# THE INVESTIGATION OF SECONDARY CELL WALL DEPOSITION IN DIFFERENTIATING *ZINNIA* CELL SUSPENSION CULTURES

by

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# ABBREVIATIONS

| | |
|---|---|
| AOPP | L-α-aminooxy-ß-phenylpropionic acid |
| Araf | α-L-arabinose furanose |
| BF | Bright field light microscopy |
| CCRC | Complex Carbohydrate Research Center |
| DCB | 2,6 dichlorobenzonitrile |
| DCPA | 2,6 dichlorophenylazide |
| DIC | Differential Interference Contrast light microscopy |
| ECL | Enhanced Chemiluminescence |
| FID | Flame Ionization Detector |
| FITC | Fluoroscein Isothiocyanate |
| GC | Gas Chromatography |
| GPC | Gas Proportional Counter |
| GRP | Glycine Rich Protein |
| GSI | glucan synthase I |
| GSII | glucan synthase II |
| HRGP | hydroxyproline-rich glycoproteins |
| IND | Induced (differentiating control cultures) |
| MS | Mass spectroscopy |
| MT | microtubule |
| NI | Non-induced (non-differentiating control cultures) |
| NMR | Nuclear Magnetic Resonance |
| PRP | Proline Rich Protein |
| TBS | Tris-Buffer Saline |
| TBST | Tris-Buffer Saline with Tween |
| TE | Tracheary Element |
| TFA | Trifluoroacetic acid |
| UDP | Uridine-diphosphate |
| GlcA | α-D-glucuronic acid |

# CHAPTER 1
# INTRODUCTION AND LITERATURE REVIEW

## 1.1 Cell Walls

### 1.1.1 Overview

The study of plant cell walls has been of great interest for many years because of the importance of the cell wall in plant development and in materials of economic utility such as fiber and wood. A plant cell wall is defined as the "layer of structural material found external to the protoplast" (Fry, 1988 p 2) in plant cells. The protective and strengthening characteristics of cell walls influence or possibly regulate the cell's shape, which in the end determines the form and adaptability of a plant (Roberts, 1989). Understanding cell walls and their components can lead to better utilization of plants and provide a possible basis for developing modified plants with increased commercial utility (Shedletzky et al., 1990).

The plant cell wall is now realized to have many critical functions in plant development and survival. These include: (a) regulating plant morphogenesis through controlled orientation of cellulose microfibrils; (b) mediating plant responses to pathogenic and environmental stress; (c) housing a diversity of complex polysaccharides, fragments of which can act as developmental signals; (d) providing an environment for the function of numerous enzymes; and (e) interacting with cytoplasmic components through linker proteins in the plasma membrane to regulate development (Roberts, 1989, 1990). Despite this increase in ascribed importance, our true understanding of wall composition and deposition is in its infancy, particularly for secondary cell walls.

Little is known about how individual cell wall molecules, including cellulose, hemicellulose, pectin, proteins, and lignin, interact with one another and possibly with other cellular components. It is known that lignin is often found in association with cellulose, but the chemical basis of their interaction is unknown. Siegel (1956) showed by *in vitro* analysis that the lignin precursor, eugenol, in combination with peroxidase and $H_2O_2$, would form a lignin-like component only when the reaction was carried out in the presence of filter paper or methylcellulose. This suggested that there was a necessary association between cellulose and lignin. The covalent and non-covalent interactions between cell wall components is an important area of study.

The signals that dictate where the cell wall components are needed in the cell wall matrix and when they need to be synthesized is also a topic of critical importance. Plant growth substances are likely to play a signaling role, in keeping with their ability to initiate,

delay or inhibit genetic expression. This raises the following obvious questions. Are these the ultimate controlling factors? Why are there intricate layers in the primary cell walls and specific thickening patterns in tracheary element secondary cell walls? Do some proteins bridge the plasma membrane and the extracellular matrix and probably act as sensors and for signaling molecules? Do these proteins identify with specific cell wall components, and if so, which ones? There are many questions and suppositions, but the real facts are lacking.

How interactions between cell wall components might affect cellular differentiation is a possible key to understanding plant development. A plant cell must follow a preprogramed differentiation process to reach its final function. If that process is disrupted, the final product or cell type is altered. An example is the disruption of normal cellulose synthesis (Delmer, 1987; Shedletzky et al., 1990) using inhibitors that apparently do not affect other cellular functions. In dicots, the adapted cells produce a weaker cell wall composed primarily of pectins (Shedletzky et al., 1992); and, therefore, the cell cannot be expected to perform its normal function. One example is the water conduction in xylem tracheary elements (TEs) that require considerable strength in their cell walls. The inhibition of cellulose synthesis in these cells would probably lead to a loss of transpiration in a plant due to the collapse of the TEs.

These questions and observations only reveal the obvious complex and intricate nature of one cell. The disruption of any stage of differentiation within a cell may be compensated for to allow survival, but the intended destiny for that cell is not likely to be achieved. Understanding how cell wall components interact during cell wall deposition in a cell will better our ability to understand how cells rely on every cellular component in some way. The possibility of using this knowledge to utilize simple cell wall perturbations to develop stronger, larger, or more palatable and digestible plants is a goal worth pursuing for the future.

### 1.1.2 Primary cell walls

A primary cell wall is the thin (0.1 to 0.2 μm diameter) extensible cell wall of a growing plant cell. In angiosperms it is usually high in hemicelluloses including xyloglucan (20-40 %) and cellulose (20-30 %) and is rarely lignified (Fry, 1988; McCann and Roberts, 1991). Most primary walls include protein. The disruption of H-bonding between cellulose microfibrils and the other components of the primary cell wall would allow the cell to enlarge in response to an increase in turgor pressure in the vacuole. The gel-like qualities that pectin gives a primary cell wall also allow for more flexibility.

### 1.1.3 Secondary cell walls

A secondary cell wall generally is deposited to impart strength and rigidity necessary for some special cell functions when the cell has stopped growing or elongating. Some fibers deposit secondary wall everywhere except the tips where they continue to elongate, but usually secondary cell wall deposition signals the end of cell growth and even death. The secondary cell wall is deposited to the inside of the primary cell wall and usually contains high amounts of cellulose (40-95%) and, in most instances, lignin. In angiosperms, xylan is another major component of the secondary cell wall. There is usually less cell wall protein present compared to primary cell walls and little or no pectin and xyloglucan (Fry, 1988).

### 1.1.4 Cell wall components

#### 1.1.4.1 Cellulose

Cellulose is a polymer of D-glucose monomers maintaining a strict ß-1,4-linkage (Fry, 1988). This linkage involves a $180^{o}$ rotation of each monomer creating a straight and rigid chain with very little flexibility. The degree of polymerization varies within cell wall types and among species. Primary cell walls have chain lengths (degree of polymerization) of around 500-1000 residues (Blaschek et al., 1982), while secondary walls may have lengths of up to 14,000 residues (Fry, 1988). The straight alignment of the ß-1,4-glucan chain allows a high potential for association with other ß-1,4-glucan chains via H-bonding, which leads to the formation of strong crystalline microfibrils (Roelofsen, 1965). This feature causes cellulose to be the major remaining residue upon alkaline or hot acid extraction of plant cell walls. Most microfibrils have a diameter of between 3.5-30 nm (Cote, 1977; Stafstrom and Staehelin, 1988; Varner and Lin, 1989), although their width varies at different stages of development and between species (Haigler, 1991). The natural role of cellulose microfibrils is analogous to that of steel rods used to reinforce a wall, imparting rigidity and strength. This is demonstrated when the cellulose synthesis inhibitor, 2,6-dichlorobenzonitrile (DCB), allows the swelling and bursting of algal cells under their own turgor pressure (Richmond, 1984).

Cellulose is extremely stable due to its strong H-bonds and crystalline nature. It must be attacked by specific enzymes in nature (referred to as cellulases) to facilitate its degradation. Fungal cellulases are the most commonly known because of the role of fungi in the decomposition of plant material (Wood, 1991), but there are also cell wall bound cellulases found in bacteria (Rapp and Beermann, 1991) and plants (Maclachlan and Carrington, 1991).

Because of its crystalline nature, cellulose is thought to be assembled at the extracellular surface of the plasma membrane. Although only circumstantial evidence is available, the cellulose synthase is proposed to be a six particle protein complex in higher plants, referred to as a rosette. Rosettes have been observed by freeze-fracture in various species at most levels of the endomembrane system (ER, Golgi cisternae and vesicles) through which normal protein synthesis, modification and transport take place (reviewed in Emons, 1991). From the Golgi, the vesicles containing rosettes (only one has been observed per Golgi vesicle thus far) must be targeted in some way to the appropriate location on the plasma membrane. Membrane fusion will then take place and the rosette will be incorporated into the plasma membrane (Haigler and Brown, 1986). It has been demonstrated that microtubules (MTs) associate with vesicles (Vale, 1987; Fosket and Morejohn, 1992), and they may play a role in directing these vesicles to the appropriate locations within a plant cell. It has also been observed that some vesicles contain rosettes of varying sizes and configurations, which has been attributed to recycling of the rosettes from the plasma membrane after they have performed their function (Emons, 1985; Schneider and Herth, 1986). It would be interesting to know if these vesicles were targeted to a degradative pathway (e.g., to the vacuole in plants or some organelle similar to lysosomes in animal cells).

When the cellulose synthase is inserted into the plasma membrane, it is then believed to be in position to carry out cellulose synthesis. The activated monomer, UDP-glucose, derived from nucleosides in the cytosol, probably is polymerized by the cellulose synthase at the extracellular surface of the plasma membrane (Delmer, 1987). It is proposed that each particle of the rosette may serve as a group of cellulose synthases, and UDP-glucose is made available to the enzymes through a central channel in each particle (Emons, 1991). The movement of the rosettes through the plasma membrane is thought to be generated by the force of crystallization, which literally pushes the rosette through the fluid membrane as the microfibril elongates (Giddings and Staehelin, 1988). Although the mechanism of how the rosettes are guided is still unknown, it appears MTs may direct the movement of the rosettes by underlying the plasma membrane and establishing membrane fluidity channels that restrict the rosette pathway (Giddings and Staehelin, 1988).

Cellulose synthesis can be indirectly observed by monitoring the incorporation of $^{14}$C-glucose into the cell wall, but cellulose synthase activity itself is yet to be assayed independently *in vitro* to any reasonable level, which is an ever present hindrance in cell wall research. A recent study by Li et al. (1993) has proposed the isolation of cellulose synthase activity in cotton (*Gossypium hirsutum*), but this result awaits confirmation by

others to be substantiated. There has also been the isolation of ß-1,4-glucan synthesis in Golgi-localized membranes of pea (Ray, 1979), now classified as glucan synthase I (GSI). The synthesis of ß-1,3-glucan at the plasma membrane has been classified as glucan synthase II (GSII). GSI is responsible for the synthesis of the ß-1,4-glucan backbone of xyloglucan (Brummel and Maclachlan, 1989), but it is probably not the synthase responsible for cellulose biosynthesis (Read and Delmer, 1991).

1.1.4.2 Hemicelluloses

Hemicelluloses include heteropolysaccharide cell wall components that are not cellulose or pectins. This name does not appropriately describe the group because the non-cellulosic polymers are not half cellulose (Meier, 1964), but this does provide a classification separate from the other groups (Fry, 1988).

There are many variations in the composition of hemicelluloses, but most have the characteristic of closely associating with cellulose in the cell wall due to the ability of cellulose to form H-bonds readily (Hayashi et al., 1987). This association provides an interconnecting matrix for the cellulose microfibrils, serving in the same way cement does in the wall of a building. Hemicelluloses are not extracted in cold water but are removed from the cell wall by alkaline treatment. The level of H-bonding is believed to determine if they are removed with weak or strong alkaline concentrations (Fry, 1988).

The configuration of hemicellulose polymers is centered around their "backbone" linkages. Hemicelluloses, including xylan and xyloglucan, contain a ß-1,4-sugar linked backbone and branches containing other monosaccharides. There are also ß-(1,3),(1,4)-glucan polymers found normally in the cell walls of grasses, but they are of minor importance in dicots (Goldberg, 1985; Bacic et al., 1988). The chain lengths in hemicelluloses vary (<1000 residues), but are always shorter than cellulose. Many hemicelluloses may have less than 100 residues/chain. The most common monomers present in hemicelluloses are glucose, xylose, mannose, galactose and sometimes arabinose. These monomers are produced from nucleosides in the cytosol and then transported into the ER or Golgi for use in the synthesis of hemicelluloses (Northcote, 1977).

Specific synthases in the endomembrane system synthesize the appropriate polysaccharides (Dalessandro and Northcote, 1981) with the final products transported via Golgi vesicles to the plasma membrane. Upon fusion, the contents are released into the extracellular matrix by exocytosis to associate with the other cell wall components.

Xylan is a hemicellulose containing the sugar xylose as the major monomer in its ß-1,4-xylose backbone. Xylans constitute about 20% of the primary cell wall in grasses, but are of little consequence in dicots, making up at most only 5% of the primary wall material. Xylans make up close to 20% of the secondary wall material in both grasses and dicots (McNeil et al., 1984). The ß-1,4-xylose backbone carries a variety of monomers in different side chain arrangements (Fry, 1988); the most common are single α-L-arabinose furanose (Araf) and/or α-D-glucuronic acid (GlcA) residues. Of the xylans in dicots, glucuronoxylans are the most abundant and have been considered a "plastifying" matrix material (Nieduzinski and Marchessault, 1972). The number of GlcA side chains on the ß-1,4-xylose backbone determines variations in and reactivity of the glucuronoxylans (Reis et al., 1992). Glucuronoxylans also associate closely with cellulose microfibrils, but if GlcA side chains are abundant, they become less able to H-bond to cellulose (Fry, 1988).

Xylan synthesis is a membrane bound process that can be assayed *in vitro* and increases during the period of xylem differentiation of the vascular cambium of sycamore and poplar trees (Dalessandro and Northcote, 1981). Xylan degradation is a process in which specific enzymes attack the ß-1,4-D-xylose linkages of the polymer. Xylanases have been isolated from different sources (Mondou et al., 1986; Kluepfel et al., 1990), and purified xylanases are used to localize xylan in plant cells (Vian et al., 1983; Ruel and Joseleau, 1984).

Xyloglucan is a hemicellulosic polysaccharide with a ß-1,4-glucan backbone. This hemicellulose comprises 20-25% of the primary walls of dicots (Hayashi, 1989; Edelmann and Fry, 1992) and only 1-5% in monocots (Fry, 1988). Though the monocots have less xyloglucan, the amount of cellulose is also proportionately lower and, therefore, the ratio of xyloglucan to cellulose maintained in the monocots is similar to dicots (Hayashi, 1989). The presence of xyloglucans in secondary cell walls has not been demonstrated, and a corresponding decline in xyloglucan synthesis is known to take place as secondary cell wall deposition increases (Delmer et al., 1985).

The ß-1,4-glucan backbone of xyloglucans is identical to cellulose and contains 300 to 3000 residues (Fry, 1989). However, side chains of α-xylose are attached 80-90% of the time to the 6-O-position of the glucose (McNeil et al., 1984). The side chains of xyloglucans seem to branch off in an ordered pattern with a series of three substituted glucans followed by one unsubstituted glucan (Fry, 1988). In studying the severing of the ß-1,4-glucan backbone by a cellulase that attacked the unsubstituted glucan residues of xyloglucan in cultured sycamore cells, it was found that two major blocks were present in high percentages, a nonasaccharide and a heptasaccharide, indicating a polymer of regularly

repeating blocks (Valent et al., 1980). The xyloglucans of dicotyledons contain D-glucose, D-xylose, D-galactose and L-fucose with the L-fucose attached terminally to the D-galactose (Bauer et al., 1973). These terminally fucosylated xyloglucan side chains can be recognized and bound by certain lectins in *Ulex europaeus* and *Lotus tertragonolobus* (Hayashi and Maclachlan, 1984), but the significance of this *in vivo* is still not understood because lectins are not found in the primary cell walls of most dicots (Hayashi, 1989).

The structural role of xyloglucans in primary cell walls may involve their chemical association with cellulose microfibrils. Their possible role in control of cell enlargement was suggested by Albersheim and co-workers using sycamore cell suspension cultures (Bauer et al., 1973; Keegstra et al., 1973; Talmadge et al., 1973) and is still under investigation (Fry, 1989). Hayashi and Maclachlan (1984) demonstrated that xyloglucan can attach to cellulose microfibrils by H-bonding, which has also been demonstrated *in vitro* (Bauer et al., 1973; Valent and Albersheim, 1974; Hayashi et al., 1987). The understanding is that xyloglucan binds to newly synthesized microfibrils (Bauer et al., 1973; Moore et al., 1986; Hayashi et al., 1987; Hayashi, 1989), which was supported by the accumulation of most of the xyloglucan into the medium of tomato cell suspension cultures treated with the cellulose synthesis inhibitor DCB (Shedletzky et al., 1990). The absence of cellulose microfibrils may have prevented the xyloglucan from incorporating into the cell wall. However, Edelmann and Fry (1992), using DCB-treated stem segments of etiolated pea seedlings, suggested that only a part of the xyloglucan is actually H-bonded to cellulose and that the remainder must be held in the wall by association with other polymers, including itself. The results are not completely comparable, however, because Shedletzky and co-workers (1990) used cell suspension cultures that may not respond in the same manner as the whole tissues used by Edelmann and Fry (1992).

### 1.1.4.3 Pectins

Pectins are highly charged methylated polymers composed mostly of α-1.4-galacturonans organized into two types of "blocks" (Jarvis, 1984). Unbranched α-D-galacturonic acid residues form "smooth" blocks that are linked within the same molecule with hairy blocks of α-D-galacturonic acid containing backbone residues substituted with other sugars (Fry, 1988). Rhamnose residues are periodically found in the galacturonan backbone and may play some role in the esterification of some homogalacturonan blocks and not others (Tuerena et al., 1982; deVries et al., 1983). Pectins are synthesized in the Golgi complex, transported to the plasma membrane, and then deposited in the primary wall matrix, with a cessation in pectin deposition occurring at the onset of secondary wall

deposition (Northcote, 1963). Pectins are easily hydrated due to their acidic residues and also have a great affinity for cations. Calcium commonly binds ionically to the unmethylesterified blocks of the galacturonan chain causing the pectins to "gel." Pectins are common in plant tissue and form the cement that holds adjoining plant cells together. They also provide a pliable "gel-like-matrix" that allows the cell wall flexibility necessary for cell enlargement or shape alteration. Pectins are easily extracted from a cell wall by using water or chelating agents, which remove the gel-stimulating cations.

#### 1.1.4.4 Cell wall proteins

There has been an increasing interest in the study of plant cell wall proteins. Researchers have provided meaningful evidence about the structure and localization of these proteins within cells and tissues, but little is understood about their specific biological roles. Recent reviews (Keller, 1993; Showalter, 1993) have covered the three major classes of structural cell wall proteins that have been studied extensively: hydroxyproline-rich glycoproteins, glycine-rich proteins, and proline-rich proteins. Current knowledge of tissue-specific and species-specific localization or expression of these three structural cell wall proteins classes has been compiled in a table by Showalter (1993). Though no specific tissue or cell type has been completely analyzed using all probes for these protein types, inferences have been made between the various species studied.

The most studied hydroxyproline-rich glycoproteins (HRGPs) in dicots are classified as extensins. Dicot extensins are characterized by the Ser-$(Hyp)_4$ repeat motif, which also has been described in monocots (Stiefel et al., 1990) and gymnosperms (Bao et al., 1992; Fong et al., 1992). This group of basic glycoproteins (isoelectric point of approximately 10) is thought to be involved in various plant functions, predominantly in defense from pathogens and in cell wall cross-linking (Showalter and Varner, 1989). The rapid cross-linking that insolubilizes extensin in primary cell walls remains consistent in all species, but how extensin specifically associates with the cell wall is unknown. Extensin has been proposed to cross-link with carbohydrates (Keegstra et al., 1973) or to interact ionically with negatively charged pectins (Keller, 1993). Such ionic interactions could also relate to defense mechanisms, since negatively charged surfaces of some plant pathogens could be recognized (discussed by Showalter, 1993). The interaction of extensin with transmembrane proteins in tobacco culture cells has been proposed to stabilize cortical microtubules (Akashi and Shibaoka, 1991), theoretically providing another developmental role.

The second class of cell wall proteins studied are the glycine-rich proteins (GRPs). Though many glycine rich proteins (50-70% glycine) are found in plant cells, only the wound induced expression of GRP-1 from petunia (Condit et al., 1990) and GRP 1.8 from bean (Keller et al., 1988) have been shown to be wall associated. The petunia GRP-1 was first localized to the cell walls in the vascular tissue of the phloem or cambium (Condit et al., 1990) and xylem vessels and fibers (Ye et al., 1991), but a recent study (Condit, 1993) using confocal microscopy indicates that this protein may actually remain associated with the plasma membrane more than the cell wall itself. The French bean GRP 1.8 was associated mostly with tissues destined for lignification and may serve as a possible nucleation site for lignin fixation (Keller et al., 1989, Ye, 1991 #109). Recent studies have provided a new perspective on the possible role of GRPs in the developmental process of protoxylem (Ryser and Keller, 1992; Condit, 1993). Using indirect labeling for the GRPs, these researchers have shown the initial presence of GRP in protoxylem after cell autolysis has begun. They propose that the GRP is imported from surrounding cells (possibly xylem parenchyma for GRP 1.8). If these GRPs are involved in lignification, this import of protein would seem reasonable as lignification (as indicated by intensity of phloroglucinal staining) may continue after TEs have autolysed (Taylor & Haigler, unpublished results). The bean GRP 1.8 seems to be made insoluble after it becomes incorporated in the cell wall (Keller et al., 1989), probably because of association with other tightly bound wall molecules. It has been suggested that GRP is most abundant in hydrolyzed primary walls of xylem cells, with its presence or absence in secondary wall thickenings inconclusive (Ryser and Keller, 1992).

The last cell wall protein group consists of proline-rich proteins (PRPs). Members of this family have similarities to HRGPs by containing Hyp, but they lack the Ser-$(Hyp)_4$ repeat and have a consistent Pro-Pro sequence (Keller, 1993). PRPs have been found in a monocot (Jose'-Estanyol et al., 1992) and in many dicots (Ye et al., 1991; Wyatt et al., 1992). In dicots, the PRPs are localized in vascular tissue and co-localize with GRPs in some cell types, such as xylem elements and fibers. The exact function of PRPs is not known, but they may have a part in the lignification process (Showalter, 1993).

#### 1.1.4.5 Lignin

Lignin is a complex polymer of aromatic alcohols that is polymerized *in situ* after precursors are inserted into the wall milieu, probably by fusion of Golgi vesicles with the plasma membrane. Lignin intercalates around the polysaccharide and protein components of the cell wall, contributing hydrophobicity and strength (Lamb, 1981; Lewis and

Yamamoto, 1990). The increase in size by higher land plants was made possible by lignin's ability to resist the compressional forces of a growing plant, which complements the ability of cellulose microfibrils to resist the tensil forces (stretching) produced during the movement of liquids within the plant (Mauseth, 1988). Lignin is most abundant in wood and is usually associated with vascular tissue in higher plants, making it one of the most abundant organic compounds on earth (Gould, 1983). Lignification in whole tissue begins at the cell corners in the middle lamella between the primary walls of adjacent cells (Wardrop, 1981) and is detected soon after in the secondary walls (Hepler et al., 1970). Lignin is very stable after it is incorporated into the cell wall and remains there until the cell decays (Goodwin and Mercer, 1983). Therefore, lignin provides an inert protective covering (similar to a sheet of plastic) for the other wall components. This allows some protection against consumption by herbivores and attack by pathogens (Swain, 1979). It is known that lignins can vary within the different wall layers in a cell (Lapierre et al., 1991), between the various cell types within one organism (Hoffman et al., 1985), and in response to different hormonal stimuli (Aloni et al., 1990). Precursors to lignin have also been observed to continue to incorporate into the secondary walls of autolysed TEs (Pickett-Heaps, 1968).

Lignin is formed in the wall by the free-radical polymerization of predominantly three aromatic alcohols, *p*-coumaryl alcohol, coniferyl alcohol (found mainly in conifers), and synapyl alcohol, whose ratios vary from species to species (Pearl, 1967). These precursor alcohols arise from the conversion of phenylalanine (produced by the shikimic acid pathway) in the cytoplasm of a cell. This phenylpropanoid pathway converts the amino acid phenylalanine to aromatic acids, which are then converted to CoA esters (Grisebach, 1981). These esters are then reduced by NADPH to form the aromatic alcohols that cross the plasma membrane (possibly in the form of glucosides) and permeate the cell wall where, with the assistance of specific enzymes, polymerization takes place.

L-phenylalanine ammonia-lyase (PAL) has been extensively studied and controls the first step in the production of the aromatic alcohols from phenylalanine. The purification of PAL from barley (Koukol and Conn, 1961) led to the discovery of isozymes (summarized in Iiyama et al. 1993). PAL activity and expression increases before lignification and is maintained at a high level during differentiation in *Zinnia* mesophyll cells (Fukuda and Komamine, 1982; Lin and Northcote, 1990). Along with two other enzymes from the lignification pathway, 4-coumarate:CoA ligase and cinnamyl alcohol dehydrogenase, PAL can be used as marker enzymes for the onset of lignification in TE differentiation (Fukuda and Komamine, 1982; Church and Galston, 1988a).

The peroxidase enzymes have long been thought to catalyze the polymerization of the aromatic alcohols *in situ* within the cell wall (van Huystee, 1987; Lewis and Yamamoto, 1990). More recently, a similar role for laccase has been proposed (Bao et al., 1993). Several isozymes of peroxidase exist in a plant cell (see recent studies by Sato et al. 1993 and Gillikin and Graham, 1991), with some occurring in the cell wall. These isozymes form $H_2O_2$ from NADH and $O_2$ (Akazawa and Conn, 1958), which then combines with an H from each of two aromatic alcohols, evolving $H_2O$. This creates a free radical form of the aromatic alcohol, ready to combine spontaneously with various polysaccharides or alcohols in the lignification process (Mader et al., 1980). In the cytoplasm of a cell, $H_2O_2$ is detrimental and peroxidase immediately degrades it, showing multiple functions of the different isozymes.

How polymerized lignin is restricted to particular wall locations, such as the secondary thickenings but not the primary wall of primary xylem TEs, is not yet known (O'Brian, 1981). Indeed, it has been commonly hypothesized that the localization of lignin is dependent on specific interactions of its precursors or enzymes involved in its polymerization with particular polysaccharides or proteins in the wall matrix (Hepler et al., 1970; Hepler et al., 1972; O'Brian, 1974; Roberts et al., 1985; Keller et al., 1989; Yamamoto et al., 1989; Ye and Varner, 1991). This hypothesis is supported by evidence that: (a) the polymerization of a lignin-like molecule can occur *in vitro* in the presence of eugenol, hydrogen peroxide, peroxidase, and a polysaccharide matrix (Siegel, 1962) as quoted in Hepler and Fosket, 1970); and (b) lignin or its precursors can become covalently bound to polysaccharides and proteins that are commonly found in cell walls (Wardrop, 1981; Whitmore, 1982; Lewis et al., 1989; Yamamoto et al., 1989).

### 1.1.5 Characterization of cell wall components

In analyzing cell walls and their individual components, a method must be used that will degrade the complex wall and release the individual components as fragments of polymers or the monomers themselves for separation via chromatography (Fry, 1988). The carbohydrates in cell walls can be easily characterized qualitatively by acid hydrolysis, which produces free monosaccharides of glucose, fucose, xylose, mannose, and galactose (Lindberg et al., 1975), and separated by thin layer chromatography (TLC). Trifluoroacetic acid (TFA) is recommended for total hydrolysis of cell wall polymers, giving good release of neutral, non-cellulosic and cellulosic sugar residues. This method cannot be used to quantify the sugar residues or to determine from which polymers particular monosaccharides arose.

Determining the covalent linkages characteristic of the acid-released monomers before release from the polymer is best accomplished by methylation analysis (Valent et al., 1980; Waeghe et al., 1983). This procedure methylates all the free -OH groups in a polysaccharide, which means that non-methylated -OH groups subsequently found on the monomers must represent sites of previous glycosidic linkages to other sugars (Fry, 1988). The methyl groups are not removed during acid hydrolysis, so the methyl-sugars can be analyzed by gas-chromatography/mass-spectrometry (GC/MS).

