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Studies on Submerged Cotton Fiber Growth: Induction and Characterization, Effects of Congo Red and Auxin

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Studies on Submerged Cotton Fiber Growth: Induction and Characterization, Effects of Congo Red and Auxin

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Induction of growth of submerged cotton (*Gossypium hirsutum* L.) fibers from cultured ovules has been investigated for the first time. Both exogenous plant hormone levels and the age of the ovules at induction play important roles in induction of submerged cotton fiber growth. The diameter of submerged fibers was about same as that of air-grown fibers but was smaller than that of fibers grown *in vivo*. Submerged fibers were shorter in the fiber length, stronger in the tensile strength, and they had thicker secondary cell walls and smaller crystallite sizes compared with air-grown fibers and fibers *in vivo*. Helical secondary cell wall thickenings were exclusively found in submerged fibers.

Congo Red is a natural dye that has a high affinity for the biopolymer cellulose. The addition of Congo Red to the culture medium had an influence only on submerged cotton fibers and not on air-grown cotton fibers. When Congo Red was applied in the early primary wall stage, fiber cell elongation was inhibited, but amyloplast production was induced. When Congo Red was applied in late primary wall or early secondary wall

stage (about 14-16 DPA), the effects were less severe, but a significant increase in birefringence of secondary cell walls was observed. In both conditions of treatment with Congo Red in the primary wall and the secondary wall stages, a "nodulation" occurred on the wall surface. Neither cellobiohydrolase CBH I or CBH II had affinity for the external wall materials, implying that there was no cellulose present or binding sites for CBH had been occupied by Congo Red. X-ray diffraction data showed that Congo Red decreased the crystallite size of cellulose in submerged cotton fibers.

The preliminary investigation with auxin (indole-3-acetic acid) depletion in the culture medium was to study whether or not amyloplasts were produced under this condition. No amyloplasts were observed in submerged fibers grown in the auxin-depleted medium, but cellulose microfibrils in the secondary cell wall were greatly disorganized. Possibly, indole-3-acetic acid might play an important role in regulating the arrays of microtubules, which, in turn, may help to organize the patterns of cellulose deposition.

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Chapter 1: Induction and characterization of submerged cotton fiber growth

Summary

Growth of submerged cotton (*Gossypium hirsutum* L.) fibers from cultured ovules has been investigated. The results indicate that exogenous plant hormone levels regulate the induction of submerged fiber growth. In addition, the age of the ovules at induction is important. Fiber cell diameter, wall thickness, and length of submerged fibers were measured and compared with air-grown fibers and fibers grown *in vivo* (i.e. fibers produced by cotton plants grown in the greenhouse). Various cell wall thickening patterns were observed among submerged fibers while only one predominant cell wall deposition pattern was produced and observed in air-grown fibers and in fibers produced *in vivo*. The diameter of submerged fibers was about the same as that of air-grown fibers but was about 22% less than that of fibers grown *in vivo*. It appears that the secondary cell wall thickenings are initiated earlier in submerged fibers. Cell wall thickness of submerged fibers at 41 DPA was 51% greater than that of fibers grown *in vivo*, whereas the cell wall thickness of air-grown fibers was 42% less than that of fibers produced *in vivo*. The cell length of submerged fibers was approximately half that of fibers grown *in vivo*, and the air-grown fiber length was about two-thirds of fibers grown *in vivo*. The age of ovules at induction appears to have effects on the outcome of the air-grown fiber cell length but does not seem to affect the length of submerged fiber cells. Crystallite sizes of cellulose in submerged fibers were smaller, while the tensile strength of submerged fibers was stronger compared with air-grown fibers and fibers *in vivo*. To produce submerged fiber growth, the optimal age of ovules at induction was found at 0 DPA. The optimal medium (with a GA_3 (gibberellic acid) concentration of 0.5 μM and an IAA (indole-3-acetic acid) range of 5-20 μM)- depends on the time of ovule induction (-2 DPA to +2 DPA). We conclude that defining the conditions leading to submerged cotton fiber growth offers investigators great potential to: (a) directly monitor growth and make precise, detailed measurements during fiber growth and development; (b)

produce cellulose and fibers *in vitro* more efficiently than earlier ovule culture methods, and; (c) better understand signal transduction and gene expression leading to the growth, development, and programmed cell death in the life history of the cotton fiber.

1.1. Introduction

Cotton, *Gossypium*, is a genus of the *Malvaceae* family. About 50 species have been recognized in this genus, but only four species are economically important for their fibers. Cotton can be grown in tropical and subtropical regions of the world. The primary cultivated cotton, *G. hirsutum* L. is a tetraploid (4n=52), but a majority of the wild species are diploid (Small and Wendel, 2000). The cotton plant, a dicotyledon, typically has a central stem and many branches. The flowers have five separate petals, and the stamens are united into a column surrounding the style. The ovary develops into a capsule which is a dry structure that splits open along four or five lines. Inside are the seeds (or ovules) with the fibers attached. Each fiber is actually a single-cell hair that grows from the epidermis of the seed coat; the cell wall becomes thickened by adding layers of cellulose.

Cotton fibers are unique plant trichomes (Glover, 2001). Unlike many branched leaf trichomes, they are linear cells. Upon maturation, these linear fibers often are twisted, thus they can be spun into yarns. These single-celled fibers can reach around 30 mm in length with a diameter of about 20-25 μm for upland cotton (*G. hirsutum*) (De Langhe, 1986; Feng and Brown 2000). The length and width of cotton fibers vary among species. Mature cotton fibers contain up to 94% cellulose by dry weight (Meinert and Delmer, 1977). Apart from the economic importance, the tremendous increase in cell size and the exclusive cellulose chemical composition of cotton fibers make this a very valuable system to study cell differentiation, cell elongation, and synthesis and deposition of cell wall material synthesis. Moreover, the quality of the fibers is important in the effectiveness of the manufacture of cotton textile products. Fiber length, strength, and fineness are the most important physical properties. Understanding cotton fiber development at the level of molecular biology and genetics will allow researchers to genetically modify cotton not only for improving cotton fiber quality and yield, but also for overcoming a number of field cultivation problems caused by insects, weeds, and low temperature (Morin et al., 2003; Scott et al., 2001; Kornyeyev et al., 2003). A number of

reviews discuss cotton growth, development, cytology, physiology, and cell wall extension (Basra and Malik, 1984; Ryser, 1985; Berlin, 1986; Seagull, 1990; 1993).

Investigation of fiber development *in vivo* is very difficult because the fibers are encapsulated with a thick ovary coat. Fibers grown *in vitro* overcome the above problems and facilitate not only the examination of fiber growth but also manipulations of growth conditions during fiber formation (Beasley and Ting, 1974; Triplett, 1999).

Development of cotton fiber

Stages

Fiber development can be divided into four overlapping stages: initiation, elongation, secondary wall deposition, and maturation (Jasdanwala et al., 1977; Naithani et al., 1982). Fiber initiation begins 1 d preanthesis up to 1 or 2 DPA (days postanthesis). Elongation follows initiation immediately, and continues up to about 20 DPA. Overlapping with the late elongation stage, secondary wall deposition begins at about 16 DPA, and lasts for about 25-40 d. At the time of fiber maturity, about 50-60 DPA, cotton bolls undergo dehiscence along the locular suture lines. Upon dehiscing, the seeds with attached fibers are exposed; and the fiber cells lose water, collapse and become twisted.

