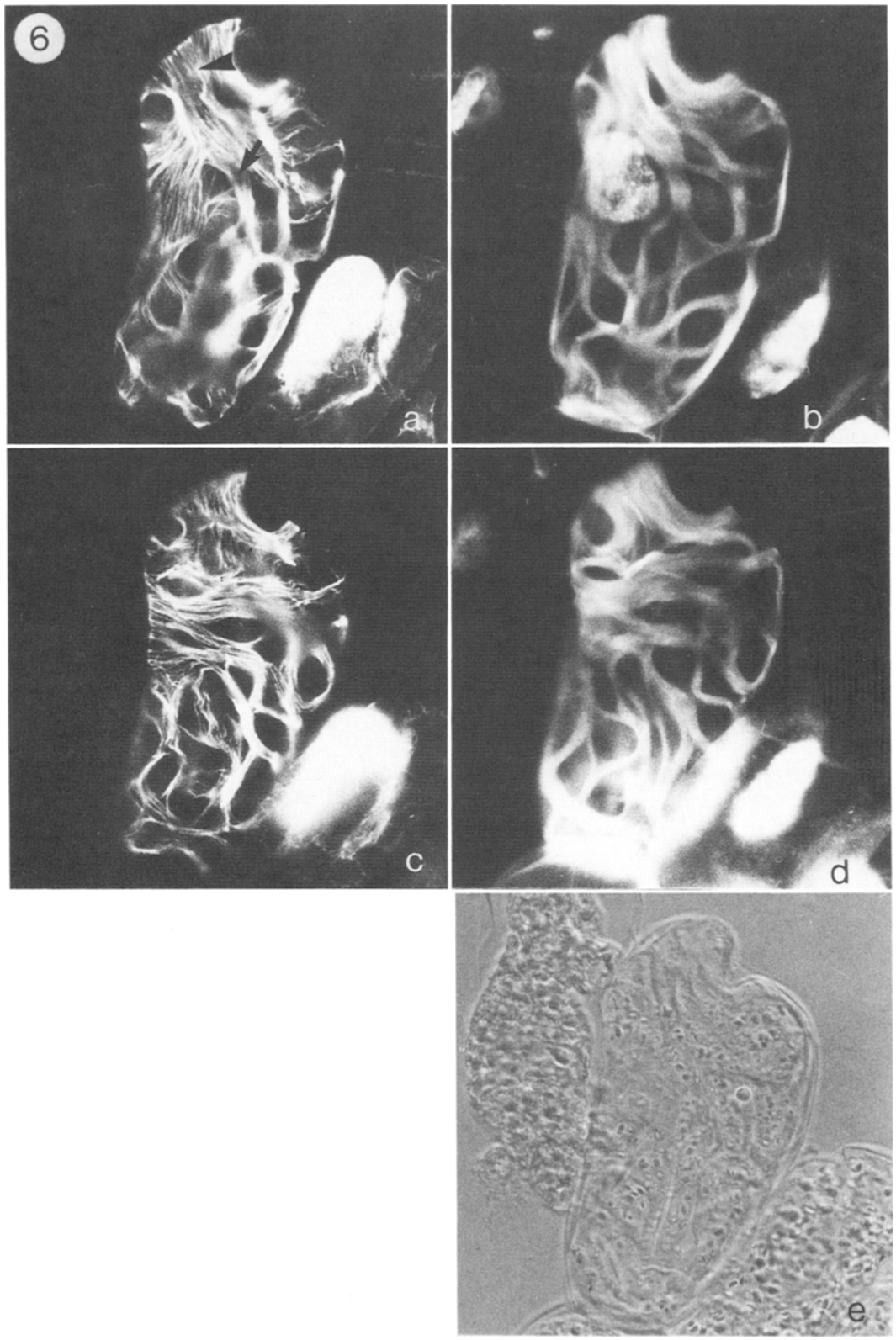

Fig. 6. Two focal planes of a tracheary element which differentiated in taxol and shows longitudinal, angled and transverse patterns within the same cell. *a* Microtubules in upper focal plane—note change in orientation. Dispersed microtubules (arrowhead) in upper left portion of cell laterally associate to form distinct groups (arrow). *b* Calcofluor white 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 white 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 these cells will become as visible as those in Fig. 4 *c*. × 756