## 1.2 Tracheary elements

### 1.2.1 Overview

Cells with only a primary cell wall do not possess the strength required for some plant functions, such as the movement of water from roots to leaves. Water is conducted by specialized xylem cells in a plant referred to as tracheary elements (TEs), which function after death and autolysis of the cytoplasm. TEs are able to retain their shape under the stress of water movement because of secondary wall development. During the differentiation process from a cell with only a thin extensible primary cell wall to a TE with a thicker unextensible cell wall, a characteristic pattern of secondary cell wall thickenings is produced. These thickenings contain large amounts of cellulose and xylan (in angiosperms), which are followed by incorporation of lignin (Thornber and Northcote, 1962). Cellulose microfibrils are arranged parallel to one another within the thickenings. These differentiated cells are easily identified by their patterned secondary wall thickenings, which vary from annular or spiral (protoxylem) to reticulate or pitted (metaxylem) (Mauseth, 1988). Protoxylem refers to the TEs in growing and elongating tissue, whereas metaxylem develops in older, non-elongating tissues.

It has been found that individual xylem cells can have: (a) areas of radically different wall composition within the same cell (Catesson, 1989); (b) thickening patterns that reflect the developmental state of the tissue (Buvat, 1989); and (c) varying extent and location of primary wall hydrolysis to allow water flow between adjacent cells (O'Brian, 1981). These variations have important effects on the ability of plants to grow at the maximum rate under optimal conditions and to tolerate water and freezing stress without interruption of water conductance (Tyree and Sperry, 1989). Each plant species contains TEs with characteristics most suitable for its particular habitat, making it possible to identify a tree by the structure of its xylem (Preston, 1988). These differences also make it evident that the wall deposition process must be under precise cellular control.

### 1.2.2 Systems for research on tracheary elements

*In vivo*, TEs arise from the procambium (primary growth) and cambium (secondary growth) (Torrey et al., 1971). They also develop after wounding as parenchyma cells surrounding a severed vascular strand redifferentiate into TEs (reviewed in Fukuda, 1992) (Fukuda, 1992). The location of TE development in plants makes it difficult to observe experimentally, which raised the necessity of developing a practical system for studying TE differentiation. This was accomplished using a single cell suspension culture of isolated mesophyll cells from seedlings of *Zinnia elegans* (Kohlenbach and Schmidt, 1975; Fukuda and Komamine, 1980b). Up to 60% of the living cells in this system can be induced to differentiate semi-synchronously into protoxylem-like TEs followed by the non-synchronous formation of metaxylem-like TEs (Falconer and Seagull, 1985). The induction of cells to differentiate was found to require the addition of cytokinin and auxin (Roberts, 1976; Aloni, 1987; Roberts, 1988; Fukuda, 1989; Roberts et al., 1992).

Other cell sources have been explored, but none provide the high percentage of semi-synchronous differentiation found in *Zinnia* mesophyll cells. Callus tissue has been studied in several species (reviewed in Fukuda, 1992), with the main disadvantage that TEs form in low frequency and in asynchronous clumps. Similarly, isolated explants of parenchyma tissues from numerous species have been cultured on semi-solid nutrient medium containing hormones, but the synchrony and frequency of TEs was not optimal (Dalessandro and Roberts, 1971; Philips and Dodds, 1977). The explants also introduced interference from differentiating phloem and other cells since the tissues were not entirely homogeneous. The parenchyma cells of leaf discs of *Zinnia* were not induced to differentiate in appreciable amounts until the epidermis was removed, allowing more exposure to hormone penetration or indicating that a wounding effect is important in the production of TEs in the *Zinnia* system (Church and Galston, 1989) as well as in explants.

### 1.2.3 *Zinnia elegans* single cell system

Studies involving the *Zinnia* single cell suspension cultures have been performed in many different laboratories looking at various aspects of cell determination and differentiation, including cell wall assembly (Fukuda and Komamine, 1982; Burgess and Linstead, 1984; Falconer and Seagull, 1985; Haigler and Brown, 1986; Sugiyama et al., 1986; Kobayashi et al., 1987; Church and Galston, 1988b; Ingold et al., 1988; Roberts and Haigler, 1989; Thelen and Northcote, 1989; Lin and Northcote, 1990; Taylor et al., 1992). This cell system has been the subject of several review articles (Fukuda, 1989; Fukuda, 1992; Church, 1993). The mechanical isolation of the mesophyll cells from the

first leaves of *Zinnia* seedlings is thought to provide a wounding effect on the cells (Fukuda, 1992) giving the initial stimulus for redifferentiation with the presence of auxin and cytokinin necessary to provide the final stimulus to complete the process (Fukuda and Komamine, 1980b). The need for auxin persists throughout differentiation, whereas the influence of cytokinin seems isolated to the early determination stage. [This view is supported by studies that link auxin with increased gene expression in dividing and elongating cells (Theologis et al., 1985; Boot et al., 1993) and also by the isolation of auxin receptor proteins in membranes of various plants (Fukuda, 1992)]. Another plant hormone that may be a part of the differentiation process is the growth sterol brassinolide, which accelerated TE production when applied exogenously to *Zinnia* cultures (Iwasaki and Shibaoka, 1991) and also overcame the inhibitory effect on differentiation by uniconazole, a drug that inhibits the biosynthesis of brassinosteroids. Ethylene, a natural product of wounding in plants and considered a plant hormone, may also play a role in differentiation, because the application of ethylene inhibitors to *Zinnia* cultures inhibited TE formation (Fukuda, 1992). There is much left to learn about how all the hormones may be related in what seems to be a complicated signaling process.

In induced cultures of *Zinnia* mesophyll cells, some cells will redifferentiate to TEs while others will not. Little is known of why this difference exists, but the cells that do differentiate enter into an irreversible sequence leading to cell autolysis and death. The induced TE development follows the same pattern and timing as in whole tissue (Burgess and Linstead, 1984) and there is a necessary prerequisite synthesis of RNA and proteins (Fukuda and Komamine, 1983). Cell division is not necessary for TE development because many undivided cells become TEs (Kohlenbach and Schmidt, 1975; Fukuda and Komamine, 1980a). Tracheary element formation was also blocked when DNA synthesis was inhibited by several different inhibitors (Fukuda and Komamine, 1981; Sugiyama and Fukuda, 1990; Sugiyama et al., 1990), one of which inhibited an enzyme associated with DNA excision repair (Sugiyama and Komamine, 1987). It was proposed that this excision repair activity may be necessary to promote the differentiation of mesophyll cells to TEs (Fukuda, 1989). In analyzing cytoplasmic proteins by two-dimensional electrophoresis, Fukuda and Komamine (1983) found little difference in the abundantly expressed proteins between induced and control cultures, though the expression of some polypeptide seems increased in the induced cultures. This could indicate that important proteins are not necessarily expressed or repressed, but that protein activity is quantitatively controlled to facilitate differentiation. Later, a 34-kD protein, determined to be a nuclease, was isolated by *in vitro* translation of mRNA from differentiating *Zinnia* cultures that was not found in

non-differentiating cultures (Thelen and Northcote, 1989). The activity of the enzyme was detectable 12 h before visible secondary walls could be seen by light microscopy and peaked during secondary wall deposition. Thelen and Northcote (1989) proposed that this nuclease could be a marker for the onset of xylogenesis. Therefore, it is more likely that 2-D gel analyses are not sensitive enough to detect expression of important proteins since cDNA for unique proteins in induced cultures, i.e. a nuclease, can be isolated by subtractive library methods (Varner et al. 1992) and detected by Rapid Amplification of cDNA Ends (RACE) (Frohman et al., 1988) strategies (L.Koonce, personal communication).

The signals that direct the differentiation of mesophyll cells into TEs results in the characteristic secondary cell wall patterns. These are delineated by the organization of parallel cellulose microfibrils within the thickenings that are normally transverse to the cell's long axis. The mechanisms determining the specific localization of the microfibrils and other cell wall components to these thickening sites are not well understood. Microtubules have been implicated in the development of secondary wall thickenings because of their localization under the thickening ridges and bundling in a pattern that predicts the future sites of the thickenings even before secondary wall deposition commences (Hepler and Newcomb, 1964; Hardham and Gunning, 1979; Burgess and Linstead, 1984; Falconer and Seagull, 1985). The use of the microtubule antagonist, colchicine, caused the aberrant deposition of microfibrils and unpatterned secondary walls without effecting cellulose biosynthesis in *Zinnia* (Fukuda and Komamine, 1980b) and other plant cells (Hepler, 1981). These observations along with the series of studies by Falconer and Seagull (1985,1986 & 1988), Fukuda and Kobayashi (1989) and Hogetsu (1991) suggest that microtubules are responsible for the patterning of secondary wall thickenings in plant cells, which was previously discussed in a review by Gunning and Hardham (1982). However, recent results in our lab suggest that microtubules may exert their effect very early (Haigler and Koonce, 1992), with previously undefined mechanisms serving to perpetrate the pattern in their later absence.

Another cytoskeletal member, actin, has also been shown to be involved in some manner in organizing secondary wall patterning (Kobayashi et al., 1988). Actin filaments were reorganized in *Zinnia* cells just prior to differentiation (Kobayashi et al., 1987) and located between the microtubules. Also, the disruption of actin using cytochalasin B caused the thickenings of TEs to remain in a longitudinal orientation relative to the long axis of the cell (Kobayashi et al., 1988). This indicates that actin plays a role in microtubule orientation, which in turn would affect secondary wall thickening orientation and patterns.

Since microtubule banding predicts the location of future secondary cell wall thickenings, the next question would be, "what determines the location of the microtubules?" One possible candidate could be calcium. Calcium has been found to be an integral component in normal plant cell functions (Hepler and Wayne, 1985) and directly activates enzymes that are specifically involved in the modification of the cytoskeleton (Steer, 1988). Studies of *Zinnia* cultures (Roberts and Haigler, 1989; Roberts and Haigler, 1990) showed evidence suggesting that both an increase in membrane-associated calcium in differentiating TEs and differentiation were suppressed when calcium channels were inhibited. Also, of the TEs that did develop, the thickenings were abnormally thin. Calmodulin was found between the thickenings of *Zinnia* TEs (Fukuda and Kobayashi, 1989) and has been colocalized with microtubules of cells undergoing change (Wick et al., 1985; Wick and Duniec, 1986), further implicating calcium as a possible participant in secondary cell wall localization.

As in TEs of other dicots (Thornber and Northcote, 1962), *Zinnia elegans* TEs contain xylan as the major non-cellulosic polysaccharide (Ingold et al., 1988), and a corresponding increase in xylan synthase activity has been observed (Suzuki et al., 1991). This xylan can be specifically labeled in secondary wall thickenings with an antibody against xylose (Northcote et al., 1989; Taylor et al., 1992) or with a cloned xylanase A and its antibody (Taylor et al., 1992).

In *Zinnia* cultures, TEs first exhibit the presence of lignin in the thickenings, as detected by red color with phloroglucinol-HCL (Gahan, 1984), several hours (5-7 h) after the thickenings are visible by light microscopy (Fukuda and Komamine, 1982; Ingold et al., 1988). The activity of the key enzyme, PAL, was shown to increase during the time of differentiation in *Zinnia* cultures (Fukuda and Komamine, 1982; Lin and Northcote, 1990) at a time correlating with lignin synthesis. The inhibition of PAL activity in *Zinnia* cultures by the application of L-α-aminooxy-ß-phenylpropionic acid (AOPP) (Amerhein et al., 1981; Smart and Amrhein, 1985b), caused secondary walls to be produced without detectable lignin (Ingold et al., 1990). This was correlated with a xylan-type polysaccharide increasing in the culture medium, giving rise to the proposal that lignin synthesis is necessary for the normal integration of polysaccharides into the secondary cell walls.

*Zinnia* TEs are programmed to die and little is known about the autolytic process during which the internal organelles degrade and polysaccharides in the cell wall unprotected by lignin are hydrolyzed (O'Brian, 1981; Fukuda, 1992). Nucleases proposed to be associated with autolysis in differentiating *Zinnia* cultures have been isolated (Thelen

and Northcote, 1989) during the later stage of differentiation, indicating that the TEs are genetically directed to degrade as they would be in whole tissue.

## 1.3 Cell wall self-assembly

### 1.3.1 Overview

The normal patterned deposition of lignin in secondary wall thickenings has been proposed to occur independently of microtubules (O'Brian, 1981; Roberts et al., 1985). This possibility would be consistent with evidence that cell wall polymers have inherent potential for self-assembly that can lead to complex three-dimensional order (Vian and Reis, 1991). The concept of a "self-assembling" cell wall remains possible because few details about cell wall assembly are known. To quote Varner and Lin (1989) "It is conceivable that the addition of noncellulosic components to an existing wall is entirely by self-assembly, with each component designed to fit appropriately with others (p 231)."

It is difficult to test self-assembly directly by *in vitro* reconstitution of the process because of strong associations between components of the cell wall. Separating the components from one another usually involves harsh treatments with alkali or other chemicals that can alter the original molecular structure and organization (Jarvis, 1992). To circumvent these problems, some laboratories are applying solid-state $^{13}C$ NMR spectroscopy to analyze molecular associations within undisturbed cell walls (Chanzy et al., 1987; Jarvis and Apperley, 1990). Atalla and co-workers are also looking at unperturbed plant tissue by applying Raman microprobe spectroscopy (Atalla and Agarwal, 1985), which uses laser technology to analyze domains of interest in a cell or tissue. Another approach could include the use of mutants with altered cell walls (Jarvis, 1992) to dissect relationships between molecular and structural changes.

### 1.3.2 Cellulose self-assembly

The self-assembly of cellulose chains to form cellulose microfibrils is accepted (Roberts et al., 1982; McCann and Roberts, 1991; Reis et al., 1992). Furthermore, in many plant cell walls, the cellulose microfibrils and interstitial matrix materials form a helicoidal pattern that is consistent with a transient liquid crystalline state (Reis et al., 1991). Reis et al. (1991) demonstrated for the first time, using the acidic xylan-coated microfibrils in cellulosic mucilage of mature seed of quince (*Cydonia oblonga* L), that an *in vitro* self-assembly of cellulose into a liquid crystal phase can occur. Such a transient liquid crystalline state in cell wall assembly could help explain the ability of growing cell walls to adapt flexibly to perturbations without being totally destroyed (Roland et al.,

1987). This pliable liquid phase has been shown to progress eventually into some of the hardest secondary cell walls found in sclerified endocarp and pericarp (Roland et al., 1989; Reis et al., 1992).

### 1.3.3 Evidence of other polysaccharide self-assembly

The ability of some cell wall components to H-bond readily to themselves or other components allows room for self-assembly under the proper conditions. The cell wall components xylan (Vian et al., 1986) and xyloglucan (Jarvis and Apperley, 1990) have been shown to have self-assembly properties. It has also been shown that ß-(1,4)-D-mannans can self-associate by H-bonding in the cell walls of palm endosperm (Chanzy et al., 1979).

The evidence of *in vivo* self-assembly is minimal due to *in vitro* studies having produced little substantial information. As seen by the lack of conclusive data to support the possibility of cell wall self-assembly, much more work must be done.

## 1.4 The use of 2,6-dichlorobenzonitrile (DCB) in plant studies

### 1.4.1 Overview

The analysis of associations between cell wall components and their possible role in the wall can be accomplished by use of inhibitors of specific wall components. One such inhibitor is the herbicide 2,6-dichlorobenzonitrile (DCB), which is thought to be a specific inhibitor of cellulose synthesis (Delmer, 1987). The intention of this review is to summarize the use of DCB on plants and plant cell systems and propose how it may be utilized to elucidate the interaction of other cell wall components with cellulose.

### 1.4.2 DCB as a herbicide

The acceptance of the herbicide DCB as a phytotoxic agent has been well known for some time (Woodford, 1958). The specific effect of DCB on a plant is still not completely clear, but cellulose synthesis is proposed to be the site of influence (Delmer, 1987). DCB inhibits seed germination and actively dividing meristematic cells (Koopman and Daams, 1965) with little direct effect on established plant parts. This indicates that DCB exerts its influence within the development of new plant cells. Because of DCB's effect on developing tissue, investigators endeavored to use DCB in various ways to determine its specific effect on a cell and how it could be applied in analyzing plant development.

### 1.4.3 DCB as a possible cellulose synthesis inhibitor

The observations that DCB promotes swelling of seedlings and inhibition of elongation (Gorter and Van der Zweep, 1964) led to the idea that DCB affects cell wall and possibly cellulose synthesis in some way. Hogetsu and co-workers (1974) first proposed DCB as a cellulose synthesis inhibitor based on their observations that DCB overcame the effect of GA on and inhibited the elongation of incubated epicotyls of Azuki bean seedlings. They also observed a decrease of 33% in the incorporation of [U-$^{14}$C] glucose into the cellulosic fraction of the tissue. Though the possible detrimental effect of DCB on respiration or general cell functions was not convincingly addressed, their results led others to consider DCB as a possible inhibitor for cell wall studies.

### 1.4.4 Observations of the effects of DCB in cells

The use of DCB on gherkin seedlings revealed possible effects on membranes and enzymes involved in cell wall synthesis with no observable effects on vesicle fusion or transport (Engelsma, 1974). Soybean protoplasts cultivated *in vitro* in the presence of DCB produced swollen and deformed cells (Umetsu et al., 1976). The plumular hooks of mung bean also exhibited excessive swelling with the inhibition of elongation in the presence of DCB. The swelling effects observed by Umetsu et al. (1976) led them to conclude that DCB altered water uptake into cells in a way similar to the effects of an accepted cellulose synthesis inhibitor, coumarin (Hara et al., 1973). They also proposed that cell swelling and inhibition of elongation may be typical effects of cellulose synthesis inhibitors, presumably due to an altered or weakened cell wall.

In tobacco protoplasts cultivated *in vitro*, it had been demonstrated that DCB inhibits cytokinesis without affecting nuclear division (Meyer and Herth, 1978; Galbraith and Shields, 1982). Partial cell plates were observed using electron microscopy, which suggested that DCB does not affect membrane fusion but some step after cell plate initiation, perhaps the period of cellulose deposition. A similar inhibition of cytokinesis by DCB is also observed in *Nautilocalyx* explants (Venverloo et al., 1984) and onion root meristem (Buron and Garcia-Herdugo, 1983). The establishment of an incomplete cell plate in *Nautilocalyx* and the failure to inhibit nuclear division in the onion root meristem matched observations in other systems. It has also been shown that DCB does not have a permanent effect (Montezinos and Delmer, 1980; Galbraith and Shields, 1982; Buron and Garcia-Herdugo, 1983; Venverloo et al., 1984). Removing DCB also removed its inhibitory effects, allowing a relatively quick regeneration of cellulose synthesis and normal cell wall development. In contrast, the removal of coumarin did not remove its inhibitory

effect on cell wall production (Hara et al., 1973; Umetsu et al., 1976). DCB has also been proposed to provide a marker at the electron microscope level because cell walls deposited in the presence of DCB have a distinctly different appearance (Richmond et al., 1983).

The possibility that DCB inhibited cellulose synthesis at the membrane level was proposed due to the observation of altered vesicles in association with dictyosomes in DCB-treated tobacco protoplasts (Meyer and Herth, 1978) and by variations in the vesicles involved in cell plate formation in DCB-treated cells (Venverloo et al., 1984; Gonzalez-Reyes et al., 1986). These results were questioned in a study of corn root cap (Shannon and Steer, 1984) in which the vesicles associated with dictyosomes did not change appreciably. This indicates the observed effect of DCB on vesicles in some studies may arise from specimen preparation or because of the developmental time at which the specimen is preserved. Another consideration concerning membrane involvement includes the observation that microtubules are not affected by DCB (Hogetsu et al., 1974; Venverloo et al., 1984). This implies that a possible transport mechanism for vesicles is not damaged. DCB was also used to inhibit cellulose synthesis in regenerating tobacco protoplasts, and this was determined to affect the cytoskeleton in some way because the nucleus failed to migrate to the protoplast periphery (Katsuta and Shibaoka, 1988). These investigators proposed that actin filaments of the cytoskeleton established a linkage with an intact cell wall that depended on cellulose. Therefore, if the cell wall is altered, normal functioning of the cytoskeleton may not occur.

DCB caused a reduction in cellulose synthesis, but not a complete inhibition over the short-term. Calcofluor White staining (a fluorescent dye for cellulose) of tobacco protoplasts (Galbraith and Shields, 1982) or $^{14}$C-glucose incorporation into the cellulose fraction of bean and cotton fiber cells (Hogetsu et al., 1974; Montezinos and Delmer, 1980) showed some cellulose production taking place, though greatly reduced. This reduction or blocking of cellulose synthesis by DCB does not affect the synthesis of non-cellulosic polysaccharides (Hogetsu et al., 1974; Montezinos and Delmer, 1980; Blaschek et al., 1985; Pillonel and Meier, 1985) over the short-term, nor is respiration in cotton greatly affected within the first two hours of the introduction of DCB as monitored by collecting evolved $^{14}CO_2$ (Montezinos and Delmer, 1980).

The ability of DCB to promote cell swelling has been used to find mutants of *Aspergillus nidulans* resistant to DCB. The mutants were cross-resistant to certain fungicides that created swelling effects similar to DCB (Chabani and Grindle, 1990). These investigators isolated a single gene from the mutants that imparted DCB resistance

and this gene matched the gene for fungicide resistance. Once obtained, the sequence of this gene may provide insight into how DCB acts in plants.

The development of DCB resistance or at least the ability for a plant cell to adapt to growth in the presence of DCB is demonstrated by the adaptation of a tomato cell line to growth in the presence of the cellulose synthesis inhibitor. These adapted cells grow in the absence of virtually all cellulose (only 1-2% cellulose present) in their primary cell walls (Shedletzky et al., 1990). The resistance or adaptability of this cell line is not founded on the ability to tolerate DCB, but to survive without cellulose. The cell wall matrix produced by these adapted cells is composed mainly of homogalacturonan and rhamnogalacturonan polymers (calcium bridged pectates). Xyloglucans are present but largely excreted to the medium, which supports the proposal that cellulose provides the major lattice for xyloglucan binding in the cell wall (Hayashi, 1989). When DCB is removed from the medium, the adapted cells return to cellulose synthesis at a rate somewhat higher than normal controls. This indicates a possible accumulation of substrates or proteins involved in cellulose production during the inhibited period that are quickly used upon removal of the inhibitor. An adapted tobacco cell line and an adapted barley cell line have recently been established by these researchers (Shedletzky et al., 1992) and the contents of their primary cell walls compared. The tobacco adapted primary walls were similar to the tomato cell line, while the adapted barley primary walls contained very little pectic material and more normal levels of hemicellulosic polysaccharides. The normal porosity of the dicot cell walls remained unchanged, while the porosity of the monocot walls was reduced. Conversely, the tensil strength of the dicot primary walls was reduced, while the monocot tensil strength was increased nearly two-fold. The connection between the cell wall and the plasma membrane was enhanced in all three cell lines analyzed.

Viewing ultrathin sections with electron microscopy, Herth (1988, 1989) observed the deposition of some cell wall component(s) in thickenings of wheat root xylem cells treated with DCB. The parallel cellulose microfibrils that are normally associated with secondary wall thickenings were absent. However, whether these thickenings were really devoid of cellulose or whether the incorporation of cellulose was simply altered was not clarified. Herth also observed an increase in the number of rosettes in the plasma membrane of freeze-fractured cells during the first few hours of incubation in DCB. The stabilization of these usually unstable rosettes supports the selective action of DCB on cellulose synthesis. Accumulation of rosettes may also relate to the enhanced cellulose synthesis found in adapted cells after the removal of DCB (Shedletzky et al., 1990).

The ability of DCB to stop or reduce cellulose synthesis at low concentrations (1-10 μM) with nominal effect on other cell activities supports the specificity of DCB in plants. The compilation of data concerning DCB effects on plant cells points to the acceptance of the herbicide as a specific cellulose synthesis inhibitor, most probably at the plasma membrane (Delmer, 1987).

### 1.4.5 The identification of a DCB receptor protein

The apparent ability of DCB to inhibit cellulose synthesis at the level of cellulose polymerization/deposition led to an investigation of the possible involvement of DCB with a specific protein/polypeptide functioning in cellulose synthesis (Delmer, 1987). A photoreactive analog of DCB was synthesized, 2,6-dichlorophenylazide (DCPA) (Cooper et al., 1987). This analog binds any associated polypeptide upon irradiation with UV light. [$^3$H]-DCPA was found to induce the same effects as DCB and could therefore be used to identify any protein(s) that DCB may bind. With this tool, an 18-kd polypeptide was isolated from cotton fiber extracts as the likely protein DCB binds *in vivo* (Delmer et al., 1987). The 18-kd polypeptide was determined not to be the catalytic protein of the cellulose synthase. The amount of the18-kd polypeptide was observed to increase during the time of increased secondary cell wall deposition in the cotton fibers. This places the protein as a possible important factor in cellulose synthesis, providing a basis for a model proposed by Delmer in which the polypeptide plays a regulatory role that can be inhibited by DCB (Delmer, 1987).

## 1.5 The use of isoxaben in plant studies

### 1.5.1 Overview

A recently developed herbicide, N-(3'[1-Ethyl-1-methylpropyl]-5-isoxazolyl)-2,6, dimethoxybenzamide (isoxaben, EL-107) (Huggenberg et al., 1982) has been shown to have cellulose synthesis inhibition abilities similar to DCB (Heim et al., 1990), providing another potential chemical for use in cell wall analysis.

### 1.5.2 Isoxaben as a herbicide

Isoxaben inhibits cellulose synthesis in sensitive plants and is used primarily as a pre-emergence herbicide against broadleaf growth in small grains, turf and ornamentals (Huggenberg et al., 1982; Schneegurt and Larrinua, 1991). Dicots are more sensitive to the influence of isoxaben than monocots, but dicots also exhibit varying degrees of sensitivity. In *Arabidopsis thaliana*, very low concentrations of the herbicide are necessary

for inhibition (Heim et al., 1989). Isoxaben has been shown to be effective in the field at lower concentrations than DCB (Thomson, 1986).

### 1.5.3 Isoxaben as a cellulose synthesis inhibitor

Isoxaben was shown to induce morphological effects in plant tissue culture cells similar to the effects of DCB, leading Lefebvre and co-workers (1987) to suggest that the mode of action of isoxaben was at the cell wall. Heim et al. (1990) were able to confirm this proposal by showing that $^{14}C$-glucose uptake into the acid-insoluble cell walls of *Arabidopsis* was inhibited similarly to the effect of DCB, but at a 40-fold lower concentration. They also tested for a non-specific effect of isoxaben and checked for effects on protein, nucleic acids and fatty acid synthesis. Their findings indicated no major physiological alterations other than a specific effect on cellulose synthesis. These results make isoxaben a potential tool in studying cell wall assembly.

## 1.6 Goals of this research

The purpose of this study was to use light and electron microscopy in conjunction with inhibitors of cellulose synthesis to evaluate how patterned secondary cell wall deposition would be affected by the absence of the microfibrillar component of cell walls. The cellulose synthesis inhibitors, DCB and isoxaben, were applied to single cell suspension cultures of *Zinnia elegans* var. Envy and compared to data obtained on control TEs. Light microscopy was used with various stains for cellulose and lignin or polarization optics to reveal the presence of cellulose. Immunological studies with antibodies to xylan, xyloglucan, and a glycine-rich protein as well as the hydrolytic enzymes, cellulase and xylanase, were used to determine the absence or presence of these cell wall components at the light level. Electron microscopy using gold labeling techniques with the various enzymes or antibodies also provided information about the times at which certain components are deposited at the cell wall and with what other possible components they are associated. Investigating the association of the individual cell wall components with each other in the developing TEs gave more insight into cell wall construction and how the individual components (cellulose, hemicelluloses, pectins, proteins and lignin) interact to create this important plant feature.

# CHAPTER 2
# ANALYSIS OF WALL POLYMERS IN NORMAL AND ALTERED TEs

## 2.1 Overview

The goal of this aspect of the research was to analyze by light and electron microscopy the composition and characteristics of secondary walls deposited in the presence and absence of cellulose synthesis inhibitors. Direct experimental evidence showing that lignin localization depends on the presence of particular cell wall polymers was not previously available until this study (Taylor, 1991; Taylor and Haigler, 1991; Taylor et al., 1991; Taylor et al., 1992). Similar results were subsequently published by another research group (Suzuki et al., 1992).