FIBER INITIATION

Cotton fibers originate from the epidermis of the cotton ovule. The outer epidermal layer of the developing cotton ovule consists of epidermal cells, guard cells with associated subsidiary cells, and cotton fibers (Lang, 1938). Epidermal cells of cotton ovules begin to differentiate into fibers approximately 3 d before anthesis (Graves and Stewart, 1988). This is based on the fact that the response of prefiber cells to the plant hormones (IAA and GA_3) is reminiscent of the response of plant-grown fiber cells on the day of anthesis (Stewart, 1975). Differentiating fiber cells can be detected at 16 h preanthesis by cell and nuclear enlargement, a reduction in the amount of electron-dense phenolic substance within the vacuoles, and an increase in cytoplasmic density (Ramsey

and Berlin, 1976). The morphological differentiation (i.e. initiation) of a fiber begins when an epidermal cell bulges and protrudes above the surface of the ovule epidermis. The majority of the lint fibers are initiated on the day of anthesis. Not all epidermal cells of cotton ovules develop into cotton fibers. The ratio of fiber initials to total epidermal cells is 1:3.7 at anthesis (Stewart, 1975). The anatropous cotton ovule is less than two millimeters in all three dimensions at anthesis. Fiber density is about 3,300 fibers per mm^2 (Stewart, 1975). Bowman et al. (2001) studied ovule fiber cell numbers in seven modern upland cottons at 2 DPA, and found the ovule fiber number ranges about 14,000-18,000 among varieties studied. Fiber initials first develop at the chalazal end of the ovules as well as at the crest of the funiculus (Lang, 1938; Stewart, 1975; Tiwari and Wilkin, 1995). Initiation gradually proceeds toward the micropyle. Five to ten days after anthesis, the second series of fiber initials gives rise to fuzz fibers or linters (Lang, 1938; Beasley 1979).

Differentiating fibers contain a denser nucleus, darker cytoplasm, and lighter vacuoles compared with non-differentiating epidermal cells (Ramsey and Berlin, 1976). Increased cytoplasmic density is due to electron-dense phenolic substances that are released from the vacuoles during fiber initiation (Ramsey and Berlin, 1976). It has been suggested that phenolic substances are involved in auxin metabolism and consequently might be involved in fiber initiation (Jasanwala et al., 1977). Such phenolic compounds could stimulate fiber initiation possibly by inhibiting IAA-oxidase, which leads to accumulation of IAA. Activity of *o*-diphenol oxidase showed an increase during fiber initiation, indicating a high level of *o*-diphenols and a low level of IAA in fibers during this phase (Naithani et al., 1981).

The nucleus and nucleolus are also enlarged in initiating fibers (Ramsey and Berlin, 1976; De Langhe, 1986; Van's Hof, 1999). The enlarged nucleus may be attributed to amplification of nuclear DNA (nDNA) (Van's Hof, 1999). There are more ribosomes and rough endoplasmic reticulum (RER) present in differentiating fiber cells

than in adjacent nondifferentiating epidermal cells, indicating a greater capacity for protein synthesis in the fiber cells (Ramsey and Berlin, 1976).

Fiber initiation is believed to be controlled genetically and regulated by plant hormones (Gialvalis and Seagull, 2001), but little is known about this process at the molecular or metabolic levels. Recent studies suggest that sucrose synthase (SuSy) may play an important role in fiber initiation (Ruan et al. 1997; 1998; 2003). In fiberless mutants, expression of SuSy mRNA and protein is undetectable in cotton ovule epidermal cells, and this correlates with a lack of fiber initiation. Transgenic cotton with sucrose synthase suppression constructs also display a fiberless phenotype. Since suppression of sucrose synthase gene expression reduces hexose levels in transgenic ovules, inhibition of fiber initiation may be caused by a decreased osmotic potential.

The cytoskeletal components are involved with fiber initiation. Seagull (1998) suggested that both microtubules and microfilaments are implicated in cotton fiber initiation. The ovule fiber number is significantly reduced with application of the microtubule disrupting agent, oryzalin. The ovule fiber number is increased with the microtubule stabilizing agent, taxol. Both the microfilament disrupting agent, cytochalasin B, and the stabilizing agent, 3-maleimidobenzoyl-*N*-hydroxy succinimide ester (MBS), induce a decrease in fiber number.

FIBER ELONGATION

Fiber elongation immediately follows initiation and is completed between 20-30 DPA, depending on the varieties. During fiber initiation, fiber cells expand isotropically, while during the elongation stage, fiber cells expand anisotropically in the longitudinal axis. The expansion is perpendicular to the ovule surface, and the cell diameter remains constant. Fibers elongate rapidly, about 1mm per day (e.g., about 40 μm/hr). The fiber elongation rate peaks at 6-12 DPA (Benedict et al., 1973; Schubert et al., 1973; Meinert and Delmer, 1977; Gokani and Thaker, 2000). The maximal elongation rate has been shown to range from 1 to 2.4 mm per day depending on varieties or species and

temperature (Benedict et al., 1973; Schubert et al., 1973; Gipson and Joham, 1969). Such a rapid expansion rate is typical in tip-growth cells such as pollen tubes, root hairs, and fungal hyphae (Geitmann and Emons, 2000).

How do fiber cells expand? One possibility is tip growth – where new wall materials are added specifically to the tip regions. Another possibility is diffuse growth – where new wall materials added along all regions of the cell. Most research on this subject supports the theory of diffuse growth (O'Kelly, 1953; Ryser, 1977; Seagull, 1990; Tiwari and Wilkins, 1995). It is hard to exclude the presence of tip growth, but there is no direct evidence to support tip growth in cotton fibers. In tip-growing cells, secretory vesicles derived from the Golgi body are concentrated in the tip region, a great number of mitochondria are distributed in the subapical region, and all the other organelles accumulate in the basal part (Steer, 1990). However, in cotton fibers, the organelles are distributed relatively evenly through the cytoplasm (Seagull, 1990; Tiwary and Wilkins, 1995). Highly packed secretory vesicles in the tip region indicate active tip synthesis because these vesicles fuse with the plasma membrane in the tip area to allow the cell to expand. The absence of this feature in fiber tips implies that tip-growth is not dominant in cotton fibers.

The morphology of the fiber tip cannot be used to predict the growth pattern (Seagull, 1990). Cotton fibers have tapered or hemispheric tips (Stewart, 1975; Seagull, 1990). Both shapes of tips are observed in younger as well as older fibers. There are no obvious indications that the tip morphology is correlated to the fiber length (Seagull, 1990). Ryser (1977) concluded that cotton fibers grow along their entire length but growth in the tip region is more intense. He fed cotton ovule culture with ^{3}H-gluocse and ^{3}H-sucrose. The result showed that radioactive label was distributed throughout the entire fiber, and the tip region was more heavily labeled, indicating both diffuse growth and tip growth occurred in the developing fibers. When the labeled ovules were transferred to non-radioactive medium, the labeling in the tip regions was diminished or disappeared, implying that tip growth is more dominant in cotton fibers than diffuse growth.

Turgor pressure drives fiber cell elongation. With a high cellular solute gradient, the cell tends to take up water from the environment, generating turgor pressure. In cotton fiber cells, the buildup of the turgor pressure is thought to be associated with accumulation of potassium and malate (Dhindsa, 1975; Basra and Malik, 1983), glucose, sucrose (Carpita and Delmer, 1981), and hexose (Ruan et al., 1997) in the vacuoles. Since plant cells are constricted by rigid cell walls, turgor pressure exerts a counterbalancing physical stress or tension in the walls. To balance the turgor-generated forces, plant cells expand by secreting cellulose and loosening the bonds between cell wall polymers. This wall loosening enables wall polymers to slip by each other, consequently increasing the wall surface area. Joshi et al. (1988) suggest that tonoplast-ATPase activity is involved in the fiber elongation or expansion process by regulating transportation of osmotically active substances (possibly potassium and malate) into the vacuoles. Transcripts of genes that regulate osmolarity, proton-translocating-ATPases and proton-translocating pyrophosphatase, reach the highest level during the climax of the fiber cell expansion (Lawrence et al., 1998). Ambient CO_2 concentration plays a role in regulating the turgor in the fiber since malate is one of products of dark CO_2 fixation (Dhindsa et al., 1975; Basra and Malik, 1983). Phosphoenolpyruvate carboxylase (PEP-Case) occurs in expanding cotton fibers. Also a higher PEP-Case concentration was found in the fibers of the long staple cultivar compared to the short staple cultivar (Basra and Malik, 1983).

Cotton fiber elongation is driven by turgor pressure, whereas the elongation rate is determined by extensibility of the cell wall (Cosgrove, 1986). Xyloglucans, cell wall components, have been thought to play a major role in wall expansion of higher plants (Taiz, 1984; Fry, 1988). Gokani and Thaker (2000) have shown that lower xyloglucan content is present in the longer staple cultivar compared to middle and short staple cultivar.