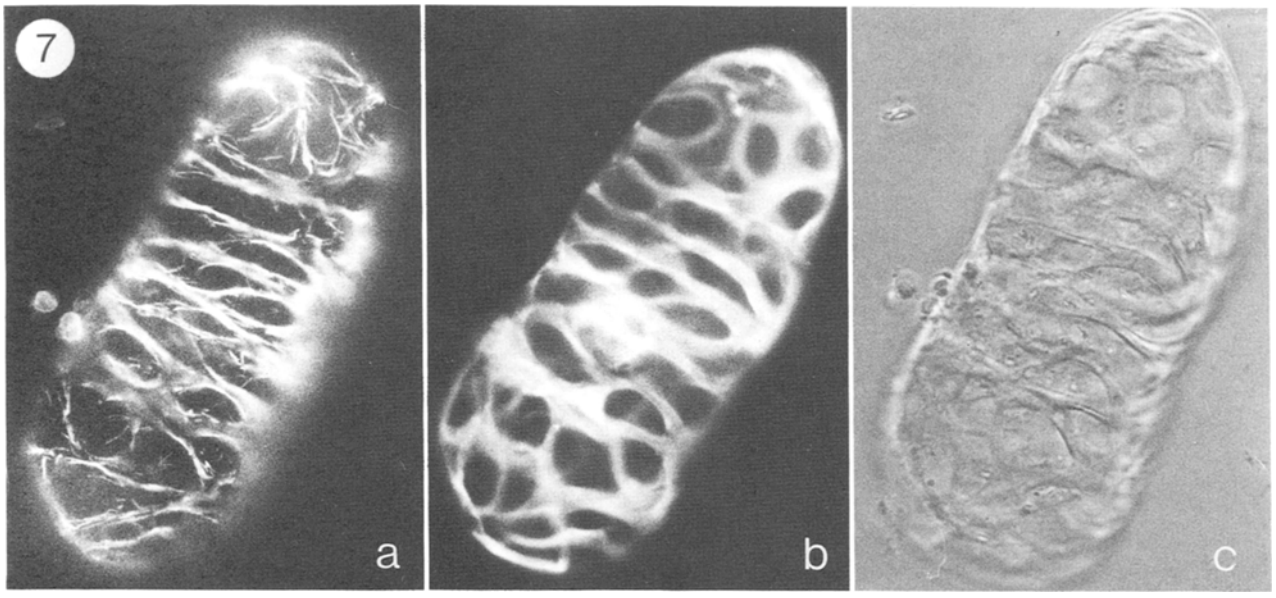

Fig. 7. Transverse secondary wall pattern of tracheary element which differentiated in taxol. *a* Microtubules are primarily oriented transverse to the long axis of the cell. *b* Calcofluor white staining of secondary wall. *c* Phase contrast of same cell showing secondary wall pattern. This tracheary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

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? 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. 2 *a*). 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,

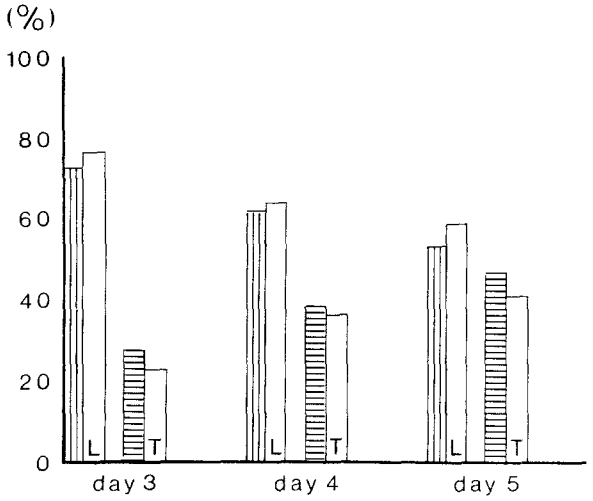

Fig. 8. Comparison of microtubule orientation and the secondary wall orientation of tracheary elements in taxol treated cultures. Microtubule orientations were stabilized in longitudinal (vertical lines) or transverse (horizontal lines) arrays by the addition of taxol on days 3, 4, and 5. Longitudinal 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

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 treacheary 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. Alternately, 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. 4–7, 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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