The distribution pattern of lignin in cell walls is closely related to physical and chemical properties of the lignocellulosic materials that are major sources of fiber and forage (US Department of Energy, 1988) as well as determining properties of the xylem critical for plant function and adaptation. Therefore, we have attempted to understand more about how lignin localization is controlled by applying to developing TEs a well-characterized cellulose synthesis inhibitor, 2,6-dichlorobenzonitrile (DCB) (Hogetsu et al., 1974; Montezinos and Delmer, 1980), and a recently proposed cellulose synthesis inhibitor, isoxaben (Heim et al., 1990).

## 2.2 Materials and methods

### 2.2.1 Cell culture

Mesophyll cells isolated from the first true leaves (about 1 cm long from 11-14 day old plants) of *Zinnia elegans* L. var Envy (G.S. Grimes Seeds, Smethport, PA) were cultured and induced to differentiate as described previously (Roberts et al., 1992) except for variations related to particular experiments as described below. Onset of differentiation, as indicated by fluorescence of cell wall thickenings with Tinopal LPW (Ingold et al., 1988) occurred between 48 and 56 h after cultures were established. Within the next 18 hours, up to 60% of the living cells differentiated into TEs.

### 2.2.2 Reagents

All chemicals and probes were purchased from Sigma Chemical Company except where other suppliers are indicated in the text. The cloned xylanase A and the antibody

raised to it were the kind gift of Prof. D. Kluepfel, Université du Québec. The isoxaben was the kind gift of Dr. Ken Burrow, Lilly Research Laboratories, Indianapolis, Indiana. The antibody to xyloglucan was a kind gift of Dr. Michael Hahn, Complex Carbohydrate Research Center (CCRC), Athens, Georgia. The GRP antiserum, which was raised collaboratively with Dr. B. Keller, was provided by Dr. C. Lamb, Salk Institute for Biological Studies, San Diego, CA.

2.2.3 Radiolabeling of cell cultures

Cells were cultured with shaking (120 rpm) in 20 ml glass scintillation vials (2 ml medium/vial) in a simplified medium (Roberts et al. 1992) at three times the normal density to maximize cpm incorporated per unit culture volume. The carbon source was changed to 10 mM glucose (shown to have no adverse effect on differentiation percentage; A.W. Roberts, unpublished results) to promote high specific activity of label. Between 60 h and 67 h when at least 20% of the cells were differentiating, as determined by observation of slightly birefringent thickenings, 7.5 μM 2,6-dichlorobenzonitrile (Fluka Chem. Co.) in DMSO was added to half of the cultures. The final DMSO concentration in the cultures was 0.07%, which had no effect on differentiation (J.G. Taylor, unpublished data). Between 0.5 to 1 h later, [U-$^{14}$C]glucose (Dupont/NEN, Boston, MA) was added to all the cultures (final specific activity, 9.6 x $10^3$ Bq/μmol) and a 2.4 cm filter disc (Whatman #1) was soaked with 60 μl 1N KOH and attached to a paper clip taped to the vial lid to trap $^{14}CO_2$ released.

Between 0.5 and 3 h after addition of label, the incubation in 3 DCB-treated and 3 control vials was stopped by addition of acetic/nitric reagent (Updegraff, 1969). After boiling for 1 h, the insoluble cell wall material was collected by vacuum filtration onto 2.4 cm glass fiber filter discs (Whatman GF/A), washed in water, and dried. All dried filter discs were placed in 2.75 ml scintillation fluid (Scintiverse BOA, Fisher Scientific, Pittsburgh, PA) and counted. KOH-soaked filters were allowed to sit overnight before counting because of transient chemiluminescence. The points shown in the graph are the average cpm/vial (each vial containing 1 x $10^6$ cells) incorporated into acetic/nitric insoluble cell wall material and $CO_2$ for three replicate vials in one experiment. Error bars represent the standard deviation of the three data points obtained from individual vials. The experiment has been repeated at least five times with similar results in every experiment. This protocol was also applied in the comparison of DCB-treated (7.5 uM), isoxaben-treated (0.3 uM in DMSO), and control cultures except the normal cell density was used and incubation in [U-$^{14}$C]-glucose was stopped at 3h.

### 2.2.4 Treatment of cells with DCB and isoxaben for microscopic analysis

Cells were cultured in the standard medium (Fukuda and Komamine, 1980) and 7.5 μM DCB or 0.3 μM isoxaben was added at 42 or 48 h.

### 2.2.5 DCB effect on microtubules

To confirm the expected lack of effect of DCB on microtubules (Venverloo et al., 1984) in differentiating TEs, we performed immunofluorescence according to published methods (Roberts et al., 1985) using a monoclonal antibody to the α-subunit of chick brain microtubules (Sigma Chemical Co., #T-9026). Cells treated with DCB at 42 h (before microtubule banding) and 48 h (after microtubule banding had occurred in many cells) and untreated controls were fixed and processed for immunofluorescence at 50 and 54 h. Therefore, the effect of DCB on microtubules was checked 2, 6, 8, and 12 h after its addition at pre- and post-microtubule banding stages.

### 2.2.6 Lignin analysis

The quantitation of lignin in *Zinnia* cultures was determined according to published methods (Johnson et al., 1961; Fry, 1988) with the following adaptations. Cell walls for analysis were sonicated 4 x 1 min on ice in 70% ethanol with a microtip using a Tekmar Sonic Disruptor and pelleted at 1000g. The pellet was washed 5 times with 70% ethanol, 2 times with 100% ethanol, and finally, before freeze-drying and storage, 3 times with a 1:1 solution of ethanol and toluene to extract all aromatic material except lignin. The lignin content of the dried sample (1.0 mg) was determined by adding the sample and 1.0 ml of acetyl bromide/acetic acid (1:3 made a day in advance) with 20 μl perchloric acid (Iiyama and Wallis, 1988) to a reactivial (5 ml), which was then sealed with a Teflon cap. The sample was then incubated at 70°C with stirring for 40 min, placed in a 10 ml measuring cylinder and cooled to 15°C, at which time 3.5 ml of the stop solution (0.9 ml 2 M NaOH + 2.5 ml acetic acid + 100 μl hydroxylamine 7 M) was added and brought to a final volume of 10 ml with acetic acid. The absorbance was measured at 280 nm using a Gilford RESPONSE UV-VIS spectrophotometer. Loblolly pine was used as a parallel control.

### 2.2.7 Conjugation of hydrolases with fluorescent probes

Cellulase (Worthington, Freehold, NJ; approx. 70% w/w protein) or cloned xylanase (from D. Kluepfel) and fluorescein isothiocyanate (Molecular Probes, Eugene, OR; 10% on celite) were mixed 1 mg: 1 mg in 0.05 M sodium bicarbonate/carbonate

buffer, pH 8.5 and stirred 6 h to overnight in a conical reaction vial at room temperature protected from strong light. The conjugate was separated from unconjugated dye by chromatography on Sephadex G-25 as previously described (Rinderkrect, 1962). Briefly, 4-5 g of pre-swollen Sephadex G-25 (Sigma, St. Louis, MO) was slowly added as a slurry to a glass column (1 cm x 30 cm) one-third full of distilled water to a depth of approximately 20 - 25 cm. The column was washed with distilled water for several bed volumes using a peristaltic pump set at 0.5 - 1 ml per minute. The column could be sealed and stored at 4° C with 0.02% azide and reused repeatedly. The conjugated dye/protein complex followed the solvent front because it was too big to interact with the bead pores while the smaller, unconjugated dye moved much slower because of its interaction with the bead pores. The bright conjugated band was quite distinct from the duller unconjugated dye band unless the amount of protein was low (as for xylanase A). In this case the fraction containing the conjugated dye/protein was collected at the time required for the solvent front to reach the end of the column (3-5 min). According to the protocol followed, the collected conjugated fraction should be free of unconjugated dye and salts with very little unconjugated protein remaining. The hydrolase conjugate was freeze dried and stored desiccated in the dark at 4°C. For testing specificity of hydrolases in double labeling experiments, conjugation with lissamine rhodamine B sulfonyl chloride (Molecular Probes, Eugene, OR) was performed similarly except that the dye:hydrolase ratio was 0.5 mg: 10 mg.

### 2.2.8 Test for specificity of hydrolases

Cellulase conjugated to rhodamine and xylanase-A conjugated to fluorescein were dissolved in 0.1 M sodium acetate buffer, pH 4.8 and added to a mixture of commercial xylan (from larchwood) and cut pieces of cotton fibers. After 20 min, the sample was pelleted in a microfuge, washed once with buffer, and resuspended in 2 drops of 1% n-phenylenediamine mountant (in 90% glycerol/carbonate buffer, pH 8.6) to suppress fading of fluorescence.

### 2.2.9 Labeling TEs with fluorescent probes

The freeze-dried conjugated hydrolases were resuspended at 1mg/ml in 0.1M sodium acetate buffer, pH 5.6. TEs were washed once in the same buffer, then suspended in one conjugated hydrolase or a mixture of the two. After incubating for 20 min (RT, dark), the TEs were washed once in carbonate buffer then mounted for fluorescence as above.

A rabbit polyclonal antibody to xylanase A was also used to localize xylanase A bound to xylan. TEs were attached to poly-lysine-coated Teflon®-well slides (ICN Biomedicals, Inc., Costa Mesa, CA), incubated in xylanase A (1 mg/ml in sodium acetate buffer as above; 20 min; RT) and washed once in phosphate buffered saline (PBS, pH 7). Then the antibody was applied (1:1000 in PBS, 0.01% azide, 1% BSA; 1 h, RT or overnight, 4°C). After washing three times, goat anti-rabbit FITC (diluted 1:200 as above) was applied (1 h, RT) as a fluorescent tag. The pre-immune serum at the same dilution as the primary antibody was used as a control. Cells were washed once in carbonate buffer and mounted for fluorescence as above.

For labeling with a rabbit polyclonal antibody to xylose (Northcote et al. 1989; Cambridge Research Biochemicals, Norwich, U.K.), TEs were incubated with primary (1:200) and secondary antibody as described above. The primary antibody was omitted as a control. Probes for xylan were also applied to fixed cells (3% freshly prepared formaldehyde, 0.05M Na-phosphate buffer, pH 7.2; 1 h RT). The fixed cells were rinsed 5 min in 0.1% Na $BH_4$/TBS to block fluorescence of aldehyde groups before application of the primary antibody or xylanase A.

Labeling with a mouse antibody to xyloglucan (Lynch and Staehelin, 1992; Zhang and Staehelin, 1992); (a kind gift from Dr. Michael Hahn, Complex Carbohydrate Research Center [CCRC], Athens, GA), was performed by incubating TEs with primary (1:100) and secondary (1:200; anti-mouse IgG-FITC) antibodies as described above. For controls the primary antibody was omitted or a mouse antibody to ß-galactosidase was applied.

A rabbit polyclonal antibody to GRP (Keller et al., 1989); (a gift from Drs. C. Lamb and B. Keller) was applied similarly with the following changes: (a ) TEs were routinely sonicated with a microtip to create breaks in cell walls, but not to destroy the cells; (b) 1:200, 1:400, and 1:800 dilutions of primary antibody were tested; and (c) stringency of washing was increased by using 3 x 15 min washes in TBST (TBS + 0.2% Tween 20) with gentle rocking. Since the pre-immune serum from the rabbit used to generate this antibody is not available (Dr. B. Keller, personal communication), 3 other pre-immune sera available in our laboratory were tested in dilutions from 1:100 to 1:1000 as controls. The primary and secondary antibodies were also replaced with TBS/BSA as controls.

Brief sonication of the differentiating cell culture with a microtip improved the percentage of TEs that labeled with all the probes without changing the patterns of labeling compared to unsonicated cells except in the case of the antibody to GRP which gave a generalized label in fixed unsonicated cells.

2.2.10 Light microscopy

Samples were observed using an Olympus BH2 microscope equipped with epi-fluorescence, POL, and DIC optics. Absence of birefringence was confirmed by stage rotation. Tinopal LPW® (a dye of the Calcofluor® type; C.I.#40622; a gift from Ciba Geigy, Greensboro, NC) was applied at 0.01% to stain cellulose. This fluorescent brightener (FBA 28) binds cellulose and sometimes other ß-linked polysaccharides (Hughes and McCully, 1975). It is interesting to note, however, that the patches of xylan described below do not fluoresce with Tinopal LPW, which is consistent with previous data showing lack of interaction with arabinoxylan (Wood et al., 1983). For observation of TEs labeled with FITC or Tinopal LPW, an optional Zeiss KP 560 barrier filter was used to block chlorophyll autofluorescence. Lignin was stained with phloroglucinol/HCl (Gahan, 1984). Cells labeled with cellulase, xylanase, or antibodies to xylose, xyloglucan and GRP were examined at early stages of differentiation (59-66 h) before autofluorescence over the thickenings became extensive. Even if autofluorescence was present in some cells, it could be distinguished from FITC labeling when the KP 560 barrier was not in place; autofluorescence was distinctly yellow, whereas FITC labeling was apple green. Cells were often stained later for lignin (99-132 h) because the color reaction intensified with culture age in control and cellulose-synthesis-inhibitor-treated cultures. For epi-fluorescence, the blue filter package (BP 490 with additional EY-455 excitation filter), the green filter package (BP 455 with additional EO-530 excitation filter), and the ultraviolet filter package (UG-1 with additional 20 nm narrow band pass excitation filter at 365 nm) were used for observation of fluorescein, rhodamine, and Tinopal LPW, respectively. Micrographs were recorded using an Olympus PM-10ADS automatic exposure device on Kodak T-MAX 400, Kodacolor 400 (not suitable for FITC images), or Ektachrome 400 film.

2.2.11 Electron microscopic observation of cell wall thickenings

Cells were fixed in 2% glutaraldehyde in 0.05 M phosphate buffer, pH 7.2, post-fixed with 2% $OsO_4$ in buffer, dehydrated in ethanol, and embedded in UV-polymerized LR White resin. To allow selection and photography of particular cells before sectioning (Reymond and Pickett-Heaps, 1983), cells were polymerized between glass slides that had been pre-treated with a liquid release agent (Electron Microscopy Sciences, Fort Washington, PA) using coverslip spacers. Differentiating cells were selected and photographed by DIC and POL optics, scored around with a diamond marking objective

(Leitz Optometric Inc., Richardson, TX), then mounted on resin blocks with cyanoacrylate glue for ultramicrotomy. Sections were mounted on Formvar®-coated grids, post-stained for 2 min in 2% aqueous uranyl acetate and 1 min in lead citrate (Reynolds, 1963), and examined in a Hitachi HU-11E electron microscope. These electron micrographs were produced with the assistance of Dr. T. Page Owen, Jr.

### 2.2.12 Electron microscopic immunocytochemistry to localize wall components

Cells were fixed in 3% formaldehyde in 0.05 M Na-Phosphate buffer, pH 7.2, dehydrated in ethanol, and embedded in UV-polymerized LR White resin on glass slides as described above. Cells were selected and sectioned as above, mounted on Formvar®-coated nickel grids, treated for immunocytochemical analysis, post-stained for 5 min in saturated aqueous uranyl acetate and 5 min in lead citrate, coated with 1 nm carbon, and examined in a Hitachi HS-9 electron microscope.

A cellulase/gold conjugate (kindly provided by Dr. R.L.Blanton, Texas Tech University, Lubbock, TX) was prepared by conjugating 15 nm diameter gold particles (produced by the reduction of chloroauric acid by sodium citrate (Frens, 1973) with Worthington cellulase and applied to ultra-thin sections of TEs on nickel grids as described by Berg and coworkers (Berg et al., 1988) with the following changes. The grids were first floated on drops of aldehyde blocker (20 mM glycine, 20 mM lysine and 20 mM ammonium chloride) for 15 min, then floated on a drop of 5% normal goat serum in TBST for 15 min to reduce nonspecific binding of the enzyme to the sections. The grids were then transferred to drops of 0.05 M citrate buffer, pH 4.6, for 10 min and washed 3 times (by dunking) in TBST followed by 2 washes in distilled water. The sections were stained as described above. A competitive substrate of carboxymethyl cellulose (CMC) and pre-absorption of sections with unconjugated cellulase were used as controls.

The labeling of sections with xylan antibody was performed according to published methods (Stafstrom and Staehelin, 1988). The sections of TEs on nickel grids were floated on aldehyde blocker (see above) for 15 min, then blocked on drops of 5% normal goat serum in TBST for 15 min and transferred to drops of the xylose antibody (diluted 1:10 in 5% normal goat serum in TBST) for 1 h RT. After the incubation in the primary antibody, the grids were washed 3 times in TBST by dunking the grids a minimum of 30 times per wash, incubated for 1 h (RT) in the secondary antibody (goat anti-rabbit, conjugated to 10 nm gold particles, diluted 1:10 in 5% normal goat serum in TBST), then washed by dunking 3 times in TBST and 2 times in $dH_2O$. The sections were post-stained as above.

The primary antibody was omitted and replaced by 5% normal goat serum in TBST as a control.

The antibody to xylanase A was used in a similar manner except that after blocking, the grids were placed on drops of xylanase A in 0.5 M citrate buffer, pH 5.6, (1.0 mg/ml) for 10 min and then washed once in citrate buffer followed by 2 washes in TBST before being incubated in the antibody to xylanase A (1:200). The pre-immune serum of the rabbit in which the antibody to xylanase A was developed was used as a control.

Labeling with the xyloglucan antibody developed in mouse was performed as above by incubating the sections in primary (1:10 and 1:100) and secondary antibody (anti-mouse IgG specific, conjugated to 10 nm gold particles diluted 1:10). Labeling with secondary antibody only and an antibody to ß-galactosidase developed in mouse were used as controls.

For GRP antibody localization, TEs were incubated with primary (1:50) and secondary antibody (goat anti-rabbit, conjugated to 10 nm gold particles diluted 1:10) as described above. The rabbit pre-immune sera used in the light microscopic GRP analysis were diluted at 1:10 and substituted for the primary antibody as controls.

The quantitation of gold particles on immunogold labeled ultra thin sections of control and DCB-treated cells was performed while viewing the specimens in the TEM. The gold localization of the xyloglucan antibody was assessed at a magnification of 30,000. The gold particles found on four random cross sections of primary walls of TEs and non-differentiating cells along a distance equivalent to 2.5 µm were counted, making a total of 10 µm of primary cell wall counted per cell in a total of 37 control and 16 DCB-treated non-differentiating cells and 9 control and 6 DCB-treated TEs. The gold localization to secondary wall thickenings of the xylose antibody and the antibody to GRP was assessed at a magnification of 50,000 and the number of gold particles found on 0.8 $\mu m^2$ of each thickening, up to 10 thickenings per TE, were counted. A total of 8 control and 10 DCB-treated TEs were counted for the antibody to xylose and 13 control and 18 DCB-treated TEs for the antibody to GRP. A Student's t-test was used to compare the mean values obtained from each count.

### 2.2.13 Western blotting of GRP

Total cell protein from a 65 h non-induced (non-differentiating) culture and induced (differentiating) cultures at t = 0, 12, 24, 36, 45, 48 and 72 h was extracted and probed with the antibody to GRP after the separation of 50 µg/lane of each protein extract on an

SDS/polyacrylamide gel (10%) followed by blotting onto Immobilon® nitrocellulose (Millipore, Bedford, MA). Immunodetection was carried out by blocking the membrane (5% non-fat dry milk, TBS, 1 h, RT), washing in TBS, incubating with antibody to GRP (1:500 in TBS/5% dry milk, 1 h RT or overnight at 4°C), washing 4 x 5 min in TBST, incubating with donkey anti-rabbit Ig-horseradish peroxidase (1:5000 diluted as above, 1 h RT), and washing 4 x 5 min in TBST and once in TBS. Detection was performed using Enhanced Chemiluminescence (ECL) and recorded on Hyperfilm ECL film (Amersham International plc, Amersham, UK).

## 2.3 Results

### 2.3.1 Cellular effects of DCB

The effects of DCB on cellulose synthesis and respiration were determined by feeding differentiating cultures with radiolabeled glucose. The incorporation of [U-$^{14}$C] glucose into acetic/nitric insoluble material at the end of 3 h was inhibited 75% in developing TEs incubated in the presence of 7.5 μM DCB compared to controls (Figure 2.1). The acetic/nitric (Updegraff) reagent digests all cell wall polysaccharides except crystalline cellulose (Updegraff, 1969). Respiration as indicated by release of $^{14}CO_2$ over 3 h was inhibited 17% in DCB-treated cultures compared to controls (Figure 2.2). Dead controls that were heat killed then carried through all manipulations showed an average cpm/vial (1 x $10^6$ cells) of 487 cpm in the cellular material remaining after acetic/nitric digestion and 238 cpm associated with the KOH-soaked filter paper. These data are representative of five replicates of the experiment.

### 2.3.2 Cellular effects of Isoxaben

The recently described cellulose synthesis inhibitor isoxaben (0.3 μM) caused a 62% decrease in [U-$^{14}$C] glucose incorporation into acetic nitric insoluble material at the end of 3 h (Fig. 2.3). This experiment was run at normal cell density to allow parallel treatment with 7.5 μM DCB, which was described earlier. In this experiment, DCB caused a 67% reduction in cellulose synthesis (Fig. 2.3). The change in respiration as indicated by $^{14}CO_2$ generation, decreased 15% and 0%, respectively (Fig. 2.4), in the presence of DCB and isoxaben. Therefore, isoxaben may have a more specific effect on cellulose synthesis than DCB.

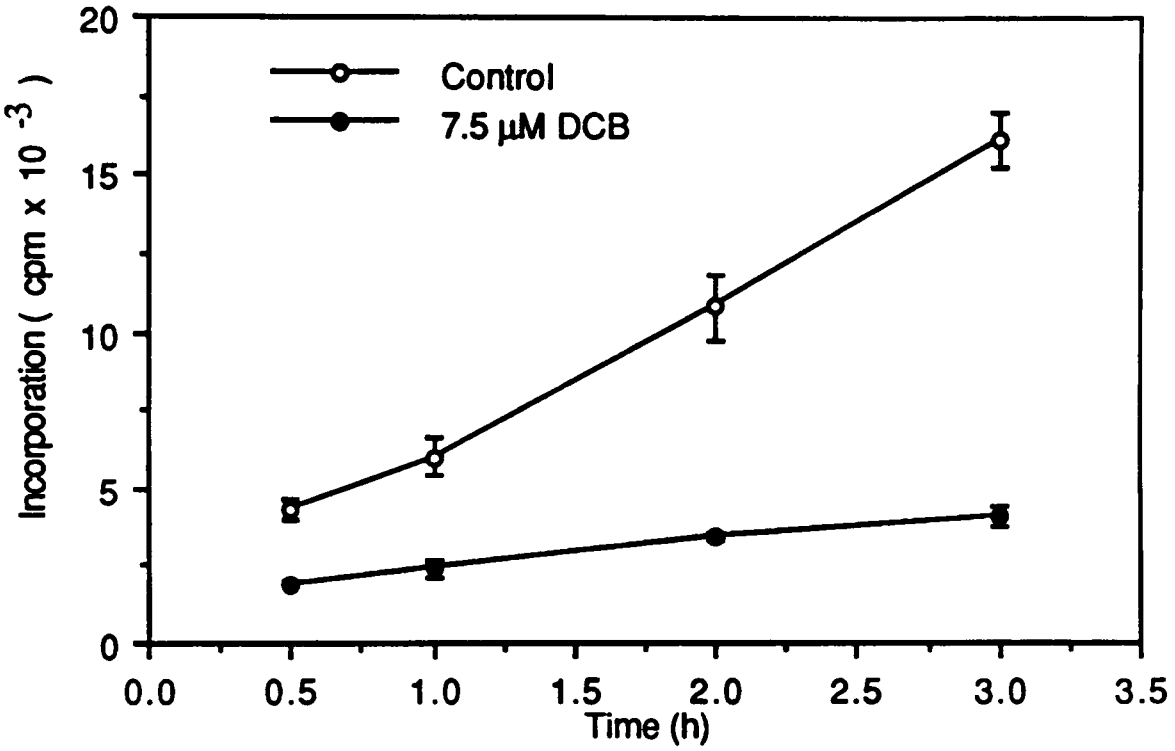



Fig. 2.1 Rates of cellulose synthesis. Average c.p.m. of [$^{14}$C]glucose incorporated over time into acetic/nitric insoluble material in one 2 ml test culture (1 x $10^6$ cells) containing differentiating TEs in the presence and absence of 7.5 µm DCB. Bars represent the standard deviation of three data points from one representative experiment.

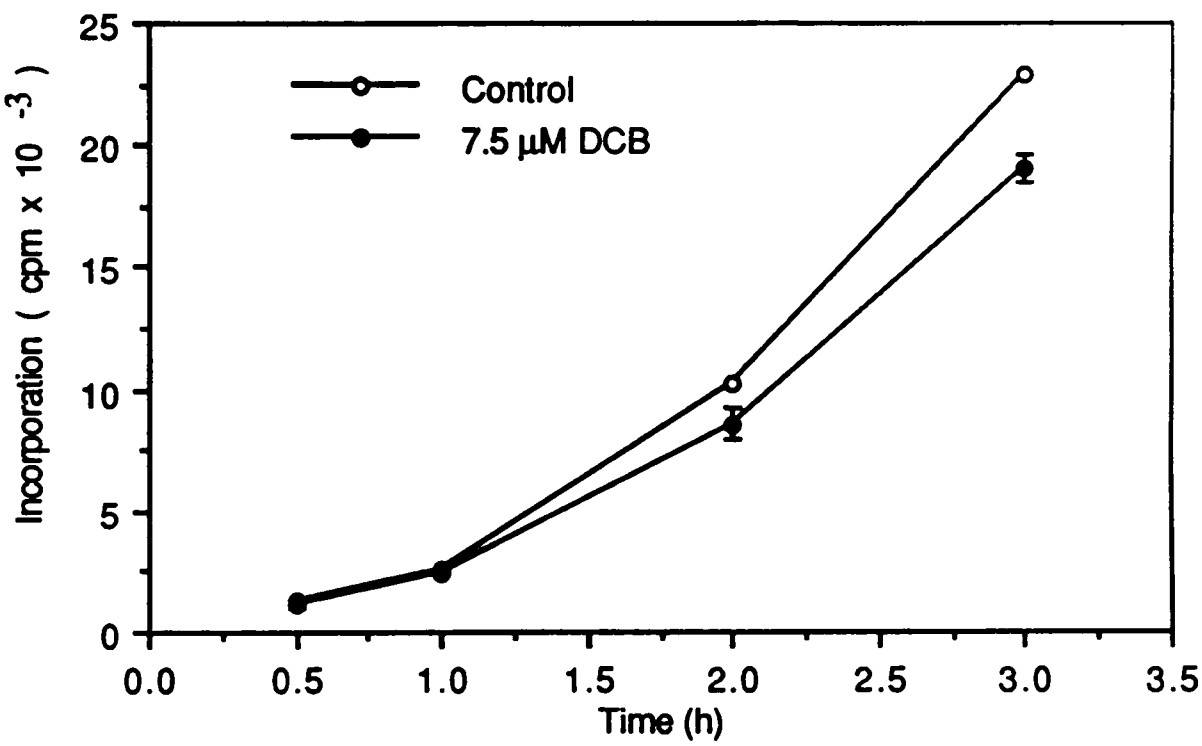



Fig. 2.2. Rates of respiration. Average c.p.m. of $^{14}CO_2$ entrapped in 1M KOH over time in one 2 ml test culture (1 x $10^6$ cells) containing differentiating TEs in the presence and absence of 7.5 µm DCB. Bars as in Fig. 2.1.