SECONDARY WALL DEPOSITION

Secondary wall deposition begins prior to the completion of fiber elongation (Benedict et al., 1973; Schubert et al., 1973; Meinert and Delmer, 1977; Naithani et al., 1981). There are several facts to support the overlap between fiber elongation and secondary cell wall synthesis: 1) ^{14}C-photosynthate incorporation increased rapidly in the developing fibers before the cessation of fiber elongation (Benedict et al., 1973; Schubert et al., 1973); 2) the rate of increase of fiber dry weight climaxed prior to the completion of fiber elongation (Benedict et al., 1973; Schubert et al., 1973); 3) the thickness of the wall began to increase between 12 to 16 DPA before the offset of fiber elongation (Meinert and Delmer, 1977); 4) the dry matter accumulation showed a lag phase relative to the maximum rate of fiber elongation that was achieved on the 10 DPA (Naithani et al., 1981).

Secondary wall deposition begins between 16-20 DPA and continues for 25-40 d. During this stage, an increase of ER-associated polysomes occurs (Westafer and Brown, 1976). The first layer of the secondary wall is called the S_1-layer. Since secondary wall deposition occurs before cessation of the elongation, the S_1-layer was thought to be extensible (Willison and Brown, 1977). Subsequent secondary wall layers deposit on the inner surface of the cell wall. The wall thickness can reach 8-10 μm when the cell reaches its programmed cell death.

FIBER MATURATION

Fiber maturation occurs about 50-60 DPA, when the bolls dehisce and the fibers lose water and dry out. Fibers become twisted upon dehydration. The twisting is possibly related to the reversals of the fiber (Waterkeyn, 1985; Ryser, 1985). Cellulose microfibril bundles arrange in a helicoidal manner (Waterkeyn, 1974). The reversals are the sites where the direction of the twist of cellulose microfibril helix changes. The feature of fiber twisting facilitates the spinning process (Basra, 1984). Fiber maturity has certain effects on the fiber physical properties, and itself is mainly influenced by the growing condition (Goynes et al., 1995).

Dynamic Changes of the Nucleus

During the dramatic increase of fiber cell volume during the elongation process, synthesis of DNA, lipid, proteins, and polysaccharides is required. A dynamic change of nucleolus size and DNA content has been recorded (Ramsey and Berlin, 1976; De Langhe, 1978; Waterkeyn, 1985) Small nucleoli fuse together during the day of anthesis, resulting in a spherical nucleolus. The nucleolus increases dramatically from 1 μm to 8 μm in diameter during the first 48 hrs coincident with the expansion and elongation of fiber initials (Waterkeyn, 1985). The nucleoli maintain this size for a few days, displaying a visible vacuolation and regular extrusions of nucleolar material. A sharp decrease occurs after reaching the maximum size of 8-10 um at about 8 DPA, followed by a gradual decrease until cell death (De Langhe 1978; Waterkeyn 1985). Besides a size change of nucleolus, the nuclear DNA content of cotton fiber increases about 24% after 2 DPA (Van't Hof, 1999).

Dynamic Changes of the Cell Wall Composition

There are two types of cotton fiber cell walls: primary cell walls and secondary cell walls. Synthesis of the primary cell wall is completed during the fiber elongation stage. The Golgi apparatus appears to be directly involved in secretion and synthesis of primary wall components (Westafer and Brown, 1976). Deposition of the secondary cell wall begins several days before the cessation of fiber elongation, and continues until fiber maturation. Based on ultrastructural analysis, the endoplasmic reticulum and plasma membrane may play an important role in the synthesis and secretion of secondary wall materials (Westafer and Brown, 1976). Thickness of the primary cell wall (0.2-0.4 μm) is relatively constant in cotton fibers. The composition of the cell wall of the cotton fiber changes throughout development (Meinert and Delmer 1977; Huwyler et al., 1979; Timpa and Triplett, 1993). During the elongation or primary wall synthesis stage, the cell wall consists of xyloglucan, cellulose, callose, pectin and proteins. The cell wall is primarily composed of cellulose, callose and proteins during the period of secondary wall deposition and maturation (Timpa and Triplett, 1993).

Xyloglucan is one type of hemicellulose. The typical structure of xyloglucan is a main chain with β-1,4-D-linked glucopyranosyl residues with side chains containing xylosyl-, galactosylxylosyl-, and fucosylgalactosyl-xylosyl units (Buchala and Meier, 1985). As discussed earlier, xyloglucans may determine wall extensibility because they are involved in cross-linking the cellulose microfibrils in the wall matrix. Deposition of xyloglucans is more likely restricted to the fiber elongation stage (Hayashi and Delmer, 1988) and has been found exclusively in the inner layer of the primary wall (Vaughn and Turley, 1999). Xyloglucan content is inversely correlated with fiber elongation (Gokani and Thaker, 2000). Huwyler et al. (1979) has shown that hemicellulose components increase greatly in absolute weight during primary wall formation and decrease during the course of the secondary wall formation. The neutral sugars, including rhamnose, fucose, arabinose, xylose, mannose, galactose and glucose (noncellulosic), compose 25 % (by weight at 10 DPA) of the fiber cell wall during the primary wall stage, 10% at 20 DPA, and 4 % (at 29 DPA) of the secondary wall (Meinert and Delmer, 1977).

Cellulose is composed of β-1,4-D-glucan chains. The high crystallinity and degree of polymerization of cellulose make it an excellent framework in the cell wall architecture. During the fiber elongation phase, the proportion of cellulose in the fiber wall is relatively low, less than 30% (Meinert and Delmer, 1977). The degree of polymerization (DP) is the term to describe the length or molecular weight of microfibrils. In the primary wall of the cotton fiber, the DP of cellulose is about 2000-4000, while it reaches about 14000 in secondary cell walls (Marx-Figini, 1982). The percentage of cellulose in the cell wall of cotton fibers continuously increases during secondary cell wall synthesis stage (Meinert and Delmer, 1977; Huwyler et al., 1979), and it can reach up to 94% of fiber dry weight at fiber maturation.

Callose, β-(1,3)-D-glucans, plays a potential role in the synthesis of cellulose (Meier et al., 1981; Francy et al., 1989). Deposition of callose occurs specifically in the innermost wall layer bordering the cell lumen (Waterkeyn, 1981), and within the same exoplasmic zone as sucrose synthase (Salnikov et al., 2003). The level of callose is low

during primary wall synthesis and rises rapidly at about the time of onset of secondary wall synthesis, then slowly decreases until the end of fiber development (Meinert and Delmer, 1977; Huwyler et al., 1979; Maltby et al., 1979; Rowland, 1984). The percentage of 1,3-glucan (in total cellulose plus 1,3-glucan) ranges from 10.5% at 20 DPA downward to 1.2% at 48 DPA (Rowland, 1984). Meinert and Delmer (1977) indicate that the 1,3-glucan (in total cellulose plus non-cellulosic glucose) ranged from about 9% (20 DPA) to 3% (29 DPA). Maltby et al. (1979) showed a peak of approximate 7% 1,3-glucan between 16-20 DPA, the value decreasing to less than 1% at cessation of growth.

Pectins are polysaccharides rich in D-galacturonic acid, and the side chains are made of D-galacturonic acid and chemically similar polysaccharides (Fry, 1988). Pectin content (in uronic acid) peaks at approximately 22% (at 10 DPA) of the primary cell wall, and a sharp decrease (2% at 20 DPA) in the uronic acid level occurs at onset of secondary cell wall formation (Meinert and Delmer, 1977). De-esterified pectins were found exclusively in the ensheathing layer of the fiber primary wall, suggesting their potential role in fiber expansion and elongation (Vaughn and Turley, 1999). However, Gokani and Thaker (2000) found that there was no obvious correlation of pectin content with fiber growth and development.