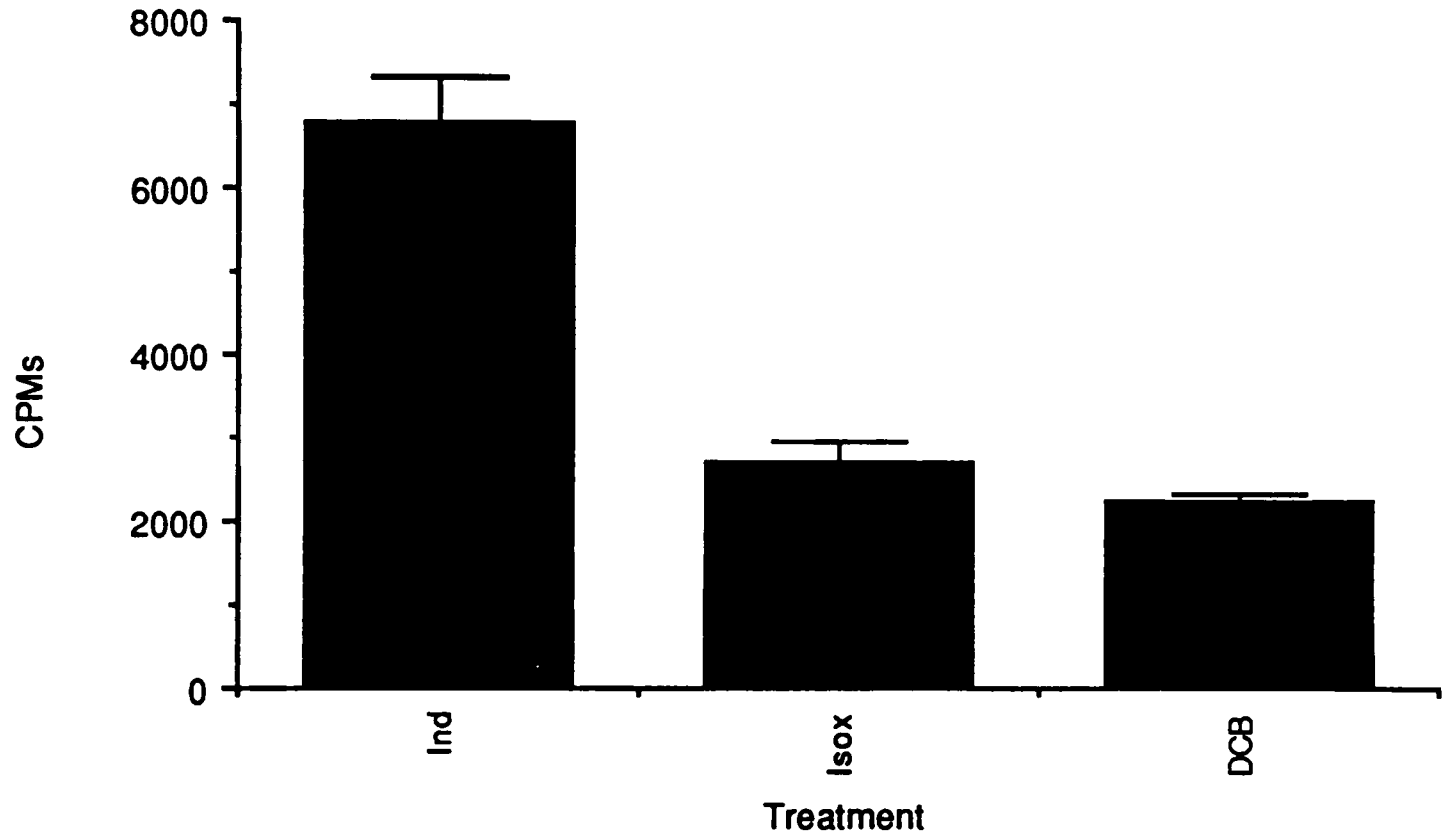



Fig. 2.3. Rates of cellulose synthesis after 4 h. Average c.p.m. of [$^{14}$C]glucose incorporated over time into acetic/nitric insoluble material in one 2 ml test culture (1 x $10^6$ cells) containing differentiating TEs in the presence of 0.3 µm isoxaben, 7.5 µm DCB and absence of each. Bars as in Fig. 2.1.

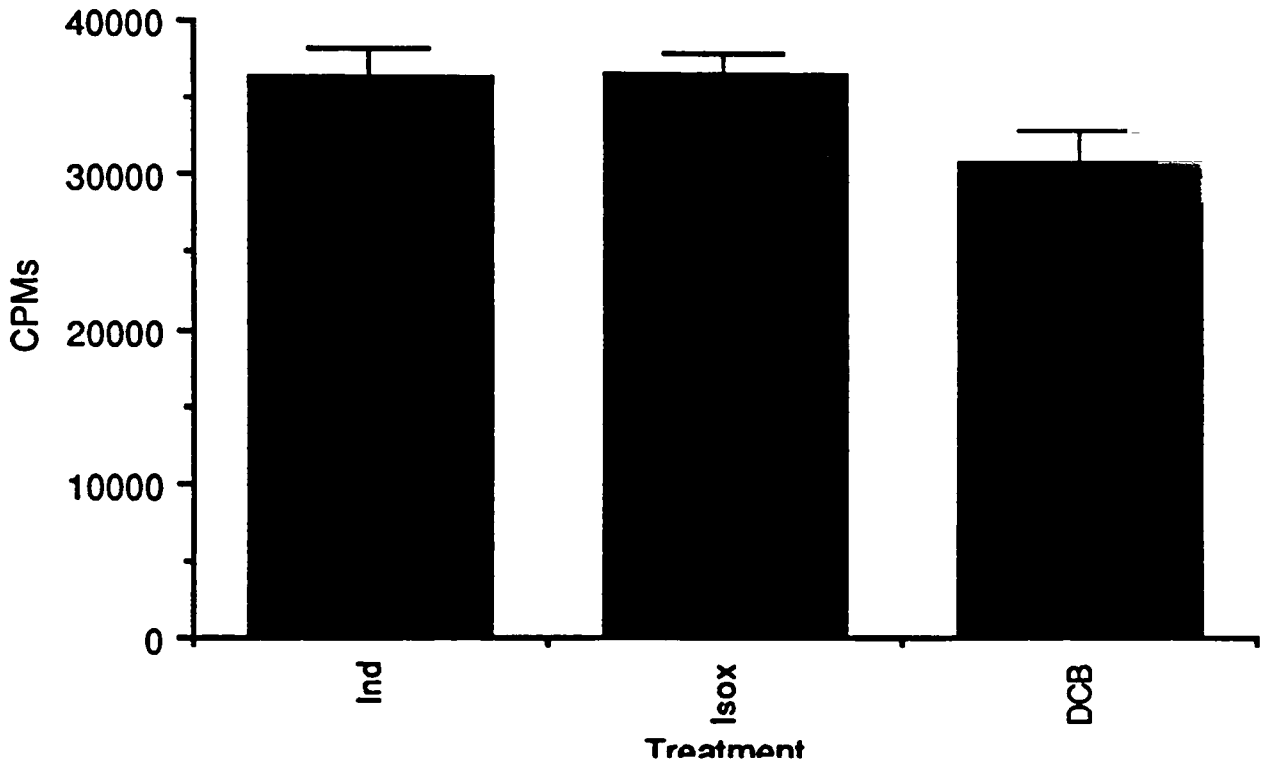



Fig. 2.4. Rates of respiration after 4 h. Average c.p.m. of $^{14}CO_2$ entrapped in 1M KOH over time in one 2 ml test culture (1 x $10^6$ cells) containing differentiating TEs in the presence of 0.3 µm isoxaben, 7.5 µm DCB and absence of each. Bars as in Fig. 2.1.

### 2.3.3 Hydrolases label their specific substrate

Xylanase-FITC bound specifically to larchwood xylan in a mixture with cotton cellulose (Fig. 2.5a) (Taylor, 1991). In addition to this it was previously shown that the xylanase was specific for xylan and had no exo- or endo-cellulase activity towards CM-cellulose (Morosoli et al., 1986). The Worthington cellulase used has been previously shown to be histologically specific for cellulose (Berg et al., 1988), even though it has slight xylanase activity (Fuchs et al., 1989). This specificity was confirmed when cellulase-Rhodamine bound specifically to cotton cellulose in a mixture with larchwood xylan (Fig. 2.5b) (Taylor, 1991).

### 2.3.4 Effects of DCB on microtubules in differentiating TEs

The microtubules in DCB-treated cultures were similar to controls in all cases; differentiating cells had banded microtubules (Fig. 2.6) and non-differentiating cells had randomly organized microtubules. More banded microtubules were observed in DCB-treated cells and controls at 54 h compared to 50 h, corresponding to the increased percentage of differentiating cells at 54 h (data not shown). Up to 12 h incubation in DCB did not cause any change in the normal rearrangements or appearance of microtubules.

### 2.3.5 DCB-treated TEs deposit some secondary cell wall material in a pattern

Control TEs with patterned, birefringent secondary walls (Fig. 2.7a and b) were selected, sectioned, and examined in the transmission electron microscope (TEM) to reveal cross-sections of thickenings typical of normal TEs (Fig. 2.7c). DCB-treated TEs with patterned secondary walls (Fig. 2.7d) that lacked birefringence (Fig. 2.7e) were similarly analyzed to reveal patterned wall material (Fig. 2.7f). The patterned thickenings of DCB-treated TEs were smaller and less regularly shaped than those of control TEs.

### 2.3.6 Detectable cellulose is reduced or absent from thickenings of DCB-treated TEs

Thickenings of control TEs detected by differential interference contrast (DIC) optics (Fig. 2.8a) contained cellulose as indicated by fluorescent staining with Tinopal LPW (Fig. 2.8b) and birefringence under polarization (POL) optics (Fig. 2.8c). Although Tinopal LPW may also stain other ß1,4-linked or ß1,3-linked glucans (Hughes and McCully, 1975), it is an early indicator of the presence of dye-accessible cellulose as is found in the thickenings of young control TEs (Ingold et al., 1988). Thickenings of

Fig. 2.5. Larchwood xylan and cotton pieces double-labeled with xylanase-A-FITC and Worthington cellulase-rhodamine. (a). Viewed in blue light for FITC. (b). Viewed in green light for rhodamine.

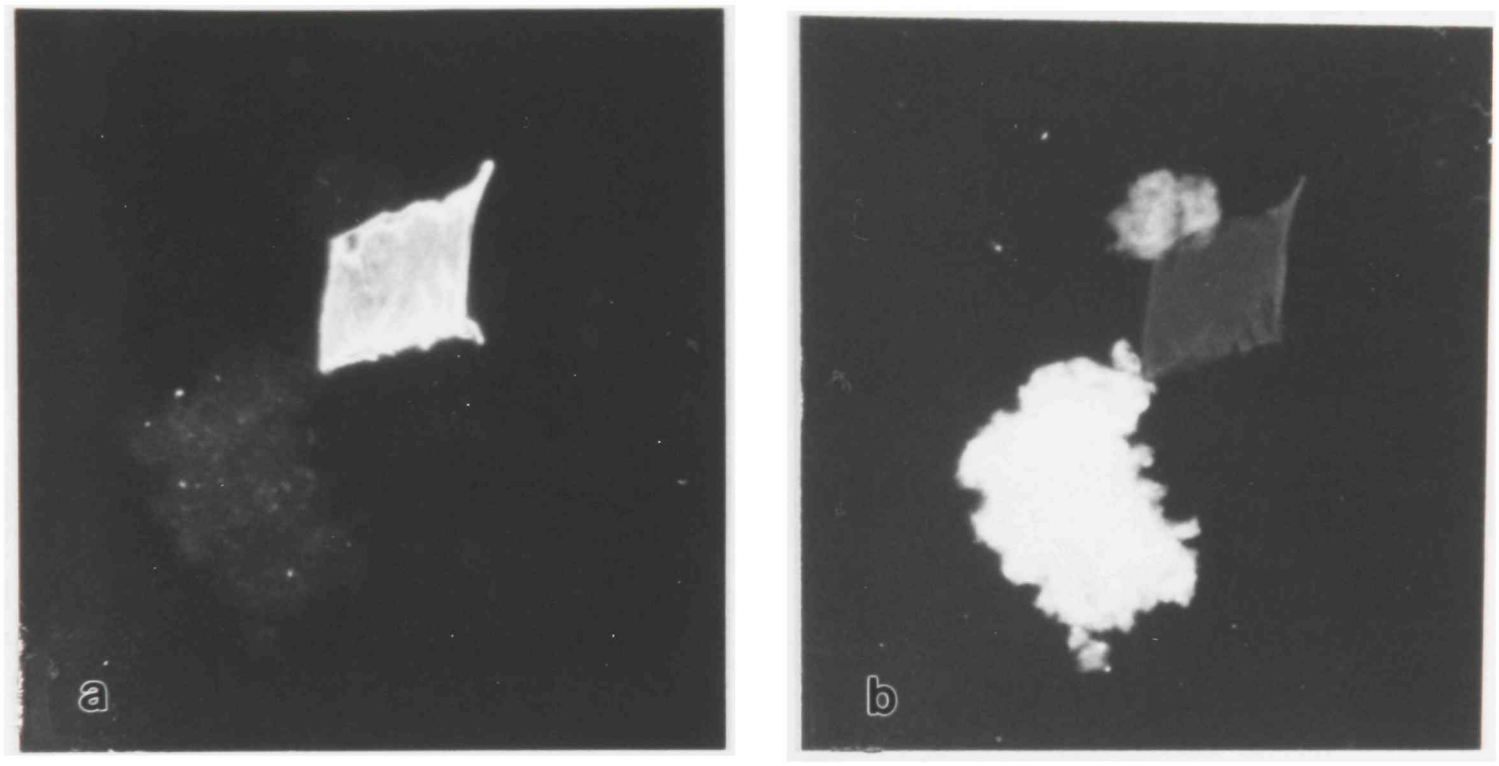

a
b

Fig. 2.6. DCB-treated differentiating and non-differentiating cells treated with a primary antibody to tubulin and a secondary antibody conjugated to FITC. Note the patterned microtubules corresponding to the patterned secondary wall thickenings in the differentiating TE and the random microtubules in the non-differentiating cells.

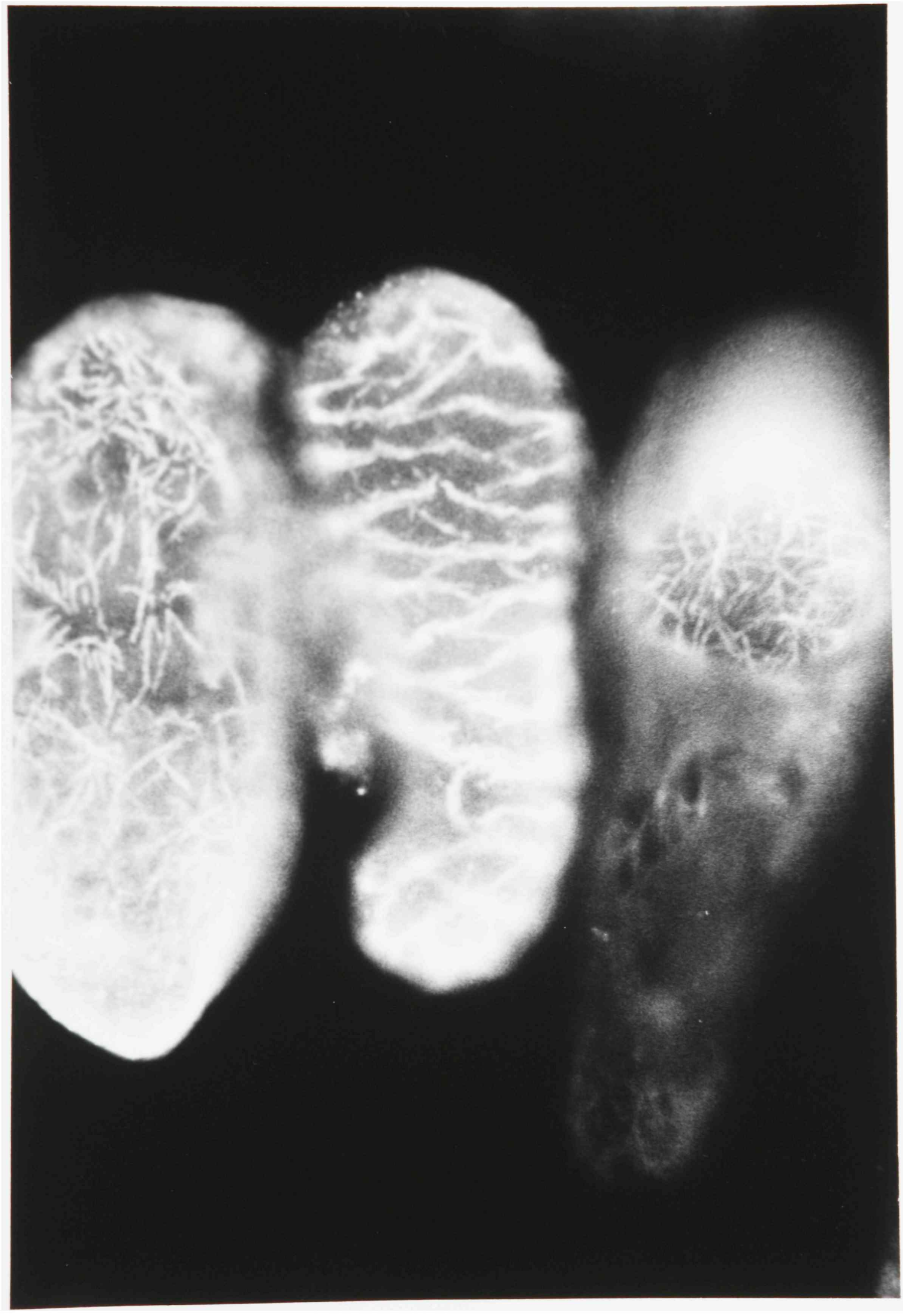


Fig. 2.7. Electron microscopy of the cell wall thickenings of a control TE at 66h (a-c). A control TE with a patterned secondary wall as shown by DIC (a) that contained cellulose as shown by birefringence in POL (b), was sectioned and observed in the electron microscope (c). Note the large, sculpted thickenings typical of control TEs. Bar = 10 μm for (a) and (b) and 5 μm for (c).
Electron microscopy of the cell wall thickenings of a DCB-treated TE at 66h (d-f). A DCB-treated TE with a patterned secondary wall as shown by DIC (d) that lacked detectable cellulose as shown by absence of birefringence in POL (e) was sectioned and observed in the electron microscope (f). Note the smaller, less precisely shaped cell wall thickenings. Bar = 10 μm for (d) and (e) and 5 μm for (f).

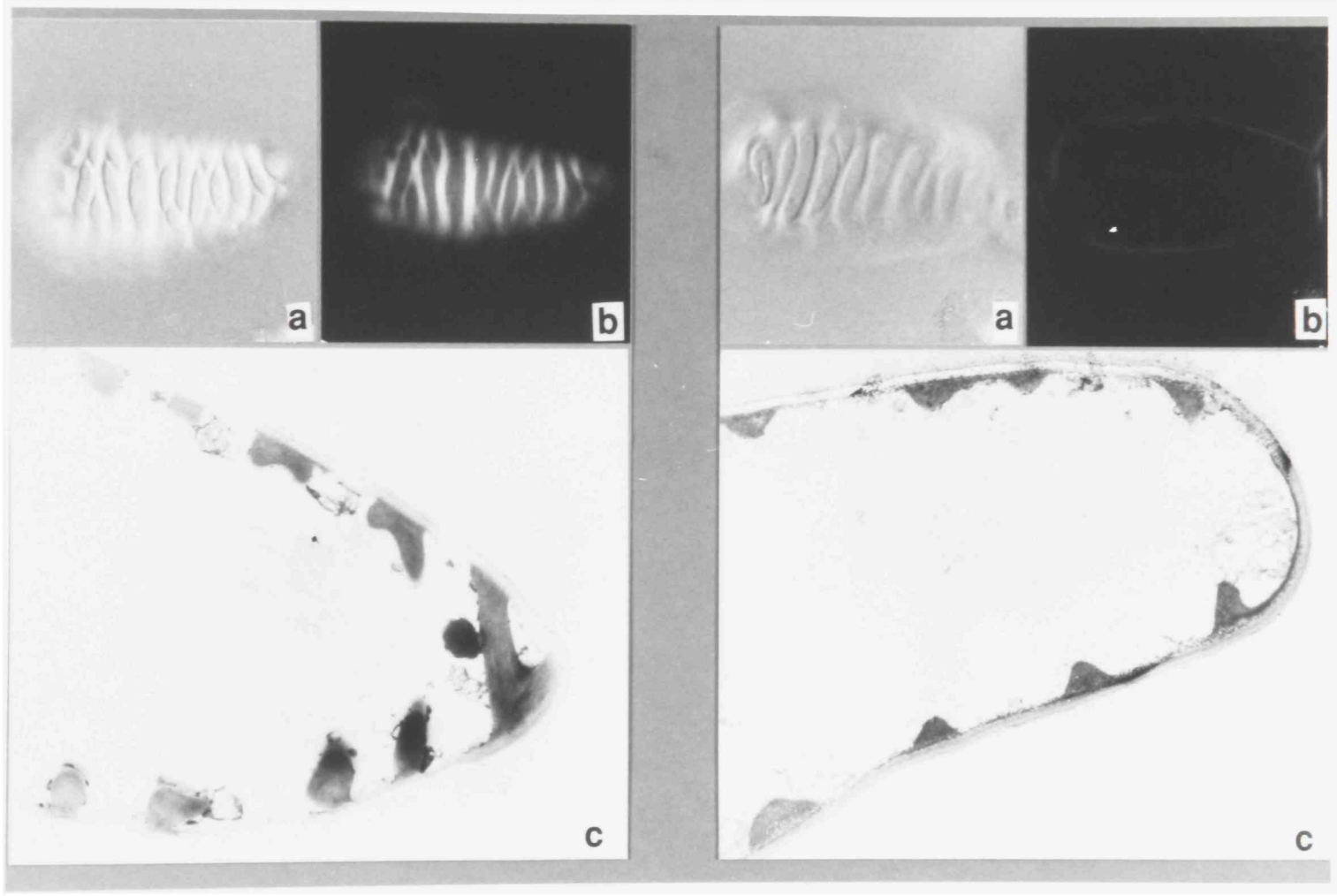
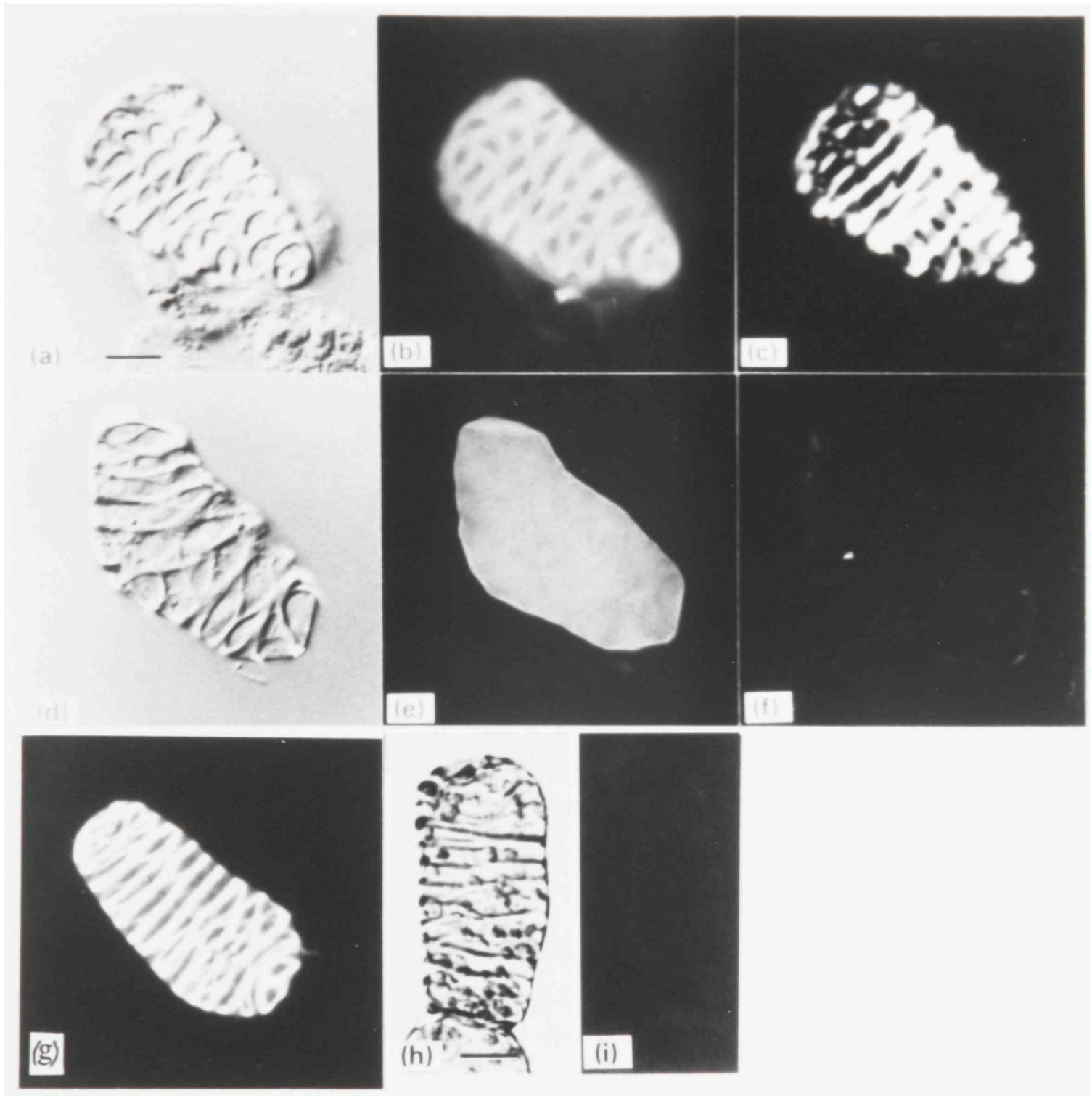

a
b
a
b
c
c

Fig. 2.8. Analysis of cellulose in control (a-c) and DCB-treated (d-f) TEs. Cellulose was present in thickenings of control cells (a-c). Control TE (at 65 h, after autolysis) with a patterned secondary wall shown with DIC (a), fluorescence after staining with Tinopal LPW (b), and POL (c). The fluorescence and birefringence both indicate the presence of cellulose.

Cellulose was not detectable in thickenings of DCB-treated TEs (d-f). A DCB-treated TE (at 65 h, after autolysis) with a patterned secondary wall shown by DIC (d). However, the pattern is not revealed by fluorescence after staining with Tinopal LPW (e) or birefringence in POL (f). The uniformly distributed Tinopal LPW fluorescence (e) indicates the presence of primary wall cellulose.

Comparison of labeling by cellulase-FITC in control and DCB-treated TEs at 64 h (g-i). Cellulase-FITC bound to the patterned thickenings of control TEs (g), but a DCB-treated TE with thickenings visible by DIC (h) showed no patterned fluorescence (i). Bars in all parts = 10 μm.


(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
(i)

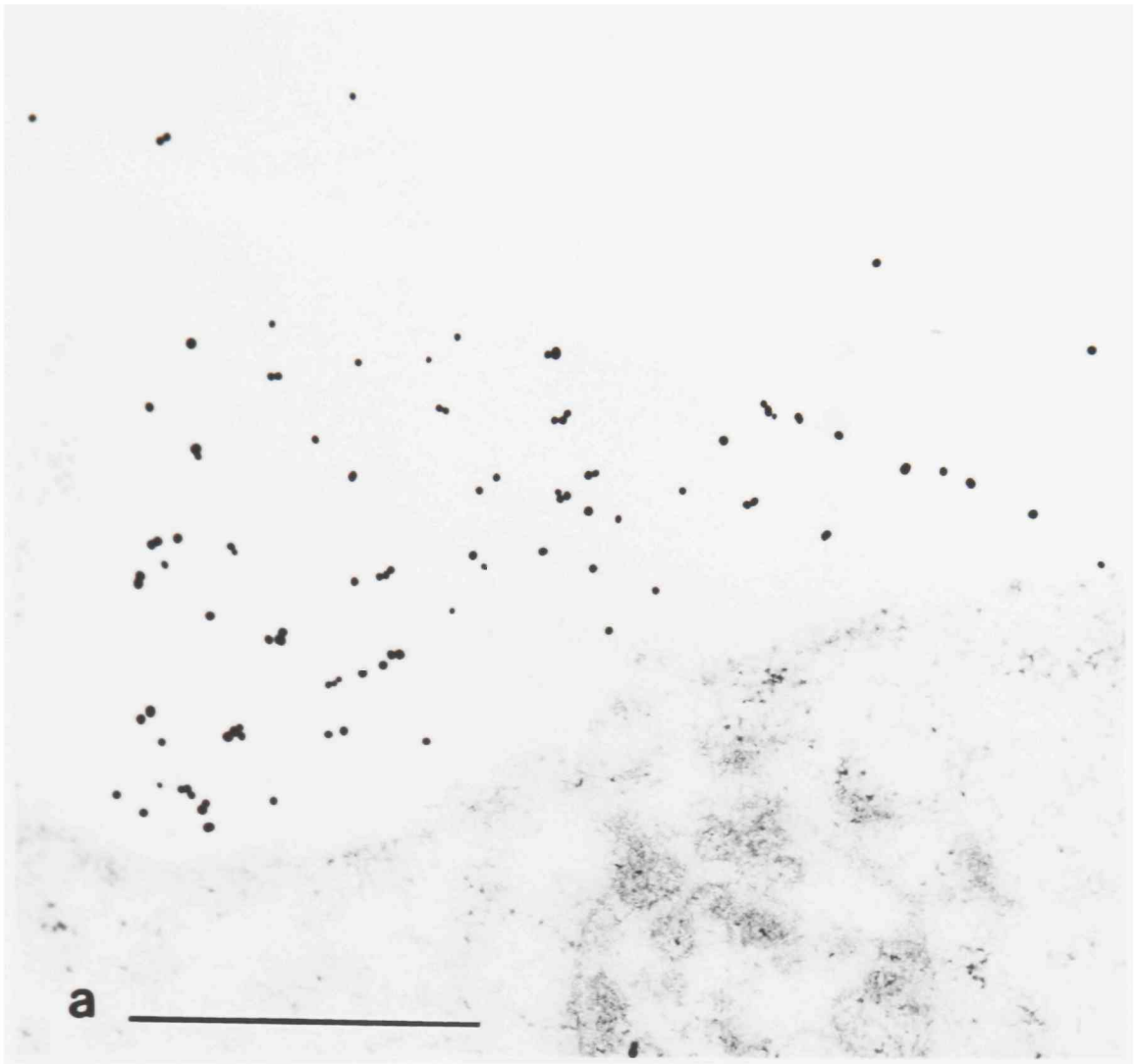
DCB-treated TEs detected with DIC (Fig. 2.8d) lacked these indicators of cellulose; the thickenings did not fluoresce with Tinopal LPW (Fig. 2.8e) and were not birefringent under POL optics (Fig. 2.8f). (The generalized Tinopal LPW fluorescence in the DCB-treated TE shown in Fig. 2.8e is due to staining of primary wall cellulose.) Thickenings of control TEs were also labeled with cellulase-FITC (Fig. 2.8g), whereas thickenings of DCB-treated TEs could not be labeled (Fig. 2.8h,i).

The cellulase/gold conjugate applied to sections of TEs and viewed by TEM labeled the secondary cell wall thickenings of control TEs (Fig. 2.9a, cells selected as above) whereas thickenings of DCB-treated TEs (cells selected as above) showed no or little gold label (Fig. 2.9b).

### 2.3.7 Detectable xylan is reduced or absent from thickenings of DCB-treated TEs

The thickenings of control TEs (Fig. 2.10a) could be labeled with unconjugated xylanase localized by indirect immunofluorescence (Fig. 2.10b), xylanase directly conjugated to FITC (data not shown), and an antibody to xylose localized by indirect immunofluorescence (data not shown). The xylanase is the product of a cloned gene from *Streptomyces lividans* IAF18 (Mondou et al., 1986). The antibody to xylose was raised against ß1->4 $Xyl_{3-8}$ purified from walnut shell xylan, characterized for specificity, and used previously to label the thickenings of *Z. elegans* TEs for analysis by electron microscopy (Northcote et al., 1989). Thickenings with xylan also stained positively with Tinopal LPW (Fig. 2.10c) and exhibited birefringence in polarized light (not shown), both of which support the presence of cellulose. The thickenings of DCB-treated TEs could not be labeled with either of these probes (results not shown, see Fig. 2.8 h,i for comparative result) unless the thickenings were also birefringent by POL or fluorescent with Tinopal LPW, indicating an "escaped" TE. Therefore, fully inhibited intact DCB-treated TEs lack accessible xylan in the altered thickenings. TEs in which cellulose synthesis was only partially inhibited by DCB or isoxaben ("escaped" TEs) could be labeled with probes for xylan, but in a different manner than controls. "Escaped" TEs, as indicated by faint staining of the patterned wall material with Tinopal LPW and/or faint birefringence, occurred if old stock solutions of the inhibitors were used and if the inhibitors were added after the onset of differentiation in some cells. Patches of xylan (Fig. 2.10e) that often bridged the primary wall space between secondary wall thickenings (Fig. 2.10d) were commonly observed in treated TEs with thickenings that contained some cellulose (Fig. 2.10f). The thickenings of such cells were either unstained or lightly stained with probes for xylan (see examples of both types in Fig. 2.10e). If the amount of cellulose in treated

Fig. 2.9. Cellulase-gold labeling of secondary wall thickenings of control (a) and DCB-treated (b) TEs. Bar = 1 μm.


a

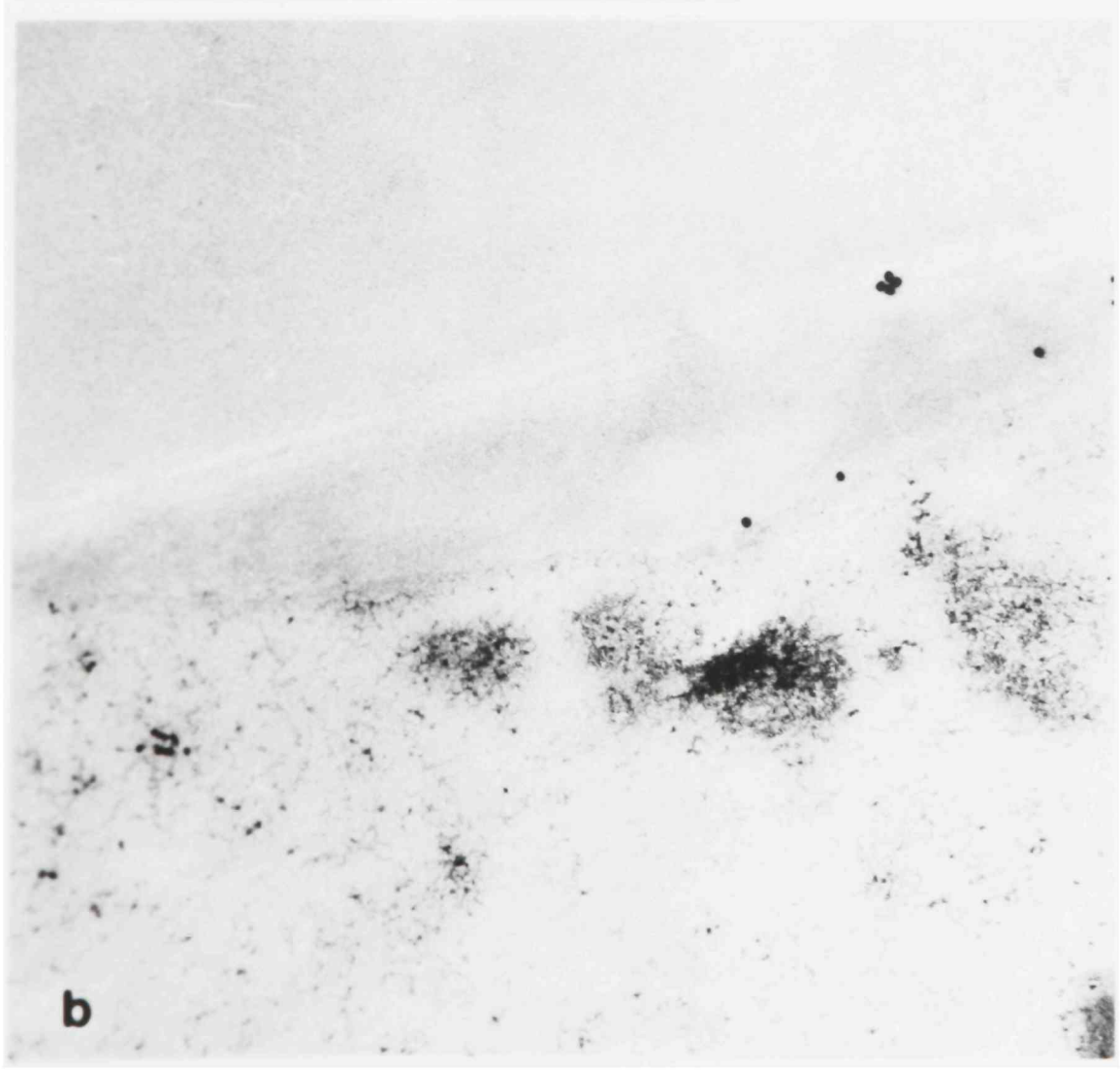
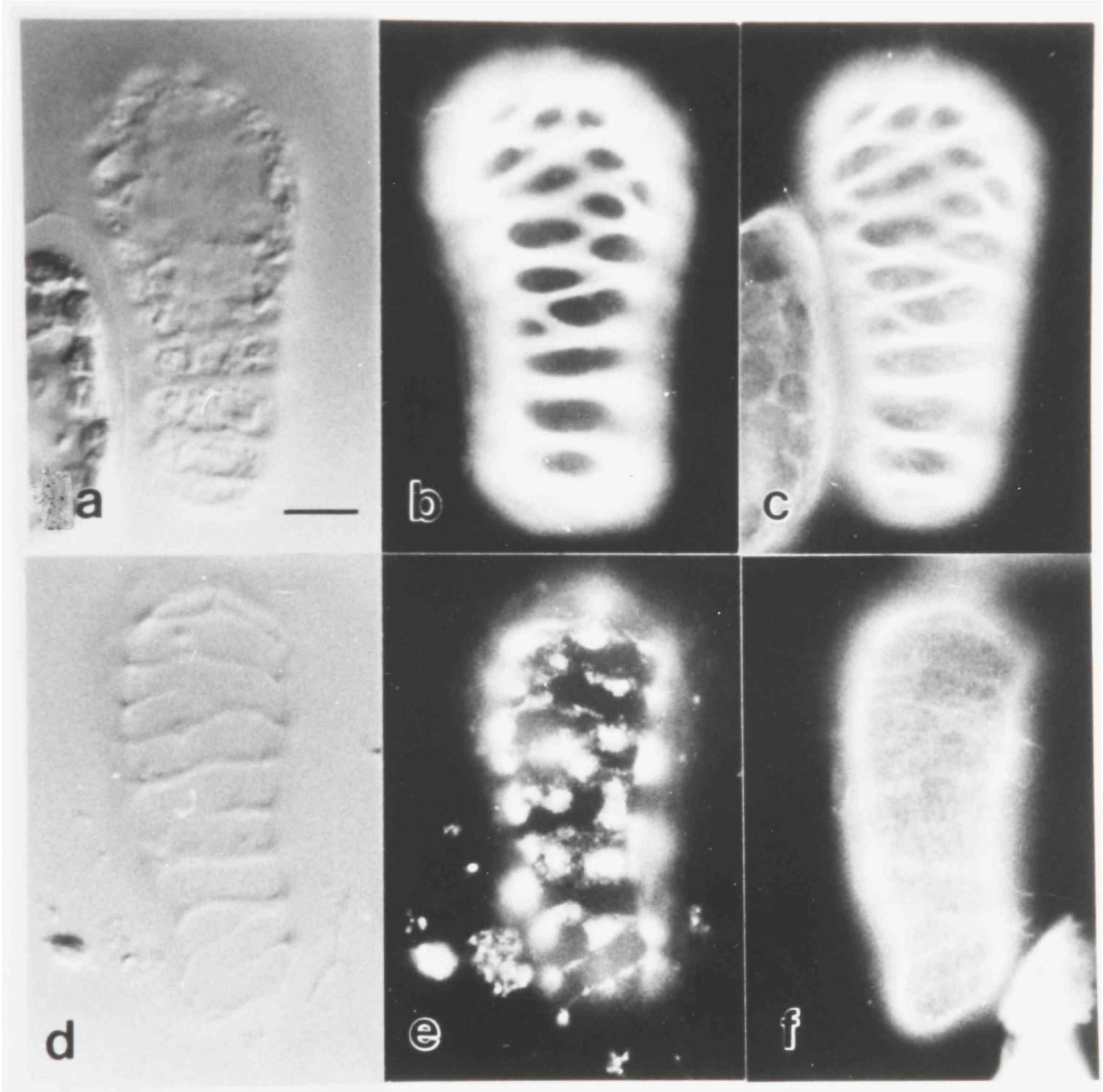

b

Fig. 2.10. Xylan labeling of control (a-c) and DCB-treated (d-f) TEs. The thickenings of control TEs visualized with DIC optics (a) contain xylan (b; localized with a cloned xylanase A and its antibody) and abundant cellulose (c). Some DCB-treated TEs with patterned secondary wall (d) show patches of xylan between the thickenings and little or no xylan over the thickenings (e; localized as above). TEs with patches commonly show evidence of partial "escape" of the DCB inhibition, as indicated by faint fluorescence of the thickenings with Tinopal LPW (f). The bar = 10 μm.


a
b
c
d
e
f

TEs was extensive as indicated by strong birefringence and strong Tinopal LPW staining, the probes for xylan corresponded to the pattern of the secondary wall as in control TEs (results indistinguishable from Fig. 2.10b). For these fluorescence assays, differentiating cells were examined at early stages when cell wall thickenings of controls stained most frequently for xylan and had low autofluorescence.

The immunogold localization of the xylan antibody applied to ultra thin sections of *Zinnia* cell cultures showed gold label on secondary wall thickenings of both control (Fig. 2.11a) and DCB-treated (Fig. 2.11b) TEs, although the label of the latter was noticeably less. A quantitative analysis of the gold labeling on the sections (Table 2.1) showed that control TE thickenings labeled significantly more ($p < 0.05$) than DCB-treated TE thickenings. Occasional observations of some DCB-treated TEs including glancing sections of the cell walls showed labeling of the exposed primary wall around the thickening (Fig. 2.11c), reminiscent of the patching observed at the light level.

Table 2.1. Immunogold labeling of secondary cell walls of TEs
Colloidal gold labeling (grains/ $\mu m^2 \pm$ sd)

| Antibody | Control | # of cells | DCB-treated | # of cells |
|---|---|---|---|---|
| anti-xylose | $18 \pm 4.3$ | 8 | $11.4 \pm 4.4$ | 10 |
| anti-GRP | $5.7 \pm 4.2$ | 13 | $1.86 \pm 1.0$ | 18 |

sd = standard deviation

2.3.8 Xyloglucan detected only in primary walls of Control and DCB-treated TEs

The antibody to xyloglucan and a secondary FITC-conjugated antibody indicated xyloglucan only in the primary walls between the thickenings of control and DCB-treated TEs (Figs. 2.12a, b, and d, e, f, respectively). As expected, the primary walls of non-differentiating cells were also labeled by the xyloglucan antibody (data not shown).

Indirect immunogold labeling with the xyloglucan antibody of ultra-thin sections of *Zinnia* TEs also showed labeling only on the primary walls in both control and DCB-treated TEs (Figs. 2.13 a and b respectively) with more gold particles ($p < 0.05$) found on the primary walls of the DCB-treated TEs (Table 2.2). The primary walls of non-differentiating control cells exhibited a more ($p < 0.05$) dense labeling (Figs. 2.13 c and d)

Fig. 2.11. Gold labeling of antibody to xylan localized to secondary wall thickenings of control (a) and DCB-treated (b) TEs. A glancing section of a DCB-treated TE (c) showing increased label on the primary wall near the thickening. t = thickening, p = primary wall, c = cytoplasm. Bar = 1 μm.

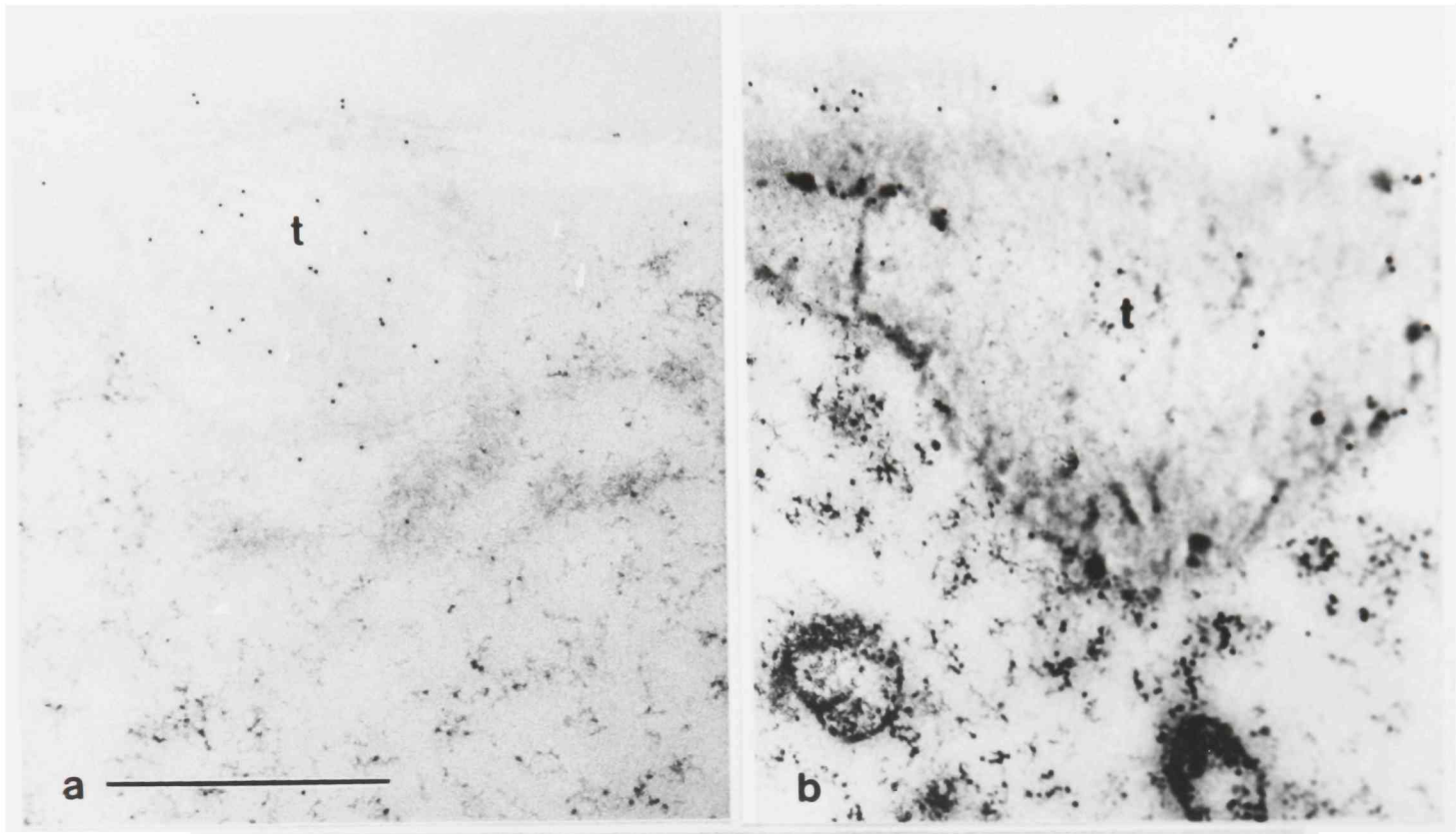

t
a
t
b
t
p
c
c

Fig. 2.12. Xyloglucan labeling of control (a, b) and DCB-treated (c, d) TEs. The thickenings of control and DCB-treated TEs visualized with DIC optics (a and c respectively) do not contain xyloglucan (b and d respectively; localized with an antibody to xyloglucan), but do contain xyloglucan between the thickenings in the primary wall (b and d respectively).

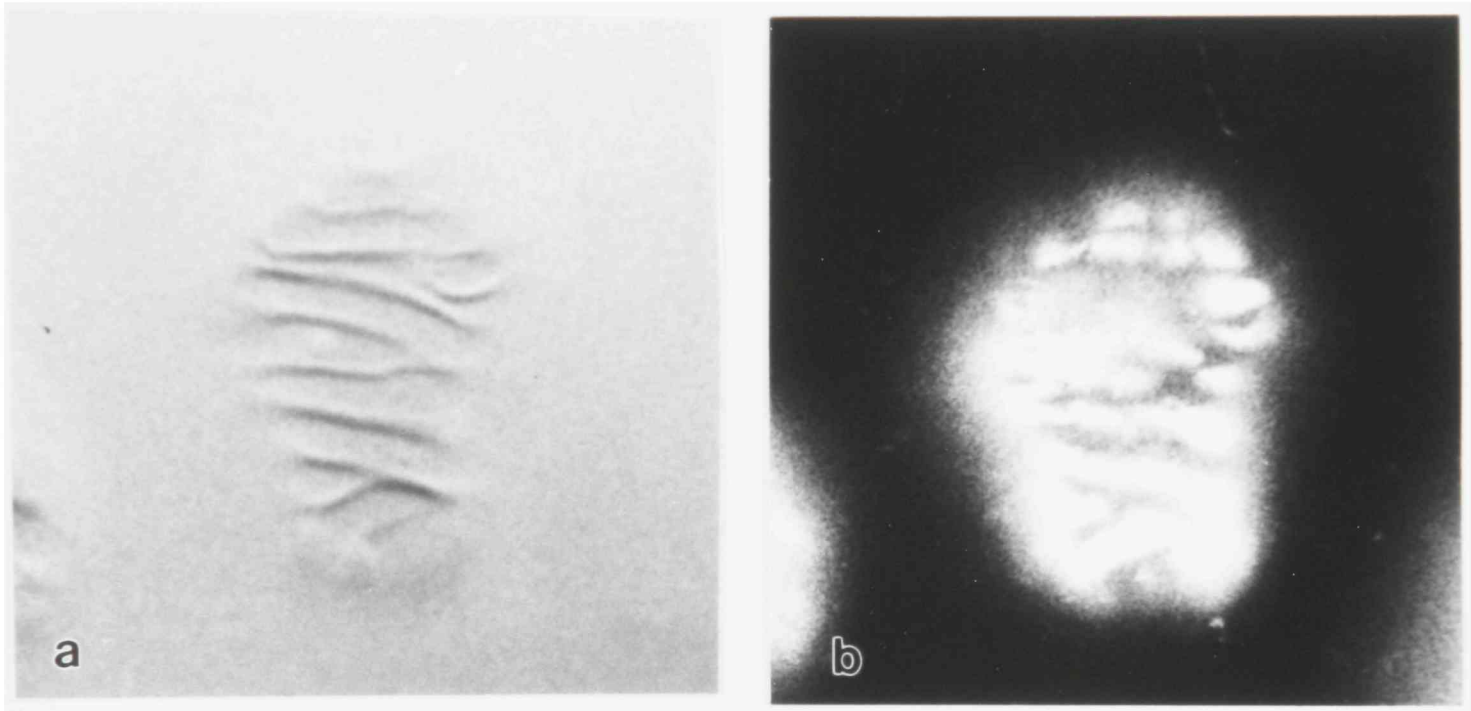
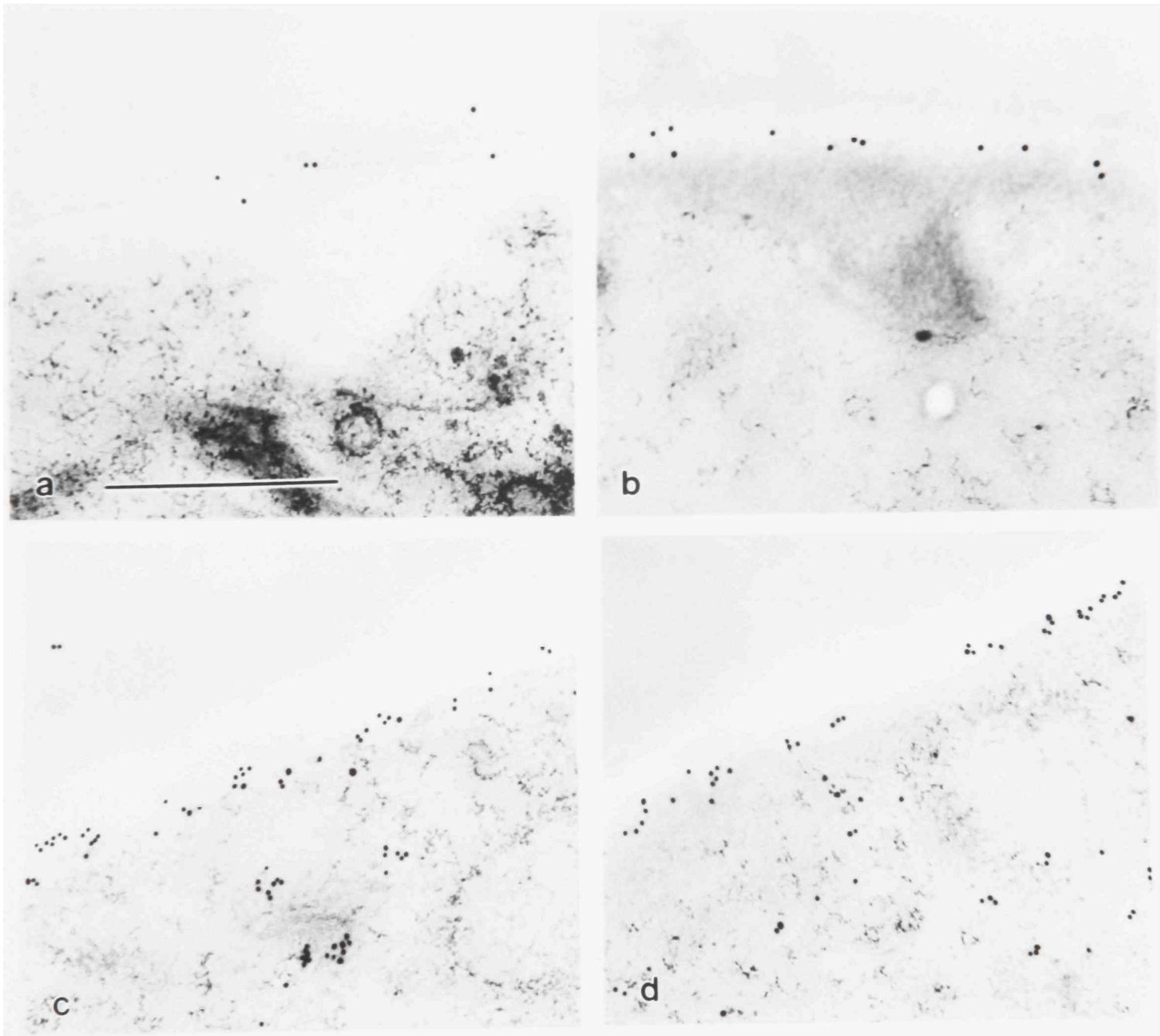

a
b
c
d

Fig. 2.13. Gold label of antibody to xyloglucan localized to the primary walls of control (a) and DCB-treated (b) TEs and to control (c) and DCB-treated (d) non-differentiating cells. Bar = 1 μm.


a
b
c
d

than the primary walls of control TEs (Table 2.2). The primary walls of DCB-treated non-differentiating cells labeled more intensely ($p < 0.05$) than the primary walls of non-differentiating control cells (Table 2.2). There was also labeling of the cytoplasm of living non-differentiating cells (Figs. 2.13 c and d) that was not as evident in TEs (Figs. 2.13 a and b), which would be consistent with the continued synthesis of primary walls in non-induced cells.