The absolute protein content in the cotton fiber wall increases during the elongation period, however, at the onset of secondary cell wall deposition, it declines sharply (Meinert and Delmer, 1977; Huwyler, 1979). The cell wall proteins in the cotton fiber peak (12.3%) between 6-10 DPA, decrease to 4.3% between 21-25 DPA, and are at 1% at the cessation of growth (Huwyler et al., 1979). Generally, extensins are the typical proteins present in the primary cell wall of plants, specifically in the outer layer of the fiber cell wall (Vaughn and Turley, 1999). They are (relative to acidic) glycoproteins, extremely rich in hydroxyproline (Fry, 1988). Meinert and Delmer (1977) suggested that extensins might not be dominant proteins because of their low percentage in the cotton fiber cell wall. The α-expansins are cell wall proteins that facilitate cell wall extension by cleaving non-covalent bonds between wall polymers. Two genes coding α-expansins

have been identified, and they are specifically expressed in developing fibers (Harmer et al., 2002).

Lipid or wax also is present in the cell wall of cotton fibers (Ryser, 1985). There are two types of lipids found in cotton fiber cell walls: cutin and suberin. Cutin is found in the cuticle of fiber cell walls, whereas suberin is found in the secondary cell wall of the green lint fibers (Ryser, 1983). Mature white cotton fibers contain 0.4-0.7% of wax (in dry weight) in the form of cutin, while 14-17% of lipid composition is present in green lint fibers (Ryser, 1985).

Cellulose Deposition and Organization

Willison and Brown (1977) suggested that a globular enzyme complex bound to the outside of the plasmalemma of developing cotton fibers may be responsible for the assembly of cellulose microfibrils. Normally adjacent microfibrils tend to form bundles via hydrogen bonding between the glucan chains. The increase of elongation of the microfibril bundles has been thought to be achieved by tip growth. New glucose residues are added to microfibril ends (De Langhe, 1986). Cellulose microfibrils in the primary wall of cotton fibers tend to orient at about right angles to the elongation axis. The orientation of cellulose microfibrils in the cotton fiber secondary cell wall is highly ordered.

The first layer of the secondary wall, S_1 layer, contains microfibrils orientet at an angle of 55^0 with respect to the fiber axis. Orientations of microfibrils in subsequent secondary wall layers display smaller angles to the elongation axis as the layer is more newly formed or closer to the lumen (Figure 1.1, after Waterkeyn, 1974). Two types of reversal structures have been identified (Waterkeyn, 1974; 1985): the crossed and the bent ones. Reversals of the "crossed" type resulted from opposite orientations of the microfibrils belonging to S_1 and S_2 layers. The direction of the microfibril helix changes within the same wall sublayer, giving rise to "bent" reversals.

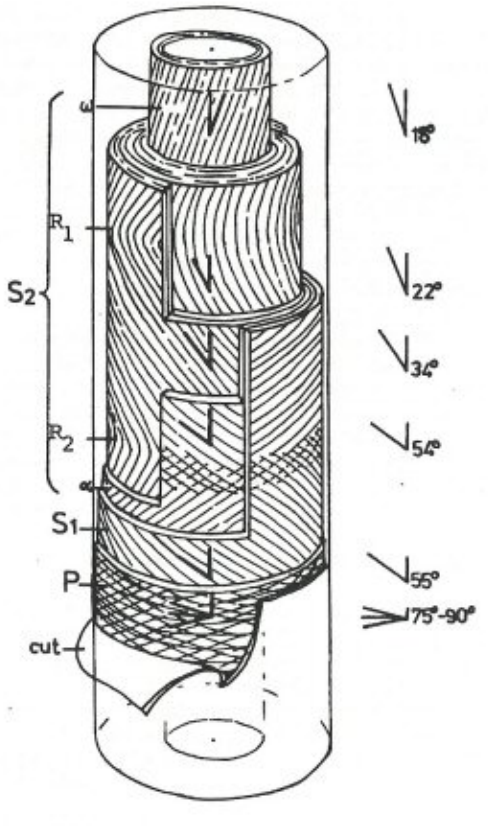


Figure 1.1. General diagram of a mature lint fiber. The primary wall has a net transverse-oriented structure (angle of 75^0-90^0). The helical angle of the S_1 layer is always similar to that of $S_{2\alpha}$ layer, but in the opposite direction. All sublayers of $S_{2\alpha}$ to $S_{2\omega}$ have the same spirality (S or Z) and all change this direction at a reversal zone. The helical angle of the subsequent sublayers decreases to $S_{2\omega}$.

Cut=cuticle
P=primary wall
S_1=S_1 layer
S_2=secondary wall, layers $S_{2\alpha}$ to $S_{2\omega}$
R_1=reversal with bended fibrils.
R_2=reversal with fibrils "crossing" or meeting on opposed fibril angle.

The alignment of microfibrils by microtubules has been proposed and is generally agreed upon for a wide group of cell types (Baskin, 2001). In cotton fibers, the parallelism between microtubules and nascent wall microfibrils also has been observed (Seagull, 1986; 1989). This contiguity even persists through the reversals (Yatsu and Jacks, 1981). Several experiments with microtubule sensitive agents have supported the alignment of wall microfibrils by microtubules (Yatsu, 1983; Seagull, 1989; 1990). When colchicine, a microtubule-disrupting agent, was applied to cotton locules (Yatsu, 1983) or ovules grown *in vitro* (Seagull, 1989), the microfibrils became disorganized and the local deposition appeared as specks when viewed with polarized light or Tinopal staining. In the absence of microtubules, the wall microfibrils display a swirled pattern through the cell with Tinopal staining or a "patchy" birefringence pattern in polarized light (Seagull

1989). The above observations support the hypothesis that microtubules control microfibril orientation cell-wide, although arrays of microfibrils depositing locally are parallel to one another.

In fiber development, the dynamic changes of microfibril arrays mimic, to a certain extent, those of microtubule patterns (Seagull, 1992). During fiber initiation and early elongation, the orientation of microtubules generally is not organized, and primary wall microfibrils deposit randomly. As fiber development proceeds, microtubule arrays undergo modification. There are three critical stages identified by Seagull (1992): 1) during the transition from initiation to elongation, microtubule arrays become organized and develop a shallow pitched helical pattern; 2) during the transition between primary and secondary wall synthesis, microtubule arrays shift from shallow pitch helices to steeply pitched ones; 3) in early secondary wall synthesis, the number of microtubules increases four times. Kloth (1989) indicated that the rapid increase in tubulin occurred simultaneously with the elongation of the fiber and an increase in cellulose synthesis. The transcripts of α-tubulin genes increase during the elongation stage and drop at the onset of secondary cell wall synthesis (Whittaker and Triplett, 1999).

It is not clear how microtubules guide the orientation of microfibrils. Microtubules and microfibrils are spatially separated, so interaction between microtubules and microfibrils must be indirect. The orientation of microtubules may be regulated by microfilaments during fiber development (Seagull 1990). The cotton kinesin-like calmodulin-binding protein (a motor protein, which traffics microtubules) may play a role in fiber cell growth by interacting with cortical microtubules (Preuss et al., 2003). In a recent review, a model is proposed that microtubules might serve as scaffolds that bind to plasma membrane proteins to orient nascent microfibrils (Baskin 2001).

Cotton fiber growth via *in vitro* ovule culture

Historical background

The initial purpose of the cotton ovule culture method was to rescue embryos produced by interspecific and intergenetic hybridization (Mauney, 1961). Self-incompatibility in some plant groups hinders the survival of hybrid embryos to maturity *in situ*. Culturing fertilized ovules *in vitro* can circumvent this natural barrier to fertility (Stewart, 1981). In the early 1970s, Beasley and co-workers conducted an extensive study on the nutritional and hormonal requirements for cotton ovule growth, primarily aiming at fiber development (Beasley et al., 1974; Beasley, 1977a). Their modified media for supporting ovule growth had several features: 1) a high salt basal medium such as Murashige-Skoog (Murashige and Skoog, 1962) rather than a low salt formulation such as White's (White, 1957); and, 2) inclusion of KNO_3 as the nitrogen source instead of NH_4NO_3; 3) a liquid medium preferred to a solidified agar medium. They also found the optimal combinations of plant hormones for culturing fertilized ovules (Beasley and Ting, 1973) and unfertilized ovules (Beasley and Ting, 1974). For the first time, they successfully grew "test tube cotton" (Beasley et al., 1971).