Table 2.2. Immunogold localization of antibody to xyloglucan on primary walls.
Colloidal gold labeling (grains/μm primary wall ± sd)

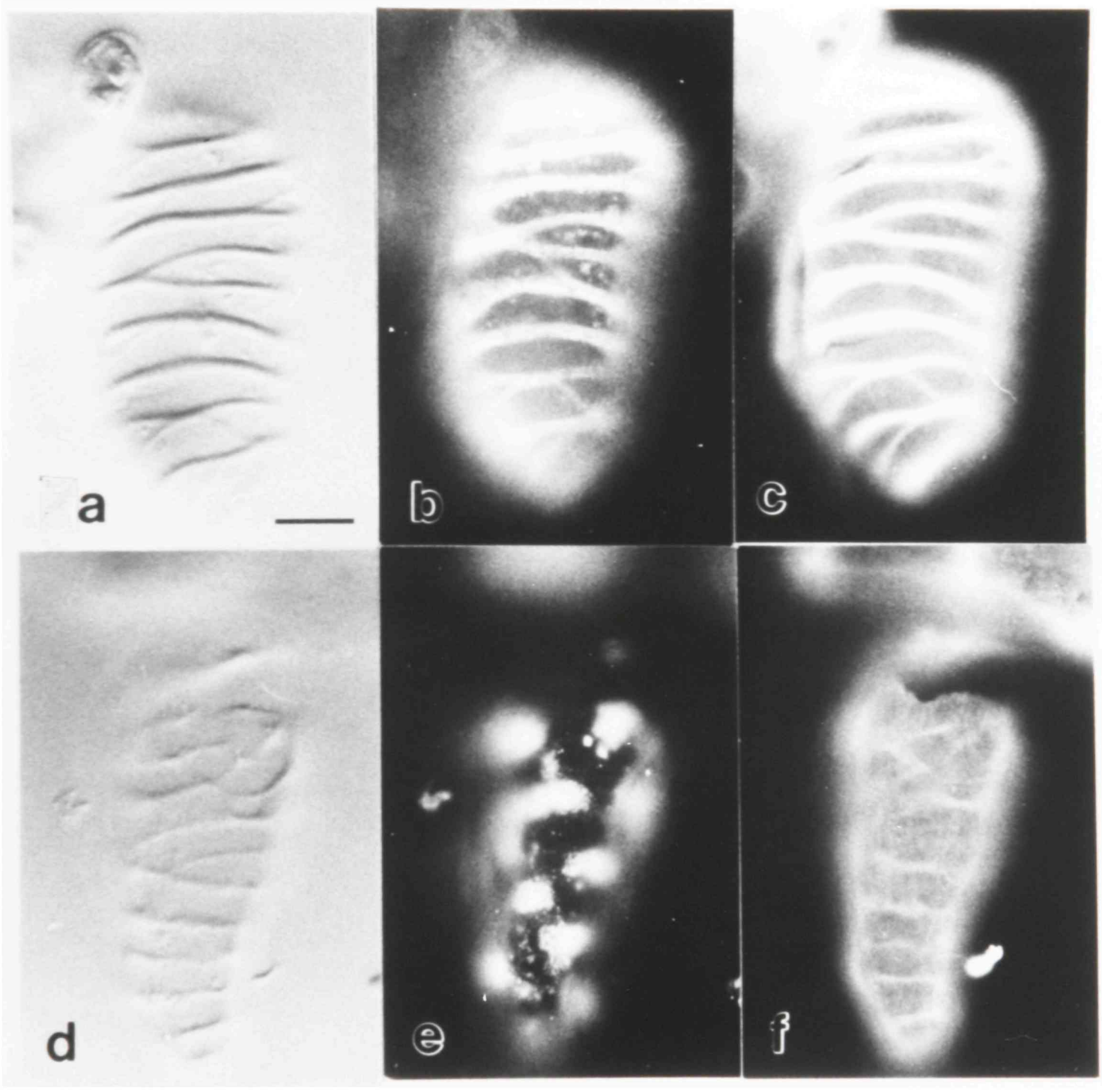
| | Control | # of cells | DCB-treated | # of cells |
|---|---|---|---|---|
| Non-diff | 9.3 ± 5 | 37 | 17 ± 5 | 16 |
| TE | 2.2 ± 1.5 | 9 | 5.4 ± 0.8 | 6 |

sd = standard deviation

### 2.3.9 Detectable GRP is reduced or absent from thickenings of DCB-treated TEs

The patterned secondary wall thickenings of control TEs (Fig. 2.14a) could also be labeled with the antibody to GRP (Fig. 2.14b), and the staining had a fine granularity compared to the smooth staining of xylan in control thickenings (compare Figs. 2.10b and 2.14b). GRP staining also differed from that of xylan by being less frequent, more adversely affected by fixation in formaldehyde before sonication, and more enhanced by sonication of the TEs. Thickenings that stained positively for GRP also stained with Tinopal LPW (Fig. 2.14c), but Tinopal LPW-positive thickenings were observed that did not stain for GRP (not shown). Many DCB- and isoxaben-treated TEs did not stain with antibody to GRP, but some with patterned secondary walls (Fig. 2.14d) showed patches between the thickenings (Fig. 2.14e). Such cells are partially "escaped" TEs as indicated by faint staining with Tinopal LPW (Fig. 2.14f) and often faint birefringence (not shown). As described for xylan, "escaped" TEs with strong indications of cellulose in the wall showed GRP staining equivalent to controls (results indistinguishable from Fig. 2.14b). Equivalent results were recorded photographically at 1:800 dilution of the GRP antiserum, although staining was brighter at 1:200 and 1:400.

Fig. 2.14. GRP labeling of control (a-c) and DCB-treated (d-f) TEs. The thickenings of control TEs visualized with DIC optics (a) contain putative GRP (b; localized with an antibody to GRP) and abundant cellulose (c; localized with Tinopal LPW). Some DCB-treated TEs with patterned secondary wall (d)show patches of GRP between the thickenings, but no GRP over the thickenings (e; localized as above). TEs with patches commonly show evidence of partial "escape" of the DCB inhibition, as indicated by faint fluorescence with Tinopal LPW (f).


a
b
c
d
e
f

When the primary or secondary antibodies were omitted, cells were either black or showed dull green background fluorescence. In previous immunolocalization studies (Keller et al., 1989; Ye et al., 1991; Ryser and Keller, 1992), the control had been used of a pre-immune serum from a different rabbit than the one used to generate the anti-GRP. Three rabbit pre-immune sera available in our lab gave very different results when applied and followed by FITC-tagged secondary antibody: (a) pre-immune 1 was correlated with green fluorescence of some thickenings at 1:100, but not at 1:1000; (b) pre-immune 2 was correlated with strong cytoplasmic fluorescence at 1:100, which was very dim at 1:1000; and (c) pre-immune 3 was correlated with smooth green fluorescence of some thickenings at 1:100, 1:200, and 1:1000. The pre-immune for xylanase A antibody showed no fluorescence and resembled treatments with secondary antibody only. At the TEM level, pre-immune 1, used as the primary antibody, produced a generalized gold label throughout the cells (TEs and non-differentiated cells). Pre-immune 2 and the pre-immune for xylanase A antibody did not label the cells.

Immunogold localization of GRP in ultra-thin sections of *Zinnia* TEs viewed by TEM showed gold labeling on the secondary wall thickenings of control TEs (Fig. 2.15a), whereas little or no gold label was found on the thickenings of DCB-treated TEs (Fig. 2.15b). The quantitative analysis of gold label on GRP-treated ultra-thin sections of *Zinnia* cells showed control TEs labeled significantly more ($p < 0.05$) than DCB TEs (Table 2.1).

### 2.3.10 Western blotting of GRP

Using the sensitive method of chemiluminescent detection, no GRP was detected in extracts of *Zinnia* mesophyll cells just after isolation for culture at t = 0 or at 12, 24, and 36 h. By 45 h, a band at about 48 kD was detected, which persisted until 72 h (Fig. 2.16). This period of detection spans the normal period of high differentiation. Less intense higher molecular weight bands sometimes become visible later in culture that may represent cross-linked molecules of GRP in the cell wall, as has been previously proposed for later stages of xylem development in tissues (Keller et al., 1989). A culture of non-differentiating cells taken at 65 h showed only a weakly labeled band near 60 kD.

### 2.3.11 Lignin content of DCB-treated TEs is similar to control TEs

Lignin absorbance from 1 mg of control cell walls was slightly higher than from 1 mg of DCB-treated cell walls (Fig. 2.17). From these absorbances, the lignin content in control and DCB-treated cell walls at 96 h was estimated to be 84 µg (8.4% w/w) and 63 µg (6.3% w/w) respectively, based on the parallel analysis of loblolly pine. The pine

Fig. 2.15. Gold labeling of antibody to GRP applied to control (a) and DCB-treated (b) TEs. Note the light labeling of the control thickening compared to virtually none on the DCB-treated TE. Bar = 1 μm.

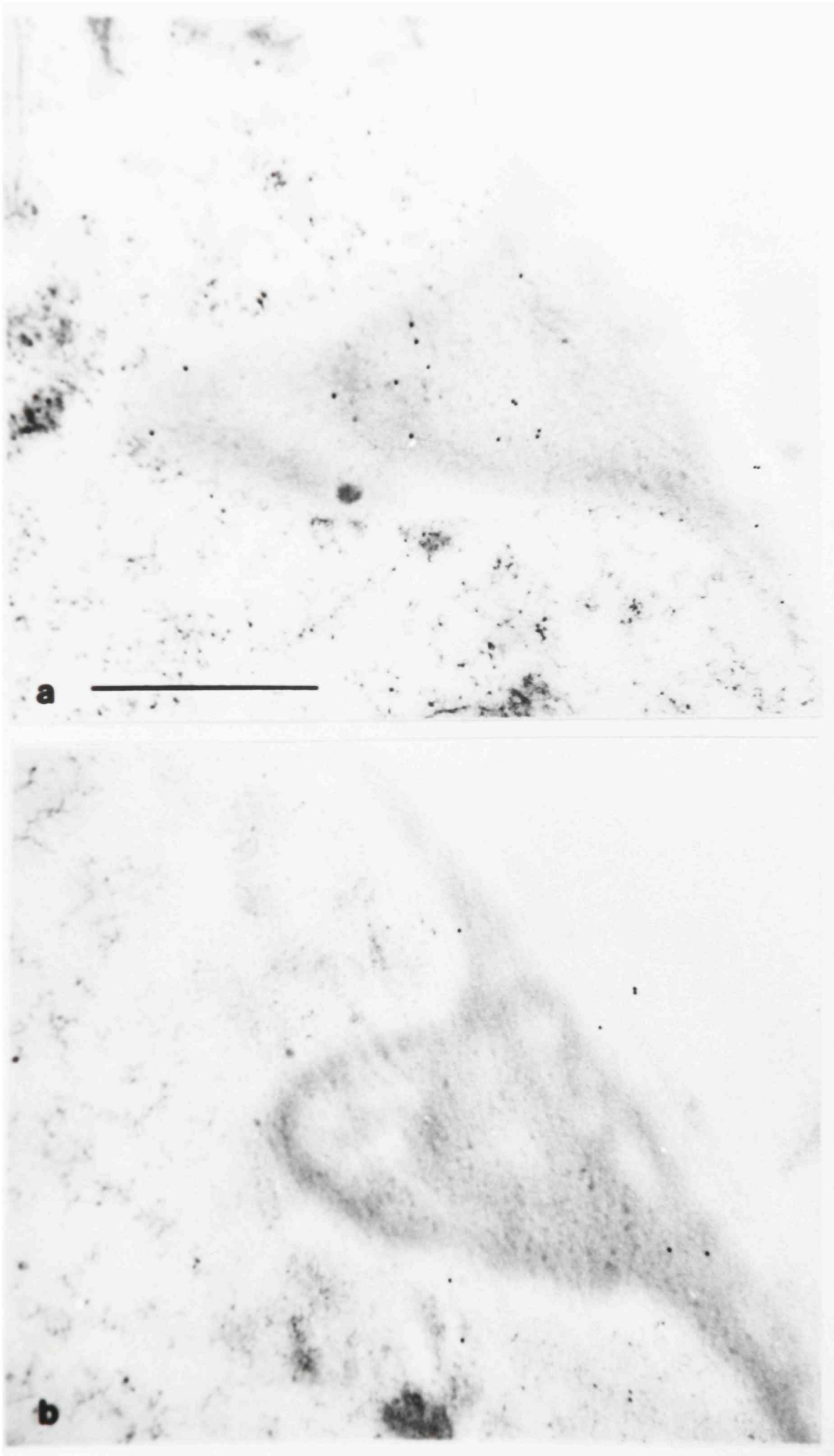
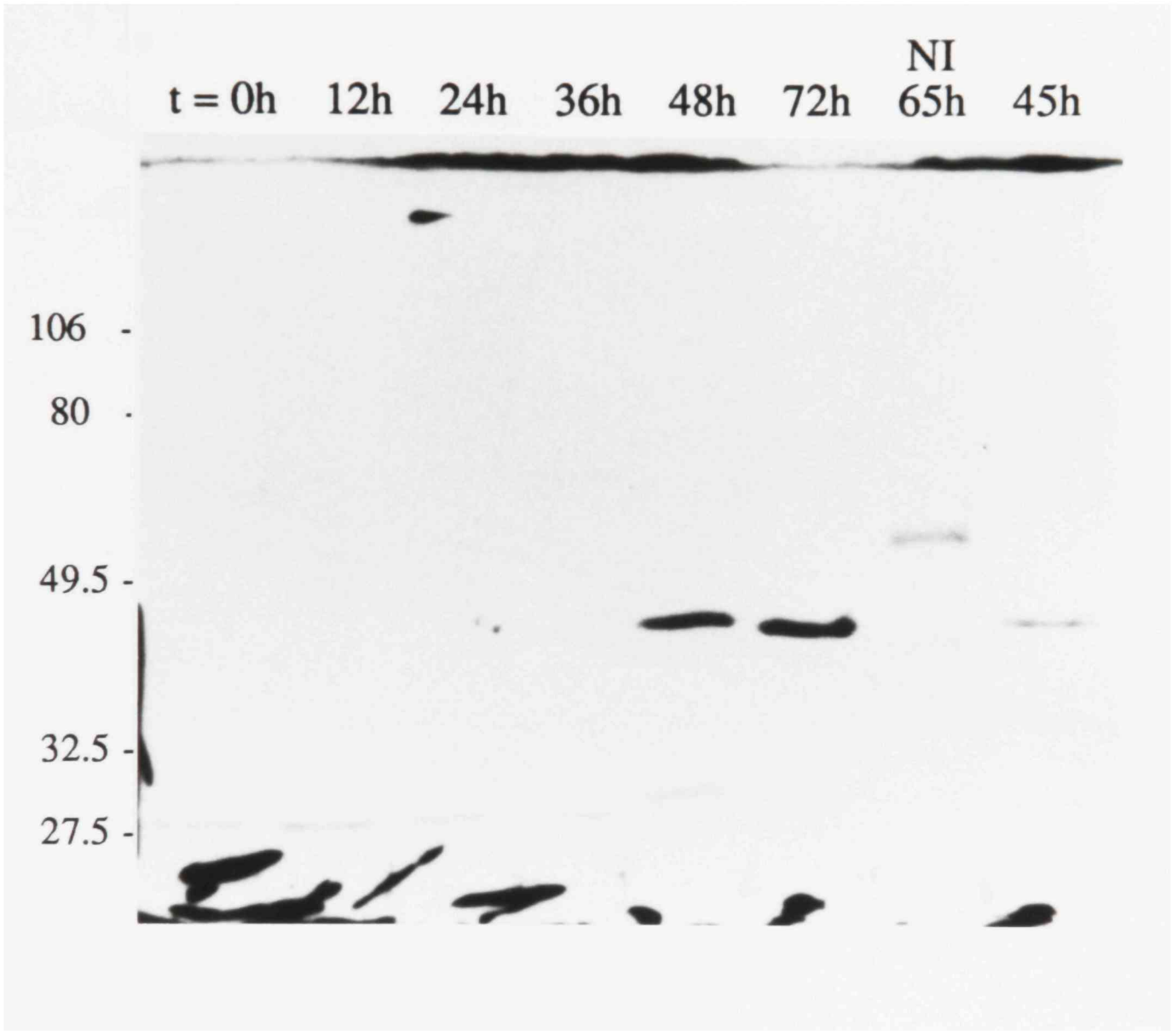

a
b

Fig. 2.16. Western blot of whole cells extracts of mesophyll cells prior to (beginning at t=0 h of culture) and during tracheary element differentiation (t=48 h to 72 h of culture). A single band near 48 kD is detected by GRP antiserum during the differentiation period.


t = 0h
12h
24h
36h
48h
72h
NI
65h
45h
106
80
49.5
32.5
27.5

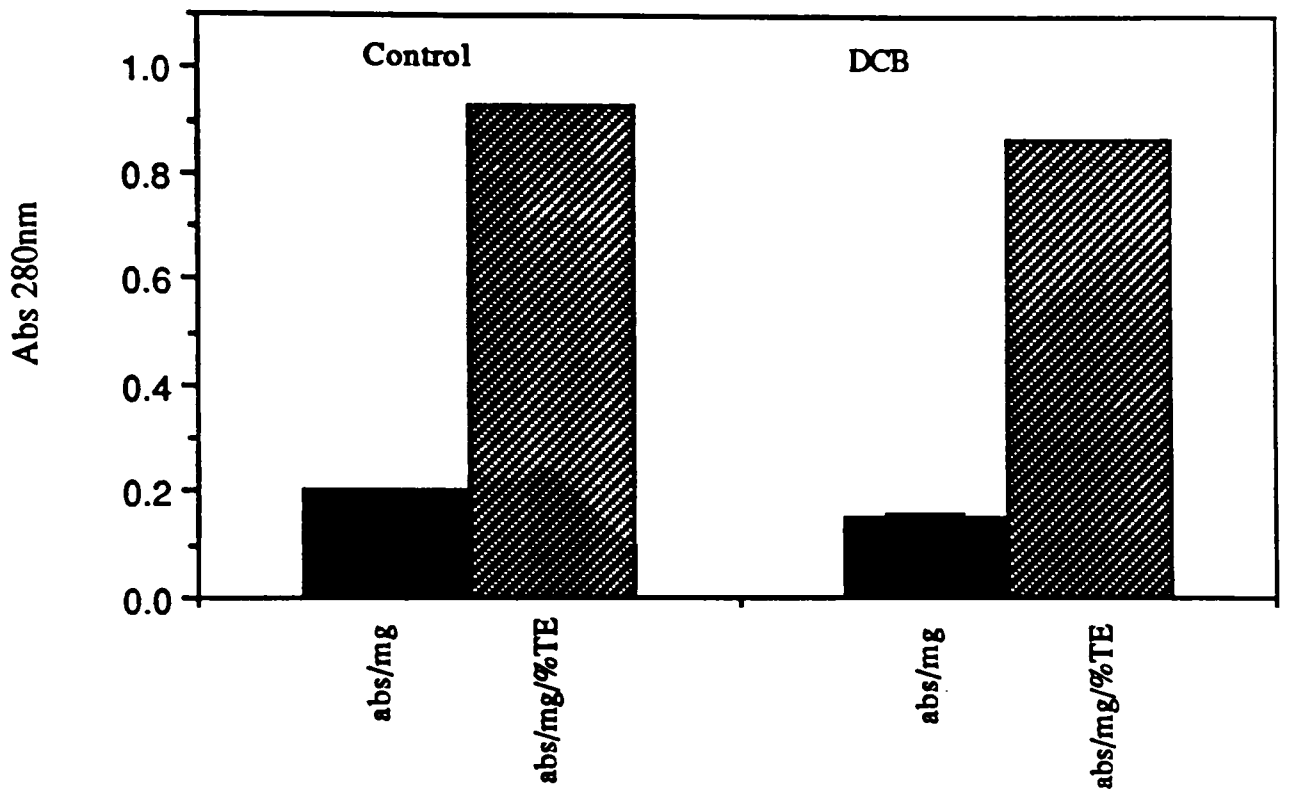



Fig. 2.17. Lignin content of cell walls of differentiating control and DCB-treated cultures of *Zinnia elegans*. The amounts of lignin per mg dried cell walls and the percentages of TEs were determined at 96 h. The absorbance/mg is expressed in the solid columns and the absorbance/mg/percent TEs is expressed in the striped columns.

standard (1 mg) contained approximately 246 µg of lignin (25% w/w), similar to results determined previously for loblolly and other pines (Johnson et al., 1961; van Zyl, 1978). When absorbances were adjusted for percent TEs in control and DCB-treated cultures, compensated values (absorbance/mg/%TE) of 0.9031 for control cell walls (21.5% TEs) and 0.8611 for DCB-treated cell walls (17.5% TEs) were derived (Fig. 2.17).

### 2.3.12 Lignin is dispersed in DCB-treated TEs

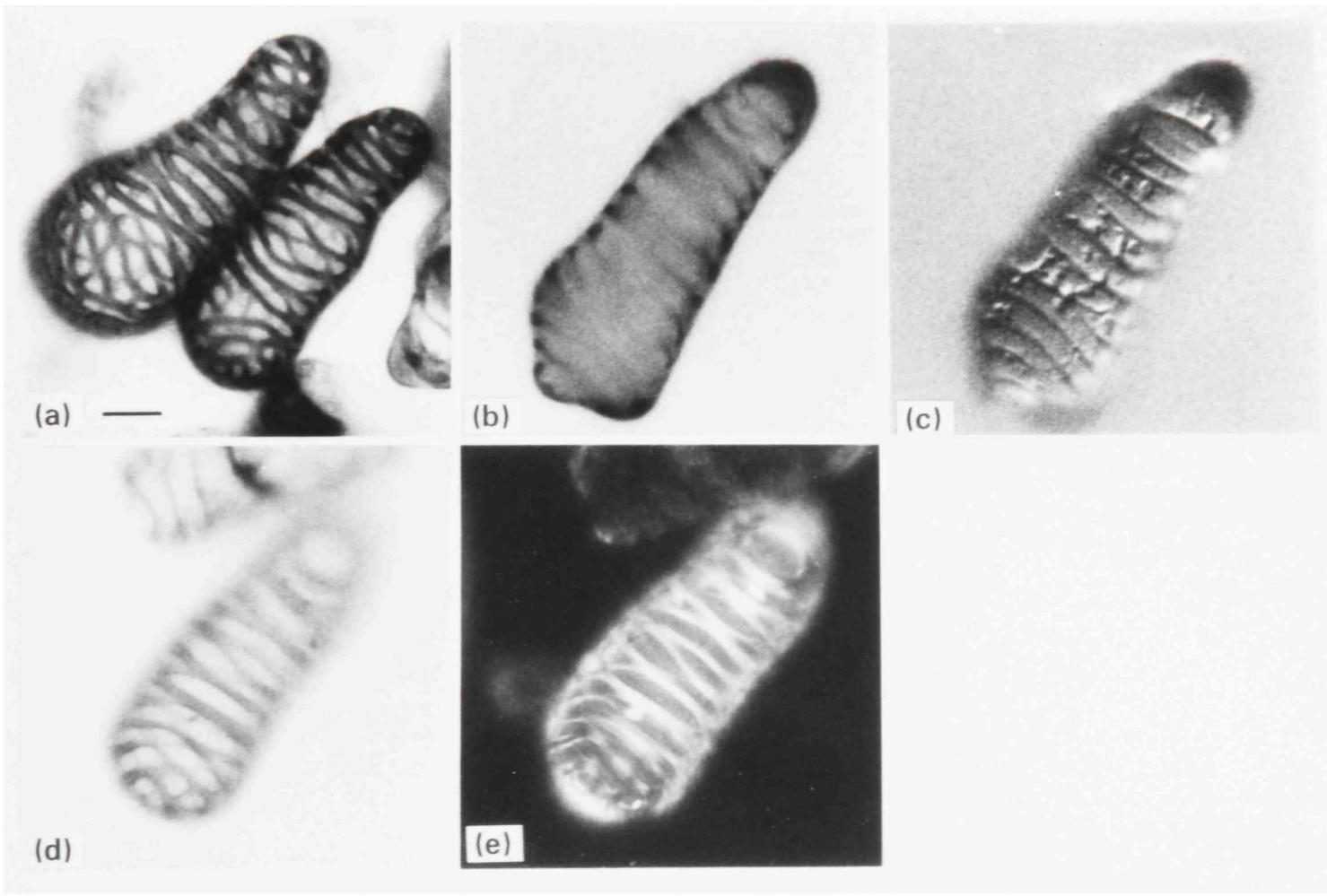
Phloroglucinol/HCl reacts with coniferyl aldehyde and cinnamaldehyde groups in lignin to yield a cationic chromatophore (Wardrop, 1981). The patterned secondary cell wall thickenings of control TEs stained readily with phloroglucinol/HCl (Fig. 2.18a). In contrast, phloroglucinol/HCl imparted a generalized red stain to DCB-treated TEs (Fig. 2.18b), indicating dispersed lignin. DIC confirmed that the dispersed lignin existed despite the presence of patterned wall material in DCB-treated TEs (Fig. 2.18c). Some cells exhibited darker phloroglucinol/HCl staining over the thickenings, probably because the cell wall was thicker and could contain more of the dispersed lignin. DCB-treated TEs with dispersed lignin did not exhibit birefringent thickenings (data not shown).

Escaped TEs had patterned lignin (Fig. 2.18d) even though DCB was present in the medium during lignin deposition. However, the cell wall thickenings of such escaped cells were also birefringent (Fig. 2.18e), indicating substantial content of oriented cellulose microfibrils. If 7.5 µM DCB was added after cellulose accumulation was detectable with POL optics in the thickenings of some TEs, mature TEs of two types were finally observed. Some had patterned lignin and birefringent thickenings, whereas others had dispersed lignin and non-birefringent thickenings.

### 2.3.13 Isoxaben also inhibited cellulose synthesis and caused dispersed lignin

Isoxaben (0.3 µM) caused morphological changes in developing TEs that were indistinguishable from those caused by DCB. Isoxaben-treated TEs had DIC-detectable thickenings that lacked birefringence and fluorescence after staining with Tinopal LPW or probes for xylan. Most had dispersed lignin as indicated by phloroglucinol/HCl staining, but a few exceptions had birefringent thickenings and localized lignin (see comments on "escaped" cells above).

Fig. 2.18. Patterns of lignification in control (a) and DCB-treated (b,c) TEs.
(a) Localized lignin in control TEs. Control TEs (at 132 h) stained with phloroglucinol/HCl exhibited red coloration in the pattern of the secondary wall, indicating the presence of localized lignin.
(b-c) Dispersed lignin in DCB-treated TEs. A typical DCB-treated TE (at 132 h) stained with phoroglucinol/HCl that exhibited red coloration over the whole cell (b), indicating dispersed lignin. DIC microscopy confirmed that the TE had patterned secondary wall thickenings (c), but these were not birefringent (data not shown).
(d-e) Rare TEs that synthesized cellulose in the presence of DCB also had localized lignin. A rare DCB-treated TE (at 99 h) stained with phloroglucinol/HCl exhibited red coloration in the pattern of the secondary wall (d), indicating the presence of localized lignin. However, analysis by POL showed that the thickenings were also birefringent (e), indicating oriented cellulose microfibrils. Bars in all parts =10 μm.


(a)
(b)
(c)
(d)
(e)

## 2.4 Discussion

### 2.4.1 DCB and isoxaben inhibit cellulose synthesis in developing TEs

Consistent with its expected action (Hogetsu et al., 1974; Montezinos and Delmer, 1980), the herbicide DCB inhibited cellulose synthesis in developing TEs. DCB is known to inhibit cellulose synthesis with little or no inhibition of synthesis of non-cellulosic polysaccharides, nuclear division, DNA synthesis, protein synthesis, respiration, or *in vivo* labeling patterns of UDP-glucose, phospholipids, or nucleoside phosphates (reviewed by (Delmer, 1987). It has been previously shown and confirmed by us that DCB does not disrupt microtubules (Venverloo et al., 1984), which are involved in determination of polysaccharide patterning in TEs (Falconer and Seagull, 1986; Hogetsu, 1991). An active, photo-reactive analog of DCB binds to an 18 kd protein in cellulose-synthesizing plant cells (Delmer et al., 1987). DCB also causes changes in the frequency of plasma membrane particle rosettes associated with cellulose microfibril deposition in higher plants (Herth, 1989). Despite these correlations, no clear mechanism for how DCB inhibits cellulose synthesis has been elucidated. The effects of DCB are reversible, with cellulose synthesis resuming upon its removal (for example, see Montezinos and Delmer, 1980). Based on available evidence, DCB is considered to be a specific inhibitor of cellulose synthesis when applied experimentally at low concentration (< 10 μM) for a few hours (Delmer, 1987).

Isoxaben also proved to be an effective inhibitor of cellulose synthesis in developing TEs. This herbicide inhibits incorporation of radiolabeled glucose into acetic/nitric insoluble wall material in seedlings of *Arabidopsis thaliana* without inhibiting biosynthesis of protein, nucleic acids, or fatty acids (Heim et al. 1990). (As noted above, acetic/nitric insoluble plant cell wall material is generally assumed to be cellulose [Updegraff, 1969]). The inhibitory effect of isoxaben was like that of DCB applied to the same seedlings, although isoxaben was equally effective at forty times lower concentration. Similarly, our results showed that isoxaben was effective at twenty-five times lower concentration than DCB. Therefore, it is likely based on previous reports and our results that isoxaben is another specific and potent inhibitor of cellulose synthesis.