Ovule culture

Ovules can be obtained from plants grown in the field or greenhouse. The age of fertilized ovaries can be determined by tagging the flowers on the day of anthesis. For the ovaries before anthesis, Graves and Stewart (1988) described how to estimate the age of ovules. In spite of the age of ovules, preanthesis or postanthesis, the culture procedures are the same. Detailed procedures of culture methods, including ovary surface sterilization, ovule excision, and culture conditions are discussed in several papers (Beasley et al., 1974b; Beasley, 1977a; 1984). In addition to the media conditions, one critical factor for a supporting medium is the combination of exogenous plant hormones. Gibberellic acids (GA_3) and auxins (IAA or NAA) are the exogenous plant hormones that promote fiber growth *in vitro* (Beasley and Ting, 1973; 1974). At present, the most-referenced culture media are published by Beasley and Ting (1973) (see Appendix 1). To estimate cotton fiber growth, Beasley et al. (1974a) also established a quantitative

method. The total fiber unit (TFU) is measured by using Toluidine blue O staining, destaining and spectrophotographic analysis.

Hormonal effects

Besides Beasley and coworkers, quite a few other research groups have studied the effects of five major classes of plant hormones on cotton fiber development *in vitro*. Generally, IAA and GA_3 promote fiber growth (Beasley and Ting, 1973; 1974). ABA and ethylene have an inhibitory influence in fiber development (Beasley and Ting, 1973; 1974; Beasley and Eaks, 1979; Hsu and Stewart, 1976; Nayyar et al., 1989; Davidonis 1992), The role of kinetin in fiber production is indiscernible (Beasley and Ting, 1973; 1974; Momtaz, 1998).

In fertilized ovules, it seems that the levels of gibberellins are deficient, but production of cytokinins is sufficient, and the level of IAA is near optimal (Beasley and Ting 1973, 1974; Dhindsa et al., 1976; Momtaz, 1998). Therefore exogenous GA_3 enhances fiber growth greatly. IAA and/or GA_3 are required for unfertilized ovules to produce fibers (Beasley and Ting 1974; Graves and Stewart, 1988; Momtaz, 1998), but the youngest preanthesis ovules that could respond to these hormones should not be younger than 3d preanthesis (Graves and Stewart, 1988). Fiber growth on unfertilized ovules needs the continuous presence of IAA (Beasley et al., 1974b). In different stages of cotton fiber development, each type of plant hormone plays a different regulatory role. The action of one type of plant hormones is not independent from another. They always play regulatory roles by interacting with one another.

Both GA_3 and IAA promote fiber initiation of preanthesis and postanthesis ovules (Gialvalis and Seagull, 2001). A greater increase in fiber initiation of unfertilized ovules was found with IAA treatment than with GA_3 treatment, while for fertilized ovules, GA_3 had more capacity to increase the fiber initiation than IAA (Giavalis and Seagull, 2001). Stimulation of fiber growth by gibberellic acid occurred 48 h after anthesis (Delanghe, 1986).

Gibberellic acids are required for fiber growth in the days following anthesis rather than on the day of anthesis (Kosmidou-Dimitropoulou, 1986). Dhindsa (1978)

concluded that GA_3 mainly promotes ovule growth while IAA is responsible for fiber growth. This suggests that fiber growth may also rely on ovule growth. GA_3 stimulates the synthesis of nucleolar material and inhibits nucleolar vacuolation, while auxin is involved in the induction of new nuleolar material and stimulation of ribosome production (De Langhe et al., 1978).

Dhindsa et al. (1976) found that inhibition of ABA (abscisic acid) is effective only when employed during the first four days of culture started on the day of anthesis, indicating cotton fiber growth is sensitive to ABA during the initiation and early elongation stage. Based on the fact that ABA causes a decrease in malate content of fibers and ovules to produce callus, they suggested that ABA might inhibit fiber growth by interfering with malate metabolism. ABA and GA_3 are known to be antagonistic (Dhindsa, 1978). ABA might interfere with pathways of IAA and GA_3 biosynthesis because inhibition of ABA biosynthesis by fluridone, an inhibitor of ABA biosynthesis, resulted in an increase in fiber growth as well as IAA and GA_3 levels of cultured cotton ovules (Nayyar et al., 1989). Chen et al. (1997) suggested that ABA might not be the key inhibitor for fiber elongation since the dynamic patterns of endogenous ABA levels are similar in normal cotton and fiber differentiation mutants. In developing cotton seeds, the ABA level is negligible during the early stage, but increases at 15 DPA and peaks at 33 DPA (Gokani et al., 1998). Therefore, ABA may have a role in fiber maturation.

For the unfertilized ovules, kinetin may have additive positive regulatory effects on fiber growth along with GA_3 and/or IAA (Momtaz, 1998). The level of endogenous cytokinins drops after flowering in normal cotton while it increases in the fiber differentiation mutants, indicating cytokinins may stimulate fiber initiation and inhibit fiber growth after fertilization (Chen et al., 1997).

Stewart and Hsu (1976; 1977) applied (2-chloroethyl) phosphonic acid (CEPA) as a substitute of ethylene to cotton ovule culture. They found that CEPA caused callus formation from the micropylar end of the ovule, retardation of ovule growth and no fiber growth. Induction of callus growth by CEPA can be suppressed by a certain level of ABA or IAA, but stimulated greatly by GA_3 (Stewart and Hsu, 1977). Beasley and Eaks (1979)

introduced ethylene into cotton ovule culture from an alcohol lamp and a natural gas burner. The results were consistent with those of Stewart and Hsu (1976; 1977) except for reduced fiber growth that occurred in response to IAA. Davidonis (1992) also showed that fiber elongation was inhibited when CEPA was applied during the early elongation stage; inhibition of fiber elongation was least with simultaneous additions of GA_3 and IAA.

Nutritional effects

As discussed earlier, fiber growth *in vitro* is favored by a high salt basal medium and KNO_3 as the nitrogen source instead of NH_4NO_3. Beasley (1977b) showed that ammonium in the growth medium along with the normally present nitrate caused a greater percentage of ovules to produce fibers in response to IAA. Ammonium is an early product of the nitrate assimilation pathway. Availability of reduced nitrate is possibly promoted by addition of GA_3 to culture medium or high culture temperature because the former two factors can substitute for the ammonium supply for fiber development from cultured ovules (Beasley et al., 1979). Boron is also required for fiber elongation (Birnbaum et al., 1974). In the presence of both IAA and GA_3, boron deficiency resulted in callus formation from most areas of the epidermis, callus browning, and restriction of fiber to a small area of the upper ovular surface. Birnbaum et al. (1974) suggested that boron nutrition is related to IAA metabolism.

Cellulose synthesis

Cotton development in *in vitro* cotton ovule systems has been used for the study of cellulose synthesis in the cotton fiber. Fiber development is similar in field-grown ovules and in ovules grown *in vitro* (Meinert and Delmer, 1977). The molecular weight distribution of polymers in the 21-d culture fiber is similar to 30 DPA *in vivo* fibers (Triplett and Timpa, 1995). Response of cotton fibers developing *in vitro* to cyclic temperature change resembles those of field grown plants under diurnal temperature fluctuation (Haigler et al., 1991). When the low point was 22 or 15 ^{0}C, growth rings were evident in fibers grown *in vitro*, while they were hardly detected when the low point was 28 ^{0}C. The elongation period was prolonged as the low point in the cycle decreased.

Compared with constant 34 ^{0}C, 34/22 ^{0}C and 34/15 ^{0}C caused delayed onset of fiber elongation and secondary wall deposition (Haigler et al., 1991).

Fiber initiation and early elongation are sensitive to temperature: cycling cool temperature (34/15 ^{0}C) caused about a 1d delay in fiber initiation and about a 2d delay in early elongation compared with 34 ^{0}C constant; more time was required to reach the final length for developing cotton fibers under cycling cool temperature in comparison with the 34 ^{0}C constant condition (Xie et al., 1993).

When C^{14} glucose is fed to the culture medium of cotton ovules, both cellulose and callose are synthesized. Incorporation of radioactivity into cellulose is about twice that into callose of the fibers during the secondary wall stage (Francey et al., 1989). Carpita and Delmer (1980) used detached fibers grown *in vitro* to study synthesis of cellulose from ^{14}C glucose. They (1981) studied turnover of UDP-glucose in developing cotton fibers, and the results supported the idea that UDP-glucose was a precursor of secondary wall cellulose in the cotton fiber.

The herbicide 2,6-dichlorobenzonitrile (DCB) inhibits cellulose synthesis in cotton fibers (Delmer et al., 1987; Francey et al., 1989), but has a little effect on callose synthesis (Francey et al., 1989). The DCB receptor was identified. It may act as a regulatory protein for β-glucan synthesis in plants (Delmer et al., 1987). Ultrastructural analysis shows that DCB inhibits cellulose synthesis and increases the amount of callose (Vaughn and Turley, 2001). The herbicides isoxaben or flupoxam cause initiated cells to divide rather than elongate based on the evidence of formation of cell plates composed of pectin rather than cellulose or callose (Vaughn and Turley, 2001). Addition of gelling agents to the culture medium did not affect the fiber growth measured as TFU (Total Fiber Unit), but cause a decrease in cellulose content in developing cotton fibers (Triplett and Johnson, 1999). Immunolocalization of sucrose synthase and callose revealed a possible dual role of sucrose synthase in cellulose and callose synthesis in secondary-wall-stage cotton fibers (Salnikov et al., 2003).

Cytoplasts

Gould and coworkers developed a system to isolate and culture protoplasts from cotton prefiber cells (Gould et al., 1986a) as well as cytoplasts (or subprotoplasts) from cotton fiber cells (Gould et al., 1986b). This system has been primarily used to monitor cell wall generation and cellulose synthesis. Developing cotton fibers increase in volume and length dramatically, so it is difficult to obtain intact and nucleated protoplasts from those fibers. Cytoplasts could be isolated from cotton fibers grown *in vitro* when the culture was transferred from culture medium to cell wall digestion medium containing osmoticum. When the cell wall was weakened, cytoplasts, anucleate membrane bound vesicles derived from plasma membrane by vesiculation were released. These cytoplasts contain vacuoles, cytoplasm, cell organelles, and rarely nuclei. Normally their viability is a week or more.

Wall regeneration, synthesis of wall materials and starch by cytoplasts in culture were detected (Gould and Dugger, 1984; Gould et al., 1986b). Arrays of microtubules and microfilaments were shown in cotton fiber cytoplasts (Andersland et al., 1998). Actin and tubulin were abundant protein in extracted cotton fiber cytoplasts (Andersland et al., 1998).

Inhibitory Agents

Tunicamycin, an inhibitor of lipid-linked glycosyl transfer, inhibited fiber elongation and secondary wall synthesis when applied at the fiber elongation stage. Monensin, an inhibitor of Golgi function, suppressed secondary wall deposition but not fiber elongation (Davidonis, 1993). Addition of a specific inhibitor (EPTC) of the ER-associated fatty acid elongases to the culture media at the beginning of ovule culture resulted in complete inhibition of ovule growth; while altered suberin lamellae deposition occurred when the inhibitor was added at the end of fiber elongation (Schmutz et al., 1996).

Suspension cultures

A suspension culture was derived from cells of cotton ovule callus (Trolinder et al., 1987). When intact or sliced ovules were submerged in the culture medium,

formation of callus cells from the funicular attachment site and the cut site was induced. Under optimal culture condition (or proper plant hormone combination), these callus cells underwent elongation to form fiberlike cells (Trolinder et al., 1987). About 90% of the cells were shorter than 2 mm although the maximum length observed was 11 mm (Trolinder et al., 1987). Most of the cell walls ranged from 0.3 to 0.9 μm in diameter by 30 d in culture, indicating the primary wall stage or a low level of secondary wall deposition as birefringence was observed (Trolinder et al., 1987).

Triplett et al. (1989) studied the suspension culture of a fiber development mutant, "Ligon-lintless", and its near isogenic wild type. Both suspension culture cells had average diameters of 0.3 mm. They concluded that length of elongated cells from suspension culture seemed to be affected by the genotype of the explant tissue. To date, the suspension fiber cell culture has not revealed fibers similar in morphology to ovule-grown fibers.

The effects of plant hormones on fiber growth via suspension culture were similar to ovule culture (Davidonis, 1990; 1992). Gibberellic acid (GA_3) promoted cell elongation in suspension culture (Davidonis, 1990). Auxins and cytokinins alone produced no significant enhancement of cell elongation (Davidonis, 1990). Elongation of suspension cells was inhibited and lateral cell expansion was promoted by CEPA+GA_3. The cell width reached up to almost 90 μm in media containing CEPA+ GA_3, compared to 46 μm in the culture medium containing only GA_3.

Cell division

Cotton plants grown *in vivo* produce single-celled fibers. However, under culture conditions, in the absence of exogenous plant hormones, cotton fibers can undergo cell division, producing multicelled fibers (Van't Hof and Saha, 1997; 1998). They had the same morphology as the single cellular fibers. The number of cells in multicellular fibers ranged from two to nine (Van't Hof and Saha, 1997). Most cell division occurred in ovules cultured at 2-3 DPA. When cultured at 34 ^{0}C, about 25% of fiber cells underwent mitosis. Addition of IAA and GA_3 suppressed mitotic frequency of cultured fibers. The more cells in the multicellular fiber, the older and longer the multicellular fiber was

(Van't Hof and Saha, 1998). The diffuse growth mechanism was shown in the multicelled fibers (Van't Hof and Saha, 1998).

Submerged cotton fiber growth

Both the aerial portion and the submerged portion of cultured cotton ovules have the potential to grow fibers. Beasley et al. (1971) had indicated that the fiber growth was limited to the aerial portion of the cotton ovule. The surface of ovules submerged in the liquid medium tended to form callus (Huang and Xu, 1996; Beasley and Ting, 1973). In several related publications, submerged fibers have been mentioned (Maltby et al., 1979; Carpita and Delmer 1980) or shown in pictures (Birnbaum et al., 1974). To my knowledge, there was no study on submerged cotton fiber growth until our report (Feng and Brown, 2000).

In my research, I have discovered that the submerged growth of normal fibers from ovules is a general phenomenon among several varieties of cotton (including the cotton cultivar, Texas Marker-1, Coker 312, DP-50, and S-501). Furthermore, I have found that plant hormone levels and the age of ovules at induction appear to regulate and control the growth of submerged fibers. At this time, I cannot rule out other factors that might be involved in submerged ovule fiber growth. Here, details are presented for achieving this novel growth and results are included for the properties of submerged fibers. In addition, I have compared submerged-grown fibers with air-grown fibers and fibers produced *in vivo* on the plant.

1.2. Materials and Methods

Cotton ovule culture *in vitro*

Cotton plants of Texas Marker-1, Coker 312, DP-50, and S-501 kindly provided by Dr. A. E. Percival (USDA, ARS, SPA) were grown in the greenhouse under 14 h light per day and day/night alternating temperatures of 30°/22°C. Cotton ovaries were collected at –2 DPA (i.e. two days before anthesis), -1 DPA, 0 DPA (i.e. day of anthesis), 1 DPA and 2 DPA, soaked in 70% ethanol for 5 to 7 min, rinsed with sterile distilled water 3 to 5 times and dissected with flame-sterilized forceps. The ovules were aseptically transferred to the ovule culture liquid medium in a Petri dish (100×25mm) and cultured at 28°C in the dark. Each Petri dish contained 50 ml of culture medium. The ovules (30-40) from an individual boll were cultured in the same Petri dish. The culture medium was composed of MS-basal salts (Sigma, M-5524, see Appendix 2) (Murashige and Skoog, 1962), 15 μM IAA, 0.5 μM GA_3, MS-vitamin mixture (Sigma M3900, see Appendix 3), 80 mM glucose, and 20 mM sucrose. The basal salts, glucose and sucrose were dissolved in distilled water. The pH value was adjusted to 4.0 using 0.5 M KOH before autoclaving. Stock solutions (stored in the freezer) of 5 μM IAA and 1 μM GA_3 were prepared (both completely dissolved in a minimum amount of 100% ethanol first before adding distilled water to make the desired concentration) and sterilized with a Millipore filter. Stock solution (stored in refrigerator) of the MS-vitamin mixture (1000x) was filter-sterilized. Just before transferring cotton ovules into the medium, the three stock solutions above were added to the culture medium to make the final concentration.