Higher concentrations of DCB were not used because of increasingly deleterious effects on cell viability and percent differentiation. After 3 h incubation, DCB-treated cultures at normal and 3x cell density had incorporated 67% to 75% less [$^{14}$C]glucose into cellulose, yet they released only 15% to 17% less $^{14}CO_2$ than controls. In parallel experiments continued for 13 h, release of $^{14}CO_2$ was finally depressed only 13% in 7.5 μM DCB-treated cultures compared to controls (data not shown). These data support a

selective, although perhaps not completely specific, mechanism of action for DCB on the cellulose biosynthetic pathway in differentiating TEs. The 13% to 17% depression that was observed in $^{14}CO_2$ released by DCB-treated cultures might be explained by the toxicity of hydroxylated phenolic breakdown products of DCB produced by some plant species (Moreland et al., 1974). The effect of isoxaben on cellulose synthesis was similar to the effect of DCB, though the inhibition was slightly lower (62% vs. 67%), but its effect on respiration was nominal (0%), which indicates a possible more specific effect of isoxaben on cellulose synthesis than DCB. Interestingly, isoxaben and DCB were also able to inhibit incorporation into acetic/nitric insoluble cell wall material only about 80% in *A. thaliana* seedlings (Heim et al. 1990); the significance of this incomplete inhibition remains to be determined.

The presence of cellulose in the cell wall thickenings of control TEs was indicated microscopically by fluorescence after staining with Tinopal LPW, birefringence under POL optics, labeling with cellulase-FITC and labeling with cellulase-gold. Parallel observations of DCB- or isoxaben-treated TEs demonstrated that cellulose was not detectable by any light microscopic method in the cell wall thickenings that could be visualized with DIC light microscopy. Similarly, it was absent or drastically reduced at the TEM level. Because 7.5 µM DCB did not completely inhibit the incorporation of [$^{14}$C]glucose into acetic/nitric insoluble material, we cannot exclude the possibility that minor amounts of cellulose are present, but undetectable by the light microscopic assays, in the thickenings of DCB-treated TEs as indicated by the presence of low cellulase-gold labeling of some thickenings at the TEM level. However, the residual cellulose is not likely extensive because of the known ability of Tinopal LPW to highlight strands of cellulose in the size range of individual microfibrils on the surface of protoplasts (e.g., see Hahne and Hoffmann, 1985). The residual cellulose could also have been deposited in primary walls, not TE thickenings. This possibility is consistent with speculation that there may be at least two types of cellulose synthases, only one of which is sensitive to DCB (D. Delmer & R. Williamson, personal communication).

### 2.4.2 DCB- and isoxaben-treated TEs deposit some cell wall components in a pattern

Differentiating TEs treated with cellulose synthesis inhibitors deposited cell wall material in a pattern reminiscent of normal thickenings as indicated by DIC light microscopy and electron microscopy. Selection for sectioning of non-birefringent DCB-treated TEs insured that cells were analyzed by electron microscopy that truly lacked detectable cellulose. The smaller and less precisely shaped cell wall thickenings of DCB-

treated TEs were similar to those in wheat root TEs treated with DCB (Herth, 1989). The electron micrographs also demonstrate that disrupting normal polysaccharide deposition does not interfere with the autolysis of the cytoplasm that normally ends TE differentiation.

### 2.4.3 Xylan is not a major component of altered thickenings

Since a xylan-type polysaccharide is abundant in thickenings of *Z. elegans* TEs differentiated in culture (Ingold et al., 1988), we speculated that it might primarily compose the thickenings of DCB-treated cells. However, I was not able to label thickenings of DCB- or isoxaben-treated TEs with a specific xylanase or an antibody to xylose at any stage of development at the light microscope level, even though both probes labeled the thickenings of control TEs. At the TEM level, the thickenings of DCB-treated TEs did label for xylan, though at a density less than controls. Although there is a discrepancy between these two modes of analysis, the results do indicate that xylan is not preferentially incorporated into thickenings of DCB-treated TEs to such an extent to compensate singularly for the reduced cellulose. The difference between the light and TEM results might be explained several ways. Xylan labeling in TEM could possibly be enhanced because cellulose and xylan are normally associated with each other in the cell wall (Dey and Brinson, 1984; Reis et al., 1992), resulting in exposure of more accessible xylan antigenicic sites per molecule in sections when cellulose is depleted. Lack of comparable labeling at the light level could be attributed to viewing of treated TEs later in culture when they could first be detected. For example, at this same age, control TEs were no longer labeled at the light level, although xylan label could be revealed with TEM. Therefore, other components could have masked the xylan present until the binding sites were exposed by sectioning. The deposition of lignin could also be a factor in blocking the accessibility of xylan to probes at the light level, because it has been shown in jute fiber (*Corchorus capsularis*) (Das et al., 1981) and mesta fiber (*Hibiscus cannabilnus*) (Das et al., 1984) that xylan and lignin form strong linkages. We consider this unlikely for the DCB-treated TEs, because the cells were selected before autofluorescence of the thickenings (presumably from phenolic components including lignin) was an interfering factor. This does not exclude, however, the possibility of lignin precursors binding to xylan and holding it in the cell wall.

Xylan, which is a hemicellulose with ability to hydrogen bond with cellulose (Hayashi et al., 1987; Uhlin, 1990), is probably reduced in the thickenings of treated TEs because it cannot integrate properly into the wall structure of suspension culture cells without binding to cellulose microfibrils. The patches of xylan in TEs that partially

"escape" the effects of DCB and isoxaben and the diffused xylan in the primary walls of treated TEs (TEM) support the idea that xylan is synthesized and exocytosed into the wall mileaeu in a random manner since xylan is not normally present in the primary walls of *Zinnia* TEs. Since the same images were obtained in control and treated TEs using an antibody to xylose and a cloned xylanase A coupled with its antibody, we are confident that the results truly indicate the behavior of xylan. The pre-immune serum from the rabbit into which xylanase A was injected to raise the antibody gave negative results, increasing the certainty of the interpretation. Also, in developing the anti-xylose antibody, Northcote and co-workers (1989) showed no non-specific labeling in *Zinnia* by the pre-immune serum from the rabbit in which it was developed.

The possibility of the diffusion of xylan is consistent with the demonstration that cellulose-depleted primary walls of DCB-adapted suspension cells contain reduced xyloglucan, the predominant hemicellulose in many primary cell walls. The xyloglucan is synthesized and secreted into the culture medium (Shedletzky et al., 1990), presumably because its normal role in the primary cell wall is to bind with cellulose (see Levy et al., 1991, for review). Similarly, low levels of xylose and mannose, probably constituents of small amounts of xylan and glucomannan, were depleted in primary cell walls and enriched in the extracellular medium of the DCB-adapted cells (Shedletzky et al., 1990). One possible example of xylan being lost from its normal position in a secondary cell wall was demonstrated by Ingold et al. (1990) who showed an increase in a xylose-rich carbohydrate thought to be xylan in the medium of differentiating *Zinnia* cultures in which lignification was inhibited by the application of L-α-Aminooxy-ß-phenylpropionic acid (AOPP) (Ingold et al., 1990). It was proposed that xylan was released into the medium because lignin was not available to complete a cellulose/xylan/lignin unit (Morrison, 1974; Eriksson and Lindgren, 1977), allowing xylan to be lost from the cell wall. This proposal was based on the finding that certain lignin/carbohydrate complexes can be isolated containing cellulose and xylan. A future project would be to determine if xylan is increased in the medium of DCB-treated *Zinnia* cultures to support the idea that xylan is synthesized and exocytosed by the altered TEs rather than being incorporated into the cell walls in a normal manner.

### 2.4.4 GRP is not increased in altered thickenings

Supporting the model of synthesis, exocytosis, and diffusion of wall components in the absence of control levels of cellulose, the GRP antiserum gave similar images to the probes for xylan. However, the GRP labeling could be distinguished from the xylan images by being more granular, less frequent, and more enhanced by sonication of cells.

The significance of this later observation remains to be determined, but it suggests that putative GRP in the walls of whole cells is more easily accessed when the cytoplasm or perhaps the wall itself is mechanically disturbed.

Based on the character of the images, we are confident that the GRP antiserum recognizes a particular component of secondary wall thickenings rather than binding non-specifically. However, because the pre-immune serum from the rabbit injected with the GRP is not available as a control, we cannot assert definitively that the component localized by immunofluorescence is GRP. Interestingly, pre-immune sera from irrelevant rabbits have been used as controls in all previous localization studies (Keller et al., 1988; Keller et al., 1989; Keller et al., 1990; Ye et al., 1991; Ye and Varner, 1991; Ryser and Keller, 1992), and, of three pre-immune sera that we tested, one can stain thickenings at 1:1000. We attempted to affinity purify anti-GRP from nitrocellulose slices and were successful in eluting enough to perform a western blot under long exposure conditions of the sensitive chemiluminescent method, indicating low recovery. However, immunofluorescence tests with this purified antibody were unsuccessful, probably because of the low titer. Nevertheless, in a Western blot the antiserum recognizes a single band near 48 kD at the time of *Zinnia elegans* tracheary element differentiation, but not in the mesophyll cells used to start the culture. This timing is consistent with the localization of GRP in protoxylem, including secondary wall thickenings, of several species (Ye et al., 1991). The apparent molecular weight of the band is similar to the 53 kD band recognized in cell wall extracts and *in vitro* translation products of mRNA of French bean (Keller et al., 1990). A non-induced culture at 65 h contained a faintly labeled band at approximately 60 kD, which may represent a different GRP because this antibody also cross-reacts with an antigen found in a rice cell wall fraction (Lei and Wu, 1991). Staining of the secondary walls of *Zinnia elegans* TEs by the antiserum at 1:800 suggests that the statistically significant colloidal gold labeling of secondary walls in electron microscope sections of French bean that was sometimes observed (Ryser and Keller, 1992) may reflect reality. The lower density of gold particles found on the thickenings of the GRP sections labeled in this study as compared to the results of Ryser and Keller (1992), who had denser gold label of thickenings in hypocotyls of French bean, could be a feature of tissue versus single cell culture and also due to a more stringent washing protocol used for the *Zinnia* samples. We observed fewer Tinopal-positive control TEs that stained for GRP than for xylan, suggesting that the antiserum recognizes a component with antigenic sites that are accessible for only short times, possibly due to interaction of GRP with other molecules of the wall. This idea was further supported by the labeling of thickenings at all stages of TE

development at the TEM level. Since GRP can be synthesized as a bacterial fusion protein (Keller et al., 1988), it would be useful in further work to attempt larger scale affinity purification by column methods or to generate monoclonal or polyclonal antibodies in animals that are pre-tested for lack of binding of their pre-immune serum to TEs. In the meantime, along with others we can tentatively propose that the component with the reported behavior is GRP.

### 2.4.5 Xyloglucan is not found in altered thickenings

Xyloglucan is a normal constituent of primary walls in higher plants. Because of this, it could become a natural substitute used by DCB-treated cultures to take the place of the cellulose, xylan and GRP which were reduced or missing from the altered thickenings. This concept was very unlikely given the results of Shedletzky et al. (1990, 1992) in which DCB-adapted tomato and tobacco cell line cultures were found to have a marked decrease in xyloglucan in the adapted walls. However, preliminary GC/MS analysis of control and DCB-treated cell walls of differentiating *Zinnia* cultures indicated a possible increase in xyloglucan in the DCB-treated cell walls (see Chapter 3 for results which later disprove this) and prompted us to test for the presence of xyloglucan in the cell walls. The inability to detect xyloglucan and at best reduced levels of the other three cell wall components at any stage of development in the thickenings of treated TEs indicates something different from these four normal cell wall components must be incorporated into the altered secondary walls, the identity of which is still under investigation.

The increase of detection of xyloglucan in the primary walls of sectioned DCB-treated cells compared to controls, could be a result of antigen accessibility as mentioned before for xylan. Xyloglucan is known to associate with cellulose, and the absence of cellulose could increase the availability of xyloglucan for labeling or cause the xyloglucan to self-associate more (Edelmann and Fry, 1992), which would also increase the proportional amount of xyloglucan in the cell wall. Also, these cells are not adapted to DCB and will ultimately die, requiring the comparison to the DCB-adapted cells of Shedletsky et al. (1990, 1992) to be made cautiously. The increased label in the cytoplasm of non-differentiating cells is probably associated with primary wall synthesis (supported by the absence of cytoplasmic labeling in differentiating TEs). To determine in this study if the DCB-treated cells and non-differentiating cells are actually synthesizing more xyloglucan is not possible. In order to accomplish that analysis, a different fixation protocol would need to be used (i.e., freeze substitution) to preserve better the endomembrane system of the cells and provide a means to determine if more antibody to

xyloglucan is localized to the Golgi and vesicles of the treated cells as compared to controls.

### 2.4.6 Lignin is dispersed in DCB- and isoxaben-treated TEs

When cellulose synthesis was inhibited by DCB or isoxaben, lignin was deposited without a pattern as indicated by staining with phloroglucinol/HCl. This finding was recently supported by Suzuki et al. (1992) using *Zinnia elegans* var Canary Bird. Sometimes the thickenings of treated cells stained slightly darker than the intervening primary wall, probably because the thicker wall at that point could contain more of the dispersed lignin. The most important point is that lignin was abundant between the thickenings when cellulose synthesis was inhibited, whereas in control TEs (from this *in vitro* system and primary xylem *in vivo*) it is confined only to the thickenings. The amount of lignin present per TE remained approximately the same in DCB-treated cultures compared to controls, implying that the general dispersion of lignin is related to relocation, not a change in quantity of synthesis of lignin precursors. Therefore, the absence or depletion of newly-synthesized cellulose in the thickenings prevented the developmentally important restriction of lignin to the thickenings that normally leads to selective hydrolysis of the intervening primary wall (O'Brien, 1981).

The normal appearance of microtubules in differentiating TEs after application of DCB suggests that the dispersion of lignin is not related to disturbance of the cytoskeleton and is consistent with evidence that control of lignin localization does not depend directly on microtubules (O'Brien, 1974; Roberts et al., 1985). The dispersion of lignin when cellulose synthesis is inhibited provides evidence that the localization of cellulose in secondary wall thickenings directly or indirectly mediates the localization of lignin. Data that are consistent with such a relationship between cellulose and lignin include: (a) previous histochemical observations showing that parts of walls in TEs that do not contain lignin also contain little or no cellulose, depending on species (Benayoun et al., 1981); and (b) the observation that when microtubule antagonists cause unpatterned cellulose deposition in differentiating TEs, lignin is also unpatterned (reviewed by O'Brien, 1974). The coordinated disappearance of both cellulose and lignin from cell walls in the path of emerging lateral roots (Bell and McCully, 1970) may also reflect a direct association between cellulose microfibrils and lignin. Since plants are not known to synthesize enzymes that break oxidatively coupled phenolic dimers (Fry, 1988), the removal of lignin in normal developmental processes may occur by digestion of the wall polymers with which it is associated. However, there are likely to be as yet uncharacterized phenomena in

this process, since it is difficult to understand how hydrolases can access polysaccharides extensively surrounded by lignin.

It could be argued that the inhibitors used had an uncharacterized effect on a process other than cellulose synthesis that affects the localization of lignin, although the similar effects of two different inhibitors itself provides evidence against this possibility. Furthermore, the characteristics of TEs that differentiated in DCB or isoxaben but had localized lignin provide contrary experimental evidence. These rare "escaped" cells (<1%) always had birefringent thickenings indicative of localized cellulose if the inhibitors were added before cell wall thickenings were detectable and more frequently if they were added after onset of secondary wall deposition in some cells. Therefore, the presence of DCB or isoxaben in the medium did not cause dispersed lignin in a particular cell if cellulose synthesis in that cell was also not almost completely inhibited.

### 2.4.7 Mechanisms by which localization of cellulose might mediate localization of lignin

The deposition of cellulose in TE secondary wall thickenings precedes lignification (Ingold et al., 1990; Roberts et al., 1985), as would be required for any mechanism by which the localization of cellulose ultimately mediates the localization of lignin. Our data are consistent with the possibility that, after secretion into the wall milieu, the precursors of lignin only remain fixed in locations where particular other molecules and bonding sites are available. The target molecules might be specifically synthesized during secondary wall deposition or they might also be present in other (older) parts of the wall, but unavailable for bonding due to associations already formed. The second possibility is consistent with the concept of wall deposition in which wall matrix polymers are initially somewhat disordered at the outer surface of the plasma membrane until they self-assemble along with the cellulose microfibrils to form a stable composite wall (Haigler, 1991; Vian and Reis, 1991). Whether or not bonding sites for the molecules that directly mediate lignin localization are or remain available would be determined by the type of wall polymers that were initially present. For example, the interaction of lignin precursors with hydroxyproline relies on the presence of unsubstituted hydroxyls in the amino acid, but these are extensively blocked by glycosidic bonds in some tissues (Whitmore, 1982). Therefore, the pre-existing cellulose in the primary wall of differentiating TEs might not be able to mediate the localization of lignin because of its prior interaction with matrix materials specific to primary wall.

How the presence of cellulose mediates the localization of lignin remains to be determined. The possibility of a direct association between cellulose and lignin is indicated

by evidence that soluble precursors of lignin alter the assembly of cellulose microfibrils in *Acetobacter xylinum* (Dr. Raj Atalla, personal communication). (Altered assembly of bacterial cellulose microfibrils is induced by dyes, cellulose derivatives, and hemicelluloses that bind cellulose [Haigler, 1991; Hayashi, 1987; Uhlin, 1990]). In addition, polarized raman spectroscopy shows that cellulose and lignin are both highly oriented in cell walls (Atalla and Agarwal, 1985), but it is not known if there is causal relationship between the orientation of the two macromolecules. Also, the observation by Siegel (1956) that a lignin-like compound is only produced *in vitro* when the precursor eugenol and the peroxidase enzyme are activated in the presence of a cellulose matrix, supports the need of cellulose in lignin deposition.

It is also possible that the dispersion of lignin in DCB- and isoxaben-treated TEs could be due to the lack of another polysaccharide (like xylan) or protein that is dependent on cellulose for normal spatial integration into the wall. Polymerized lignin and hydroxycinnamic acid precursors of lignin can form ester and/or ether linkages with polysaccharides including glucoronoxylan (Morrison, 1972; Wardrop, 1981; Lewis et al., 1989; Yamamoto et al., 1989). The polysaccharide-linked hydroxycinnamic acids have been suggested to be possible nucleation sites for initiation of lignification (Yamamoto et al., 1989). Diphenolic bonds may also exist between the monomers of lignin and aromatic side chains of acidic polysaccharides or basic glycoproteins; there is evidence that lignin is preferentially associated with hydroxyproline-rich glycoprotein in cell walls of *Pinus elliottii* callus (Whitmore, 1982). Similarly, it has been speculated that the abundant tyrosine residues of the glycine-rich protein specifically found in lignifying cells might form the substratum for initiation of the oxidative polymerization of lignin (Keller et al., 1989), but this remains to be proven.

Alternatively, the isoperoxidases that are required to generate radicals from the aromatic alcohol precursors of lignin prior to polymerization (Lamb, 1981) could have a binding domain for cellulose or an associated wall component, much like some cell wall degrading enzymes have been shown to have cellulose-binding domains (see Kellet et al., 1990). Cytochemical localization of peroxidases in wound vessel members of *Coleus* demonstrates that the active enzymes are preferentially localized in secondary wall thickenings and the regions of the primary wall immediately below them (Hepler et al., 1972). Increased activity of wall-bound peroxidases is a specific feature of differentiating *Z. elegans* TEs (Fukuda and Komamine, 1982). Suzuki et al. (1992) showed the dispersal of peroxidase activity in DCB-treated TEs in a pattern similar to the dispersed lignin shown by phloroglucinol/HCl staining, further supporting the concept of the localization of

cellulose being necessary for the proper localization of other cell wall components. More data are needed to demonstrate that these or other mechanisms explain how the localization of cellulose ultimately mediates the localization of lignin.

### 2.4.8 Significance of coordinated localization of cellulose and lignin

The water-conducting efficiency of TEs is affected by the pattern of wall hydrolysis that occurs as part of the final autolysis of the cells. Only unlignified regions of the wall are hydrolyzed because impregnation with lignin makes wall components in those areas relatively inaccessible to hydrolases (O'Brian, 1981; Burgess and Linstead, 1984). Therefore, the localization of lignin is an important determinant of the final functional properties of the TEs. In *Zinnia* TEs, dispersed lignin is correlated with the absence of or reduction in cellulose, xylan, GRP, and possibly other molecules from the secondary wall thickenings. Other tracheids, such as those in secondary xylem that have lignified primary and secondary walls (Wardrop, 1981), might have lignin localization in the primary wall mediated by binding sites on hydroxyproline-rich glycoproteins (see Roberts et al., 1985; Whitmore, 1982) that are often abundant in primary walls. Developmental and taxonomic plasticity of wall composition, leading to availability or not of particular binding sites that serve to localize lignin, would provide a mechanism for the extensive variability in patterns of lignification observed in nature.

It should also be noted that localization of lignin might be required for the permanent fixation of polysaccharides into the secondary wall due to their interdependence with lignin in a structural network and/or uncontrolled hydrolytic activity. The latter possibility was offered as a possible explanation for the fate of TE secondary thickenings in seedlings treated with L-α-aminoxy-ß-phenylpropionic acid (AOPP), an inhibitor of lignin synthesis. After exposure to transpiration stress, the unlignified thickenings partly disintegrated and detached from the primary wall; the hydrolytic enzymes that would normally degrade only the unlignified primary wall might have also attacked the unprotected secondary thickenings (Smart and Amrhein, 1985a). Support for the first possibility was based on analysis of an increase in xylose content in the extracellular medium of differentiating *Z. elegans* TEs treated with AOPP (Ingold et al., 1990), but the data do not unequivocally support the claim if the stated increase in TE number caused by the inhibitor is taken into account.

## 2.4.9 Patterned secondary cell wall assembly in TEs occurs in a self-perpetuating cascade

The above data provide evidence that normal patterned wall assembly in TEs occurs in a self-perpetuating cascade in which some wall components are themselves able to mediate the localization of others. A self-perpetuating cascade, by definition, would be an energetically favorable mechanism, since cytoplasmic and/or membrane determinants of wall patterning would not have to be maintained after the first patterned component(s) were laid down. It would also account for precise spatial control of the final localization of diffusible molecules (Keller et al., 1989). Such a mechanism may be especially applicable to lignin polymerization, which occurs from diffusible precursors near the end of a terminal differentiation process when organization of the cytoplasm is declining (Lamb, 1981; Roberts et al., 1985; Wardrop, 1981). These results suggest that xylan and GRP, in addition to lignin (Suzuki et al.,1992; Taylor et al., 1992), cannot remain localized in secondary wall thickenings if cellulose synthesis is disturbed. This conclusion is reasonable based on the ability of xylan to hydrogen bond with cellulose. As stated by Reis and co-workers, "The presence of acidic xylan more or less tightly associated to microfibrils appears as a constant feature in various cellulosic helicoidal assemblies" (Reis et al., 1992 p 33). The secretion of xylan is analogous with the secretion of xyloglucan, which can also hydrogen bond with cellulose, into the medium of DCB-adapted suspension cells that lack substantial cellulose in their primary cell walls (Shedletzky et al., 1990). Because of the expected intimate and extensive association between cellulose and xylan, it is possible that the GRP, lignin, and other unidentified molecules that may also be missing from the thickenings in treated TEs are indirectly dependent on cellulose for their localization. That is, they may be directly associated with xylan or other molecules that interact with cellulose, not the cellulose itself. Definitive determination of where various molecules fit into the cascade must await more detailed determination of the molecular interactions in cell walls.

Even when partially effective DCB and isoxaben allow small amounts of cellulose, as indicated by faint birefringence and Tinopal LPW staining, to be deposited in the thickenings, the xylan and GRP still mainly diffuse away from the thickening sites. Only when indicators of cellulose become strong do these other components also associate normally with the thickenings. Therefore, particular ratios between cellulose and other molecules are probably necessary for normal assembly of the wall. This is supported by the effects of various concentrations of DCB on assembly of helicoidal cell walls in maize and mung beans, which led to the proposal that a threshold ratio between cellulose and hemicellulose had a role in determining wall structure (Satiat-Jeunemaitre, 1987). We have

also considered why patches of xylan and GRP are commonly observed in TEs that have had cellulose synthesis partially inhibited, but not in those where the inhibition is apparently complete (see Taylor et al., 1992). Perhaps a small amount of cellulose can provide a temporary anchoring site so that the molecules take longer to diffuse away. Patches of xylan often appear as blocks that bridge the thickenings sites, as if the molecules at the edge of the aggregate are bonded to the small amount of cellulose that is present. We have also noted that patches of xylan are more common and more coherent than those of GRP, which is perhaps related to the tendency of non-cellulosic polysaccacharides like xylan to self-associate (Jarvis, 1992). Experiments are planned to test directly whether larger amounts of xylan and GRP diffuse into the culture medium of treated TEs.

Recently a microtubule antagonist was applied to developing TEs in *Pisum* and *Commelina* roots, and it was shown that cellulose (as indicated by Calcofluor staining) and a non-cellulosic polysaccharide (putatively xylan, labeled with FITC-wheat germ agglutinin) were coordinately altered in distribution (Hogetsu, 1991). From these results, it was concluded that microtubules determined the localization of a non-cellulosic polysaccharide as well as cellulose. However, application of the same lectin (FITC-labeled from *Triticum vulgaris*) to DCB-treated *Zinnia elegans* TEs revealed patches between thickenings reminiscent of the results with the xylan probes (data not shown). Therefore, the results of Hogetsu can be reinterpreted to suggest that the xylan in TEs treated with microtubule antagonists merely follows the altered distribution of the newly synthesized cellulose, which is localized by a microtubule-mediated mechanism. This hypothesis is consistent with other evidence that microtubules have their primary role early in secondary wall deposition; once cellulose has begun to be deposited in the thickenings, they are no longer needed (Haigler and Koonce, 1992). The idea that the presence of localized cellulose establishes an essential framework for the localization of other secondary cell wall molecules is consistent with a "hierarchical self-assembly process" for development of wall structure outlined previously for primary walls (Satiat-Jeunemaitre, 1987).

# CHAPTER 3
# CHEMICAL ANALYSIS OF *ZINNIA* CELL WALLS

## 3.1 Overview

The chemical composition of plant cell walls can be determined through the incorporation of a radioactive label into the individual molecules (Fry, 1988). The radioactive precursor $^{14}$C-glucose is a good choice for labeling cells because it is converted into the central metabolite, glucose-6-phosphate, and incorporated into nearly all polysaccharides. This label is also reliable as it is only incorporated into living cell walls. Radio-labeling increases the sensitivity of identification methods 2-6 times, making it easier to locate and identify small amounts of polymers (Fry, 1988). Hydrolyzed, methylated, radiolabeled polymers can be analyzed by GC/MS to determine the possible linkages found between the monomers released. The amount of radiolabel detected gives an indication of the proportion of the individual sugars present, making it possible to suggest the presence of particular polysaccharides.

The following analysis of the monosaccharide composition in cell walls of DCB-inhibited and control induced and non-induced *Zinnia* cultures was performed in collaboration with Dr. Alan White, North Dakota State University, Fargo.

## 3.2 Materials and methods

### 3.2.1 Cell culture

Single cell suspension cultures were established as described in Chapter 2, except that the carbon source was changed to 10 mM glucose (Roberts et al., 1992) so that the specific activity of the $^{14}$C-glucose would be higher (Taylor et al., 1992).

### 3.2.2 Reagents

All chemicals and probes were purchased from Sigma Chemical Company except where other suppliers are indicated in the text.

### 3.2.3 Radiolabeling of cell cultures

Cell suspensions were cultured in 50 ml Erlenmeyer flasks (12 ml medium/flask) at 27°C in the dark on orbital shakers at 80 rpm. The radiolabeling of *Zinnia* cultures was performed as in Taylor et al. (1992) with the following alterations. At 42 h, 4-6 h before visible differentiation was observed, 7.5 μM of the cellulose synthesis inhibitor, DCB (Fluka Chem. Co.), was added to half of the cultures. During the period of early

differentiation (1-6% TEs in total cells as viewed by DIC light microscopy), [U-$^{14}$C] glucose (Dupont/NEN, Boston, MA) was added to the cultures (final specific activity, 9.6 x $10^3$ Bq/μmol). Control (IND) cultures showed 1-6% TEs by 56 h when the $^{14}$C-glucose incubation was initiated. The addition of 7.5 μM DCB at 42 h caused a delay of 4-6 h in the onset of visible differentiation of TEs. Therefore, DCB-treated cultures were not incubated in $^{14}$C-glucose until 63 h of culture. At the end of 4 h of labeling, the cells were collected by centrifugation (5 min x 800 G) in 50 ml plastic test tubes and kept at 4° C after the addition of 7 ml of 70% ethanol.