Study on hormone combinations and the age of ovules at induction

Five hormone combinations (0.5 μM GA_3 + 1, 5, 10, or 20 μM IAA, and 2.5 μM GA_3 + 10 μM IAA) and 5 ages of ovules at induction (-2, -1, 0, 1, 2 DPA) were used to induce submerged fibers. For each treatment, 5 bolls were cultured as described above. The percentage of submerged fibers, determined by the ratio of the number of ovules producing submerged fibers to the total number of ovules in culture at 28 DPA, was used to quantify the submerged fiber growth.

Measurement of fiber length

Ovules derived from cultures induced at 0 DPA, 1 DPA and 2 DPA were taken from the medium after 40 days of culture and rinsed with distilled water. Submerged fibers and air-grown fibers were excised from the chalazal part of the ovule, and placed into glycerol, about 80 μl, on the top of a slide. Submerged fibers were separated from each other right after soaking in glycerol; however, for air-grown fibers, it took 10 to 15 min for the glycerol to wet the fiber surface. Single fibers were drawn from the glycerol with a pair of glass needles or forceps and aligned on the slide under a dissecting microscope. A plastic ruler was placed underneath the slide to measure the length of the fiber (in mm). Fibers of 41 DPA from cotton plants grown in the greenhouse were measured in the same way as above. For each treatment, 6 ovules from 3 bolls (2 ovules from each boll) were selected randomly. Twenty fibers from the chalazal region of each ovule were measured, and five longest measurements were chosen. Thirty measurements from six ovules were made, and the data was statistically analyzed using the T test (Steel et al., 1997).

Light microscopy and image processing

Ovules were taken from the medium at various culture stages and rinsed with distilled water. Submerged fibers and air-grown fibers were excised from ovules with forceps and placed on a slide in several drops of water. The aggregated fibers were separated from each other by flushing with water from a pipette or directly using forceps (wet mounted fibers were not flattened during this process). The fibers were mounted on slides and observed by light microscopy using polarization optics. Images from an Optronics video camera (Goleta, CA) were digitized and recorded onto the hard drive of a computer using a Matrox frame grabber (Matrox Electronics System, Ltd, Dorral, Canada).

Submerged fibers and air-grown fibers from the same ovule grown for 26 and 41 DPA were observed and studied with light microscopy. For each type of fiber, 50 photomicrographs were randomly taken. *In vivo* fibers at 28 DPA and 41 DPA were treated in the same way. The dimensions, including cell wall thickness and cell diameter

of fiber cells (not the tip region) were measured using Image-Pro Plus software (Media Cybernetics, Silver Spring, MD) with calibrated standards to correct for the conversion factors between image size and real size at different magnifications. For each fiber, three measurements of outer cell diameter and four measurements of cell wall thickness (two for each side) were taken and recorded in an Excel file. The data were analyzed statistically by using the T test (Steel et al., 1997).

SEM studies on submerged cotton fibers

Cotton ovules were excised from the ovaries, and immediately put into the center of a concave glass dish that was placed in a glass Petri dish containing a few drops of 2% OsO_4. After a couple of minutes, the ovule surface turned black because of the reduced osmium vapor reacting with the tissues, then the ovules were transferred into the fixative containing 2% glutaraldehyde and 1% OsO_4 buffered with 0.1 M cacodylate buffer (pH 7.2) for 2h at room temperature, then washed with 0.1 M cacodylate buffer (pH 7.2) for 3x10min. After washing in distilled water for 3x10min, the samples were dehydrated in a 30%, 50%, 70%, and 100% ethanol series before critical point drying (Samdri-790, Tousimis Research Corp.). Dried samples were sputter coated with gold (30800, Ladd Research Industries, Inc.), and then viewed under a scanning electron microscope (Hitachi S-4500 field emission scanning electron microscope).

X-ray diffraction

Mature cotton fibers (older than 50 DPA) including fibers grown *in vivo*, air-grown fibers, and submerged fibers, were purified according to the methods previously described (Imai et al., 1999). Fibers were excised from the ovules, extracted in 1% NaOH for 2d at 70 ^{0}C, washed and neutralized with distilled water, then extracted in 0.1% sodium chlorite in acetate buffer (pH 4.9) at 70 ^{0}C. After washing in distilled water, the samples were further extracted in 2.5 N HCl for 2d at 70 ^{0}C, followed with washing in distilled water and centrifugation. Purified and neutralized samples were air-dried. A digital pattern was obtained with a Philips PW 1720 X-ray generator and a vertical goniometer camera. The digital data were processed with Jade software.

Single fiber strength test

Submerged fibers from 9 individual ovules, air-grown fibers from 6 individual ovules, and naturally matured fibers were used for fiber measurement. The cultured fibers were older than 60 DPA. Single fiber strengths of 50 fibers for each seed were measured on a Mantis single fiber instrument (courtesy, Dr. Alfred D. French, SRRC, ARS, USDA). The average of the single fiber strength for each type of fiber was calculated.

1.3. Results

Submerged fibers in various media and from various ages of ovules were induced (Figure 1.2). Ovules floating on the liquid medium also produced air-grown fibers as well as submerged fibers (Figure 1.4A). It was noted that ovules would die if they sank to the bottom of the liquid medium. The younger ovules used for initiation (-2 DPA and –1 DPA) generally had the propensity to sink in the culture medium. To avoid this problem, the ovules must be transferred gently onto the surface of the liquid medium in the Petri dish. If an ovule sank during transfer, it could be recovered by gently pulling it to the surface of the medium with a pair of sterile forceps. The chalazal region of the ovule was observed to produce a greater quantity of longer submerged fibers (Figure 1.4A).

Induction of growth of submerged fibers

In order to induce cotton fiber growth from the submerged portion of ovules, over 15 media containing various combinations of IAA and GA_3 were tested, as well as ovules of various ages at induction, including -2, -1, 0, 1, and 2 DPA. We found that 9 of the media were able to induce submerged fiber growth. Those media were: (a) 0.1 μM GA_3 + 10 μM IAA; (b,c,d,e,f) 0.5 μM GA_3 + 1 or 5 or 10 or 15 or 20 μM IAA; (g, h) 1 μM GA_3 + 5 or 10 μM IAA; and, (i) 2.5 μM GA_3 + 10 μM IAA. In testing the optimal combinations of IAA and GA_3 on submerged fiber growth and determining the best medium to induce submerged fiber growth, 5 of the above media were analyzed, including the following: (a) 0.5 μM GA_3 + 1 μM IAA; (b) 0.5 μM GA_3 +5 μM IAA; (c) 0.5 μM GA_3 + 10 μM IAA; (d) 0.5 μM GA_3 + 20 μM IAA; and, (e) 2.5 μM GA_3 + 10 μM IAA (Figures 1.1 and 1.2).

The ovules at 0 DPA at induction appeared to have more potential to produce submerged fibers in response to all of the above 5 culture conditions than the other 4 ages of ovules (Figure 1.2). The next best age to produce submerged fibers was from ovules induced at -1 DPA. Submerged fiber growth was poorly induced from 2 DPA ovules. Specifically, the optimal hormonal combinations for inducing -2 DPA ovules to grow

submerged fibers were 0.5 μM GA_3 +5 μM IAA. On the other hand, for –1 DPA cultures, 0.5 μM GA_3 +5 or 10 μM IAA is optimal. For 0 DPA cultures, 0.5 μM GA_3 +5, or 10 or 20 μM IAA is optimal. For 1 DPA cultures, either 0.5 or 2.5 μM GA_3 + 10 μM IAA is optimal. For 2 DPA cultures, 2.5 μM GA_3 +5 or 10 μM IAA is optimal (Figure 1.2).