### 3.2.4 Cell wall isolation

Cell walls were isolated according to the methods in Fry (1988). Briefly, the cells in 70% ethanol were sonicated at the maximum setting using a microtip with cooling between each burst (4 x 1 min at 4° C). The broken cell walls were then washed 5 times in 70% ethanol at room temperature (RT) followed by one wash with 90% DMSO and resuspension in 10 ml of 90% DMSO. Starch was removed by stirring the DMSO sample overnight at RT (Selvendran et al., 1985), followed by 6 washes in 70% ethanol, 2 washes in 100% acetone and drying under vacuum. The dried cell wall sample obtained should contain very little contamination from intracellular proteins and RNA as well as other polymers. This method also removes few integrated components from the cell wall itself (Fry, 1988).

### 3.2.5 Cell wall analysis

#### 3.2.5.1 Methylation linkage analysis

The sample preparation and analysis was performed according to White et al. (1993). Briefly, the cell wall samples were methylated for glycosyl linkage analysis with n-butyllithium following the method outlined in Shea et al. (1989). The $P_2O_5$ dried reaction products were dissolved in 0.2-0.5 ml DMSO and n-butyllithium was added slowly, followed by methyl iodide. The methylated samples were extracted with chloroform, washed with water, and the chloroform phase evaporated to dryness, fully hydrolyzed with 2 M TFA at 121° C for 1 h and reduced with $NaBD_4$. The resultant methylated alditols were acetylated with acetic anhydride and 1-methylimidazole as a catalyst (Blakeney et al., 1983) forming partially methylated alditol acetates.

3.2.5.2 Radiogas proportional counting and GC/MS

The radiolabeled partially methylated alditol acetates were dissolved in 100 µl acetone and the radioactivity of 1 µl was measured by scintillation counting. An aliquot of sample was evaporated to dryness, redissolved in 5 µl acetone, injected splitless on a Varian 3700 GC with 30 m x 0.75 mm SP-2330 glass capillary; column (Supelco Inc., Bellefonte, PA) at 170° C. After a 3 min delay, column temperature was raised 4° C/min to 240° C. The GC column effluents were split (10:90) between a flame ionization detector (FID), to show the presence of organic compounds, and a gas proportional counter (GPC) to detect radioactivity. The radioactive peaks were identified by comparing peaks and retention times with injections of the same samples on a Hewlett-Packard GC/MS with a 30 m SP-2330 fused silica capillary column and a similar temperature program.

## 3.3 Results

### 3.3.1 Cell culture development

The application of 7 µM DCB to *Zinnia* cultures caused a 4-6 h delay in the onset of visible TE formation. After differentiation began, the percentage increase in visible TEs during the $^{14}C$-glucose incubation period in the DCB-treated cultures was similar to the percentage increase in visible TEs during the same incubation time span in IND cultures (Table 3.1). Differentiating IND cultures, but not DCB-treated cultures, could be monitored for TE development with polarization (POL) light microscopy (indicates the presence of cellulose), which detects the development of thickenings a few hours before they become visible by BF microscopy (Table 3.1). During the labeling periods, two of the three differentiating IND experiments were incubated at a slightly earlier period of differentiation. This was indicated by a lower percentage of increase of TEs in BF but a similar increase in TEs observed by POL. Two of the DCB-treated differentiating cultures also indicated a slightly lower increase of TEs during the labeling period when viewed by BF.

### 3.3.2 Cell wall analysis

The sugar-linkage composition of the *Zinnia* cell wall samples was determined for comparison between control and DCB-treated cultures. These cultures were induced (IND) to differentiate and form secondary walls or non-induced (NI) to observe primary wall changes only. Therefore, four cell wall types were analyzed as shown in Table 3.1 and Fig. 3.1: IND, IND DCB, NI, and NI DCB. The proportional incorporation of $^{14}C$-glucose into the different sugars within walls of metabolizing cells was monitored, giving

Table 3.1. Incorporation (%) of $^{14}C$-glucose into cell walls of Non-Differentiating and Differentiating Cultures of *Zinnia elegans* (Control and DCB-treated)
(GC-MS analysis by Dr. Alan White, North Dakota State University)

| | IND | | | | | IND DCB | | | | | NI* | | | | | NI DCB | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | (56-60h)** | | | | | (63-67h) | | | | | (58-62h) | | | | | (63-67h) | | | | |
| expt. number | 1 | 2 | 3 | | | 1 | 2 | 3 | | | 1 | 2 | 3 | | | 1 | 2 | 3 | | |
| POL | 6-17 | 3-16 | 3-15 | | | <1 | <1 | <1 | | | NA | | | | | NA | | | | |
| BF | 3-13 | 1-6 | 1-4 | | | 6-15 | 3-9 | 4-11 | | | NA | | | | | NA | | | | |
| Linkage | | | | X̄ | ± SE | | | | X̄ | ± SE | | | | X̄ | ± SE | | | | X̄ | ± SE |
| t-Araf | 10.3 | 10.4 | 10.3 | 10.33 | 0.05 | 10.4 | 8.0 | 10.5 | 9.63 | 1.16 | 9.9 | 11.9 | 9.3 | 10.37 | 1.11 | 10.2 | 3.4 | 3.4 | 5.67 | 3.21 |
| 5-Araf | 10.2 | 12.7 | 13.3 | 12.07 | 1.34 | 17.5 | 9.6 | 15.5 | 14.2 | 3.35 | 16.9 | 13.3 | 17.3 | 15.83 | 1.8 | 12.3 | 11.7 | 16.6 | 13.53 | 2.18 |
| 3,5-Araf | 6.7 | 11.6 | 13.0 | 10.43 | 2.70 | 9.3 | 8.6 | 13.0 | 10.30 | 1.93 | 12.6 | 16.3 | 11.9 | 13.6 | 1.9 | 15.4 | 13.9 | 14.4 | 14.5 | 0.6 |
| t-Xyl | 3.9 | 8.2 | 7.3 | 6.47 | 1.85 | 8.7 | 9.3 | 5.1 | 7.70 | 1.85 | 15.9 | 13.7 | 15.3 | 14.97 | 0.93 | 12.6 | 14.6 | 9.9 | 12.37 | 1.93 |
| 2-&4-Xyl | 22.8 | 24.2 | 23.7 | 23.57 | 0.58 | 14.1 | 14.2 | 15.1 | 14.47 | 0.45 | 11.9 | 8.6 | 5.0 | 8.50 | 2.82 | 7.0 | 8.8 | 4.7 | 6.83 | 1.7 |
| % 4-Xyl | 66.2 | 67.9 | 67.1 | | | 63.9 | 72.6 | 71.6 | | | 61.4 | 65.1 | 62.8 | | | 60.0 | 60.2 | 62.4 | | |
| t-Glc | | | | | | 7.6 | 8.2 | 4.6 | 6.80 | 1.57 | | | | | | | 8.9 | | | |
| 4-Glc | 35.6 | 22.3 | 18.4 | 25.43 | 7.36 | 22.1 | 17.3 | 19.9 | 19.77 | 1.96 | 23.3 | 22.1 | 26.3 | 23.90 | 1.77 | 27.5 | 18.2 | 19.0 | 21.57 | 4.21 |
| 4,6-Glc | 10.4 | 10.5 | 14.0 | 11.63 | 1.67 | 10.1 | 15.8 | 8.8 | 11.57 | 3.04 | 9.4 | 14.1 | 14.9 | 12.80 | 2.43 | 6.2 | 9.1 | 13.4 | 9.57 | 2.96 |
| 3,4-Glc | | | | | | | 8.9 | 7.5 | | | | | | | | | | | | |
| Unk 4.7 min. | | | | | | | | | | | | | | | | 8.9 | 11.4 | 11.2 | 10.50 | 1.13 |

*Non-differentiating cultures.

**Indicates time of labeling. DCB-treated cultures were often labeled later than controls because DCB causes a delay in onset of differentiation.

POL indicates the % change in TEs viewed by Polarization (indicator of celluose) during the labeling period.

BF indicates the % change in TEs viewed by bright field microscopy during the labeling period.

SE = standard error from 3 separate experiments done in triplicate.

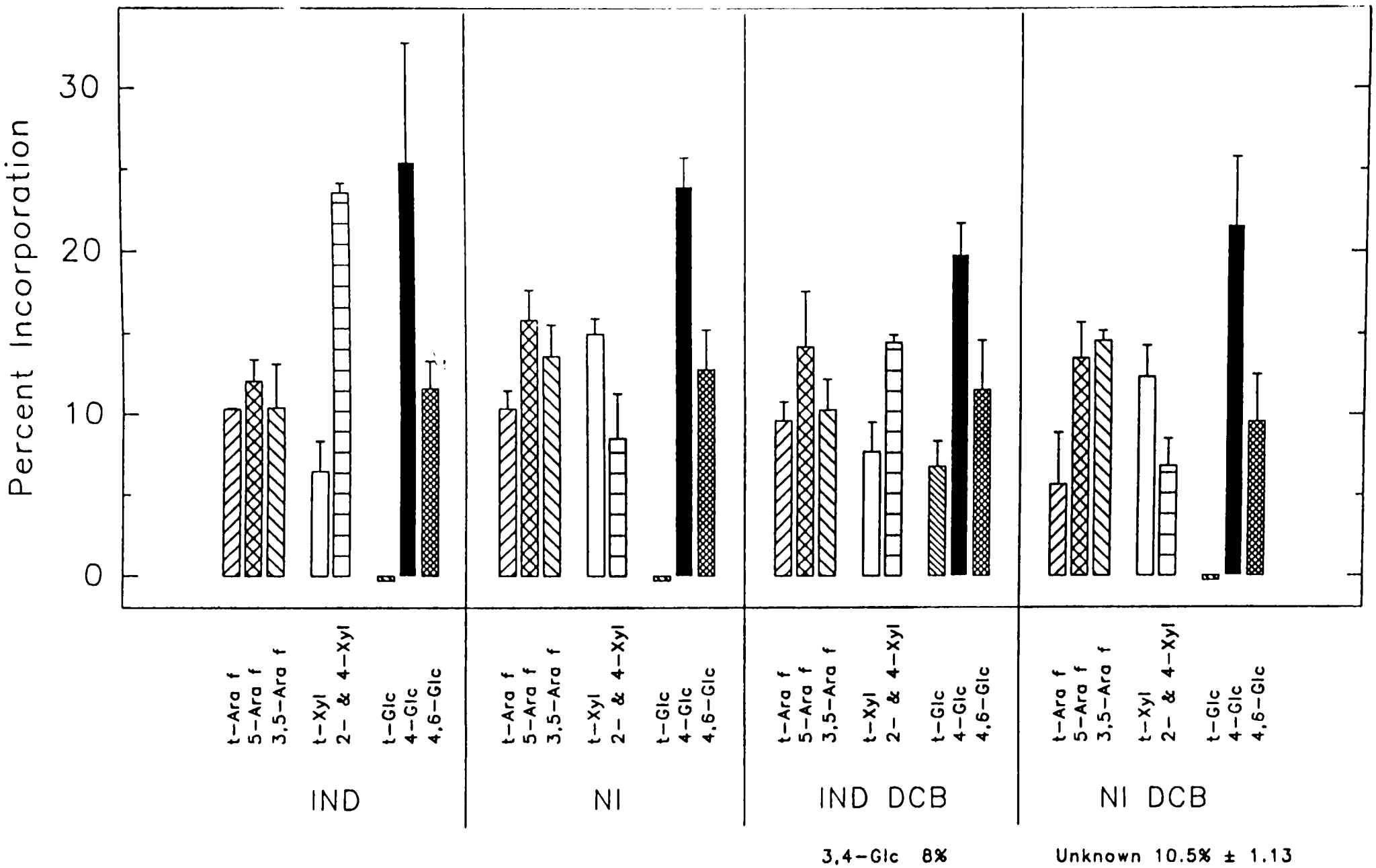



Fig. 3.1. Summary of mean linkage analysis.

an indication of what components may have been synthesized and added to the cell walls of the DCB-treated and control cells (see Fig. 3.1 for a graphical representation of the means in Table 3.1). The error bars in the graph represent standard deviations. The magnitude of these is probably increased by combining data from experiments in which cells were at somewhat different stages of TE development as evident in table 3.1.

The IND DCB cultures showed a decrease in 2- and 4- xylose (14.5%) compared to IND cultures (23.6%), with 1,4-xylose linkages (representative of xylan) making up 67% of the total (Fig. 3.1). The appearances of t-glucose (terminal glucose) and 3,4-glucose in the DCB-treated cell walls are the other obvious anomalies found when compared to IND cell walls. A reduction in 4-glucose, indicative of cellulose, occurred in 2 of 3 tests of the IND DCB cell walls. The difference does not appear statistaically meaningful in Fig. 3.1, but the data in table 3.1 indicates a difference which is explained later.

The NI DCB cell walls showed a proportional decrease in the pectin or xylan associated t-arabinose sugar (5.7%) as compared to NI cell walls (10.4%). This may be attributed to the appearance of an unknown component of significant amount (10.5%) in the NI DCB cells, influencing the comparative proportions (Table 3.1).

The cell walls of differentiating IND cultures compared to the cell walls of NI cultures revealed results similar to the IND DCB cell walls compared to NI DCB cell walls. The differentiating cultures showed an increase in the proportion of 2- and 4- xylose while the NI cultures showed an increase in the proportion of t-xylose (Table 3.1). These similarities between IND versus NI and IND DCB versus NI DCB indicate the differences seen in the IND DCB and NI DCB cultures are probably due to something other than the DCB treatment, probably the deposition of secondary walls in the IND treatments.

## 3.4 Discussion

### 3.4.1 Effects of DCB on cell culture development

In this study the *Zinnia* cultures were surveyed during the period of semi-synchronous differentiation. The DCB-treated cultures were delayed in starting visible differentiation, and the radio-label incubation time was adjusted appropriately. In a similar study of the effect of DCB on *Zinnia* cultures, Suzuki et al. (1992) analyzed the incorporation of $^{14}$C-glucose into the KOH insoluble cell wall fraction (including, but not necessarily exclusively cellulose). In their study, they did not mention if a visible delay in onset of differentiation took place in the DCB-treated cultures or not. Their experiment added DCB at the same time the radio-label was added. They claimed that DCB-treatment increased the incorporation of $^{14}$C-glucose into pectin and into the extra-cellular

polysaccharides, though their results indicate changes of only 0.1% and 0.2%, respectively, using two replicates. Their results are more in line with what we found in preliminary experiments involving the labeling of *Zinnia* cultures. We found that adding label at the same time as the DCB showed appreciable change only in the synthesis of cellulose and not in other sugars (results not shown). The reason for this is possibly due to the lack of time for a differentiating cell to adjust to the presence of DCB and enhance or establish other pathways of polysaccharide synthesis. This delay may also be an indication of how long it takes the cell destined to differentiate to adjust to the inhibition of cellulose synthesis caused by DCB. These TEs treated with DCB after the onset of differentiation label for cellulose, xylan and GRP (see results of Chapter 2) in a manner similar to IND TEs because they have already established secondary walls before the application of the inhibitor. Therefore, this study more effectively evaluates the effect DCB has on secondary cell wall assembly by studying differentiating cells during the period of altered cell wall deposition that may occur after a cell adjusts to cellulose synthesis inhibition.

The effect of 0.5-10.0 μM DCB on *Zinnia* cell cultures ultimately leads to all the cells dying by 4 days (results not shown; Suzuki et al., 1992). The TEs are programmed to die at maturity and the non-differentiating DCB-treated mesophyll cells usually show degradation of the cytoplasm followed by swelling and lysis (personal observation), the swelling presumably being a result of weakened cell walls (Umetsu et al., 1976). The percentage of dead cells in DCB-treated cultures slowly increases over time, with the normal 30-35% dead at the time of inhibitor application (42 h) to 50% dead at 60 h and ending with practically all dead cells by 96 h (results not shown).

### 3.4.2 Changes in cell walls of differentiating cultures

The cell walls of differentiating IND cultures showed expected changes when compared to the NI cultures. Crystalline cellulose (1,4-glucose) (see Chapter 2; Taylor et al., 1992) and xylan (1,4-xylose) increased in the differentiating cultures while the apparent synthesis of xyloglucan decreased (t-xylose), all in line with previous studies (Northcote, 1977; McNeil et al.,1984; Delmer, 1985). The differentiating cell walls of IND DCB cultures showed an increase in t-arabinose and 1,4-xylose in comparison to NI DCB cultures, which indicates the possible synthesis of a more water soluble arabinoxylan in IND DCB TEs. This difference may be of little significance due to the presence of an unknown component in the NI DCB cell walls. The unknown component is most likely a degradation product of some type and its early elution indicates it is not a sugar (A. White, personal communication). The percentage of the unknown component may influence the

results just enough to cause a lower representation of the percentages of other components present, giving what may appear to be a significant difference between IND DCB and NI DCB cell walls, which in reality may not be substantiated.

### 3.4.3 Effect of DCB on TE cell wall composition

The methylation analyses of the *Zinnia* cell walls gives some indication of minor qualitative changes in composition, but for the most part, only quantitative changes in the linkages of the sugars were detected between IND and IND DCB cultures. The similarity in the composition of the NI and NI DCB cell walls indicates that the differences observed in the linkage analysis of the IND and IND DCB cell walls is real and probably produced during secondary wall development. Qualitative differences are shown in the differentiating cultures by the appearance of t-glucose and possibly 3,4-glucose in DCB-treated cell walls.

The presence of t-glucose is not common in polysaccharides as it depicts a lone glucose attached terminally or as a side-branch. This linkage is also sometimes found in glycoproteins and phenolic compounds of plants (Fry, 1988). This linkage could be related to the delocalization of lignin and possibly other cell wall components in the IND DCB TEs (see Chapter 2). By maintaining a more generalized incorporation into the cell wall, molecules indicated by the presence of t-glucose may allow for linkages with the terminal glucose that may not occur in normal conditions. The possible presence of 3,4-glucose in the IND DCB TEs is uncertain. It can be a result from undermethylation, which is possible as only one methylation step was used in this analysis (Carpita and Shea, 1988; A. White, personal communication). When this linkage is real, it is usually produced from a polymer of callose (1,3-glucose) (White et al., 1993), which does not appear in the *Zinnia* cultures tested.

The quantitative changes found in comparing IND and IND DCB TE cell walls were (in 2 of 3 tests) decreased levels of linkages associated with cellulose (1,4-glucose) and (in all tests) reductions in xylan (1,4-xylose) synthesis in the IND DCB TEs. The decrease in xylan in the IND DCB TE cell walls was also shown by immunolabeling (see Chapter 2). This reduced incorporation of xylan into TEs may be a result of reduced synthesis or it may also be the result of the inability of the synthesized xylan to bind to cellulose or some other component in the cell wall, resulting in excretion into the medium as is the case of xyloglucan in the DCB-adapted cells of tomato and tobacco cell lines (Shedletzky et al., 1992). The possibility of xylan being lost into the medium in DCB-

treated cultures is currently being investigated, but conclusive results are not yet determined.

The decrease in 1,4-glucose linkages in the cell walls of IND DCB TEs is not as consistent as that shown for primary walls of the DCB-adapted cells in the study of Shedletzky et al. (1992) or for the incorporation of $^{14}$C-glucose into the acetic/nitric insoluble material (crystalline cellulose) in differentiating *Zinnia* cultures (this research) or in developing cotton fibers investigated by Montezinos and Delmer (1980). The proportion of 1,4-glucose linkages in DCB-treated TEs was relatively constant in each of the three experiments even when some variation in the number of visible TEs occurred (Table 3.1). The proportion of 1,4-glucose linkages in IND TEs was quite variable depending on the number of visible TEs developing during the time of incubation. This is seen by the first experiment (1) in which there were more DIC-detectable thickenings at the onset of radiolabeling and a subsequent higher percentage of radio-labeled 1,4-glucose incorportated. The other two IND experiments (2 and 3) would have probably shown a higher 1,4-glucose proportion if they were assayed only 1 or 2 h later than they were. This slight delay in starting the incubation time for IND experiments 1 and 2 would have probably led to a greater comparative decrease in the percentage of 1,4-glucose linkages in the IND DCB TEs, which would fall closer in line with experiment 1 (compare the first IND experiment (1) with the first IND DCB results).

There are other possible explanations for the presence of a significant proportion of 1,4-glucose linkages being found in the IND DCB TEs. One possibility is that the cellulose formed is of a non-crystalline nature. The cells analyzed by GC/MS were extracted only by 70% EtOH and 90% DMSO, in contrast to the acetic/nitric extraction used in other radiolabeling experiments. In protoplasts regenerating cell walls (Shea et al., 1989), it was shown that, of the 1,4-glucose linkages present, only a small percentage were resistant to acetic/nitric hydrolysis until late in cell wall development, indicating that most of the early cellulose was of low crystallinity. This possibility may be true for the relatively young IND DCB TEs of *Zinnia* used in these experiments, but the absence of cellulase labeling, Calcofluor staining and birefringence in polarized light (see Chapter 2), raises some reservations in accepting that as the only possibility. If cellulose were present despite these negative indications, it would have to be in a disorganized form with normal binding sites not present (e.g., through changed conformation or masking). Also, Shedletzky et al. (1990) proposed that in the altered primary walls of DCB-adapted cells, the synthesis of cellulose, and not just its crystallization into microfibrils is inhibited. Some of these 1,4-glucose linkages in IND cultures do result from xyloglucan, but the

similarity in xyloglucan synthesis between the IND and IND DCB TEs would make it insignificant. Another possibility related to xyloglucan synthesis is that the ß-1,4-glucan synthase that assembles the 1,4-glucose backbone for xyloglucan in the Golgi (Read and Delmer, 1991) may exocytose unbranched or unmodified 1,4 glucose fragments into the cell wall.

The presence of xyloglucan seems to be comparable in both IND and IND DCB TE cell walls as indicated by little variation in the proportion of linkages of t-xylose and 4,6-glucose. Xyloglucan is normally associated with primary cell wall assembly in dicots (Hayashi, 1989), and the lack of differences between the two treatments makes the primary wall the probable site for xyloglucan deposition and not the secondary wall. This supports the labeling of primary walls and the lack of labeling of thickenings of DCB-treated and IND TEs by an antibody to xyloglucan (see Chapter 2). Also, an increased proportion of pectin does not seem to occur in correlation with the increase found in DCB-adapted primary cell walls of tomato and tobacco cell lines (Shedletzky et al., 1993). These DCB-adapted cell lines showed no new linkages produced to compensate for the loss of cellulose in the cell wall, only that xyloglucan was lost to the medium and a pectin-like matrix became the major component of the adapted cell walls.

# CHAPTER 4
# SUMMARY AND CONCLUSIONS

This research demonstrated the following.

1. Cellulose synthesis was inhibited by DCB and isoxaben in differentiating tracheary elements of *Zinnia elegans*. This was shown by the lack of labeling of the thickenings by the Worthington cellulase enzyme conjugated to a fluorescent probe for fluorescence microscopy and conjugated to gold for TEM analysis. The fluorescent stain, Tinopal, also did not stain the thickenings of the DCB- and isoxaben-treated TEs. Birefringence of the secondary walls was absent by polarization microscopy and the incorporation of $^{14}C$-glucose into crystalline cellulose was reduced in the treated cells.

2. The presence of xylan was not detected in the thickenings of treated TEs by a xylanase-FITC conjugate or an antibody to xylose (specific for xylan) using fluorescence microscopy. However, xylan was found at times to form patches between the thickenings as if the xylan was diffusing away from the thickenings. At the TEM level, xylan was shown to be present, but at reduced levels, when using the antibody to xylose localized by a secondary antibody conjugated to gold. In some instances, the antibody was shown to label the primary wall around a thickening in a manner similar to the patchy labeling observed at the light level. This indicates that xylan is still produced by the TE but is not localized to its normal position in the thickenings, perhaps because of the absence of cellulose with which it normally associates.

3. Xyloglucan was not alternatively deposited into the treated TE thickenings; it could not be labeled by an antibody to xyloglucan at the light microscope or TEM levels. Xyloglucan was shown to be present in the primary walls of both TEs and non-differentiating cells of treated and control cultures. The lack of differences in the percentages of 4,6-glucose between the IND and IND DCB cell walls also indicates the synthesis of xyloglucan was not affected to any great extent.

4. GRP was reduced or absent in the thickenings of treated TEs as shown by the lack of labeling with an antibody to a bean GRP localized by FITC or gold. GRP localized by FITC was also shown to label in patches between the thickenings of some treated TEs in a manner similar to xylan. This indicates that GRP is diffusing away from its normal location in the thickening, perhaps because of the lack of cellulose or some other component to which it normally binds.

5. Lignin was not localized specifically to the secondary wall thickenings, but was dispersed into the primary wall as shown by phloroglucinol-HCL staining. This indicates

that the presence of cellulose or possibly some other absent component was needed to localize lignin to its normal position in the thickenings.

6. The DCB- and isoxaben-treated TEs deposited some abundant molecule(s) into the secondary wall thickenings that are not typical cellulose, xylan, xyloglucan or GRP. The identity of the major molecules in the thickenings of DCB-treated TEs is still unknown.

7. Carbohydrate linkage analysis of treated differentiating TEs compared to control TEs shows decreases in 1,4-glucose linkages and 1,4-xylose linkages, indicative of cellulose and xylan respectively. An increase in t-glucose, uncommon in normal plant cell walls, appears in treated TEs. This gives more support to the component(s) being deposited in the treated thickenings being altered in some way. The presence of 1,4-glucose linkages suggests that cellulose may be present, but not in the conformation detected by the accepted methods for cellulose identification.

8. These results suggest that normal patterned wall assembly in TEs occurs in a self-perpetuating cascade in which some molecules of the secondary cell wall mediate patterning of others.

The *Zinnia* culture system has proven itself an important tool in cell wall study. More work is needed to elucidate possible interactions of the cell wall components. Using an improved method of freeze-substitution of *Zinnia* cells can give more insight into the synthesis of the cell wall components by better preserving the ultrastructure and antigenicity of the cytoplasm and cell walls. Applying immunocytochemical techniques with antibodies to various cell wall polysaccharides and proteins can give much information on cell wall development. More work is also needed to analyze the cell wall domains in intact cell walls using Raman spectroscopy, $^{13}C$ NMR spectroscopy as well as other possible techniques to be developed in the future.

Future research will probably detail the role of the cytoskeleton and specialized plasma membrane domains, which would logically include the characterization of the types and real functions of cell wall proteins.

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# APPENDIX

Table A.1. Light microscopic analysis of the localization of various probes for cell wall molecules on the walls of *Zinnia* TEs

| Treatment | Inductive | DCB/ISOX |
|---|---|---|
| Phloroglucinol | 2° | 4 |
| Cellulase-FITC | 2° | - |
| Xylanase-A-FITC | 2° | - |
| anti-xylanase-A[a] | 2° | - |
| anti-xylan | 2° | - |
| Triticum, lectin to xylan | 2° | - |
| anti-XYG[b] | 1° | 1° |
| Ulex, lectin to XYG | 4 | 4 |
| Ricinus, lectin to XYG | - | - |
| Jim 4[d], anti-AGP | - | - |
| Jim 5[d], anti-pectin | 1° | 1° |
| Jim 7[d], anti-pectin | 1° | 1° |
| Mac 207[d], anti-AGP | 3 | 3 |
| anti-GRP[c] | 2° | - |
| anti-extensin[e] | - | - |
| PI 1*, 1:1000 dilution | 4 | NA |
| PI 2, 1:100 dilution | - | NA |
| PI 3, 1:200 dilution | 2° | NA |
| PI 4, 1:100 dilution | - | NA |

| | |
|---|---|
| - | No label or stain |
| 2° | Secondary wall thickenings only |
| 1° | Primary wall only |
| 3 | Apex of cells, not very definitive |
| 4 | Generalized over cell, 2° not enhanced |
| a | Provided courtesy of D. Kluepfel |
| b | Provided courtesy of M. Hahn |
| c | Provided courtesy of B. Keller and C. Lamb |
| d | Provided courtesy of K. Roberts |
| AGP | Arabinogalactan protein |
| GRP | Glycine-rich protein |
| XYG | Xyloglucan |
| PI | Pre-immune sera from different rabbits |
| * | The ability of some pre-immune sera to bind to TEs, even at high dilution, indicates need for caution in applying and interpreting results from polyclonal antisera |