In general, various hormonal combinations had different effects on induction of submerged fiber growth, whereas less variation was observed in the percentage of ovules producing aerial fibers (Figure 1.3). When 0.5 μM GA_3 was used with a low level of IAA (1μM), only a small percentage of submerged fibers developed. On the other hand, with an increase of the IAA level to 5-10μM, the percentage of ovules producing submerged fibers greatly increased (Figure 1.3). Maintaining the GA_3 level (0.5 μM) and continually increasing IAA to 20 μM IAA failed to increase the induction rate of submerged fiber growth (Figure 1.3). When the GA level was raised from 0.5 μM to 2.5 μM while maintaining the IAA at 10 μM, no increase in the induction rate of submerged fiber growth was observed (Figure1.2).

Coiling of fibers

Coiling of the fibers grown *in vivo* is a natural process, and this was observed as early as 3 DPA. For ovules cultured in the liquid medium, coiling of air-grown fibers lagged 1-3 days behind the *in vivo* grown fibers. This depended on the age of ovules being cultured. For example, ovules initiating culture at 2 DPA started to produce coiled fibers after 2 days of culture (Figure 1.4C). It was interesting that the submerged fibers were much less coiled than air-grown fibers. They appeared "non-luminous" and quite loose in comparison with air-grown fibers that were very shiny aggregates of bundles (Figure 1.4 A and B).

The cell wall thickenings of submerged fibers

1. Secondary Wall Patterns

There were several abnormal secondary cell wall thickening patterns observed in submerged fibers (not limited to the chalazal region). These were observed neither in air-grown fibers nor in fibers produced *in vivo*. Fibers that have abnormal walls account for up to 35% of submerged fibers. A typical or normal pattern of helical secondary cell wall thickening is shown in (Figure 1.4G). Abnormal fiber wall patterns from submerged fibers are shown in Figures 1.4D, J and K. Fiber cells with such a pattern have a major secondary cell wall thickening band that appears discontinuous (Figure 1.4D and J), and shallow in other cells (Figure 1.4K). This discontinuous cell wall thickening pattern occurred quite often in submerged fiber cells. Roughly 10-30% of the submerged cells possessed such a feature. This pattern was also found in air-grown fibers but only rarely (less than 1% of the fibers had discontinuous secondary wall thickenings). No discontinuous helical cell secondary wall thickening were observed in fibers grown *in vivo*. Most helical secondary cell wall thickenings appeared about 25-40 DPA. Out of 54 fibers with helical thickenings that were investigated, 32 (59.3%) displayed left-handedness, 10 (18.5%) were right-handed, and 12 (22.2%) contained both left-handed and right-handed helices. The average interval of one complete turn of helix was 78.8 μm.

Another abnormal cell wall thickening pattern was found in which the helical thickenings were almost longitudinal, and bundles of secondary thickenings appeared to be interwoven (Figure 1.4L and M). This pattern occurred at a frequency of less than 5%. Reversals were also observed in submerged fibers (Figure 1.4L). The normal cell wall thickening pattern from *in vivo* grown fibers (Figure 1.4F and I) and air-grown ovule cultured fibers (Figure 1.4E, H and O) was quite smooth and even.

2. Cell wall thickness-developmental aspects

Secondary cell wall thickenings in submerged fibers usually are initiated at 12-16 DPA. At 26 DPA, cell walls of submerged fibers are as thick as 2.27 μm, while the wall dimensions of air-grown fibers and *in vivo* grown fibers are less than 1 μm (Table 1.1). At 41 DPA, the cell wall thickness of submerged fibers reaches 4.81 μm, which is 51 %

greater than that of fibers *in vivo* and about 2.6 times of that of air-grown fibers (Table 1.2). The cell wall thickness of air-grown fibers at 41 DPA was 1.83 μm, which was even less than that of submerged fibers at 26 DPA.

Length of submerged fibers

Submerged fibers from ovules initiated at 1 DPA were as long as 18.1±2.99 mm at 41 DPA, which was 51 % of that (35.8±2.43 mm) of fibers grown *in vivo* at the same age. On the other hand, air-grown fibers from the same ovule were 23.9±2.90 mm in length, which was about two thirds of that of fibers *in vivo* (Table 1.2). Statistical analysis (*t*-test) indicated that submerged fibers are significantly shorter (at the 99% confidence level) than both air-grown fibers and fibers grown *in vivo*.

The age of ovules upon initiation of culture, such as 0 DPA, 1 DPA and 2 DPA, did not affect the length of submerged fibers (at 38 DPA) but air-grown fibers (at 38 DPA) showed a significant difference with respect to ovule age (Table 1.3). The older ovules used to initiate cultures produced longer air-grown fibers. The length of submerged fibers was about 90% of the 0 DPA air-grown fibers, 72% of the 1 DPA air-grown fibers and 60% of the 2 DPA air-grown fibers, whose length was about 73% of the cell length of fibers (at 38 DPA) in *vivo* (Table 1.3). Therefore, submerged fibers are the shortest, air-grown next, and in vivo the longest at 38 DPA.

Diameter of submerged fibers

The diameter of both submerged fibers and air-grown fibers was very similar at 41 DPA (Table 1.2). At 26 DPA, the diameter of submerged fibers was slightly less than that of air-grown fibers (Table 1.1). The diameter of fibers produced *in vitro*, either as submerged fibers or as air-grown fibers was at least 20% less than fibers grown *in vivo*. The diameter of submerged fibers at 41 DPA is 20.55±2.21 μm. The standard deviation of the average diameter of submerged fibers is ±2.21 μm, while that of fibers grown *in vivo* is 2.66±μm. Thus, among different submerged fibers, the diameter is very uniform. Spiral thickenings are not correlated with the diameter of the fibers.

SEM examination of submerged fibers

In SEM specimens, the submerged cotton fibers were not distinguishable from air-grown fibers unless the ovules had been cultured for at least 2d when the culture started at 0 DPA (Figure 1.5A-B), but it appears that initiation of air-grown fibers is more uniform than that of submerged fibers because more air-grown fibers than submerged fibers are initiated simultaneously. It appeared that submerged fibers are not of uniform length, but are longer toward the liquid-air interface The difference between submerged fibers and air-grown fibers was very distinct when the culture period was 4 d or more (Figure 1.5 C-F). The air-grown fibers formed bundles that coiled (Figure 1.5 C-E), whereas submerged fibers did not form a aggregated fiber pattern. (Figure 1.5 C-D, F). The air-grown fibers were longer than submerged fibers, indicating a greater elongation rate in the air-grown fibers. It appeared more "barren" (no fiber growth) area in the submerged portion than in the air-grown portion (Figure 1.5 E-F). Although fiber initiation has occurred in these "barren areas", fibers appeared to rest in this state and not proceed to the elongation stage (Figure 1.5 E-F). Hence, under the submerged growth condition, the fiber elongation appeared to be retarded compared with the aerial growth condition.

Cellulose crystallinity of submerged fibers

Table 1.4 shows the result of the X-ray diffraction analysis of submerged fibers, air-grown fibers and fibers *in vivo*. Three major reflection peaks (101, 10-1, 002) are typical for cellulose I. Peak parameters were obtained from three types of cotton fibers. There was no significant difference among three types of cotton fibers with regard to *d*-spacing at each of the reflection peaks. Crystallite size was determined by using the fitting analysis of Jade software. Cellulose of the submerged fibers had a smaller crystallite size than that of air-grown fibers and fibers grown *in vivo*. Air-grown fibers showed a slight decrease in the crystallite size in comparison with fibers grown *in vivo* (Table 1.4). A great decrease in intensity at the reflection peak 101 was found in both submerged fibers and air-grown fibers compared with fibers grown *in vivo*.

Single fiber strength tests

The result of the single fiber strength test is shown in Table 1.5. The single fiber strength of submerged cotton fibers was 19% greater than the control that was grown *in planta*. Air-grown cotton fibers had about 77% of the tensile strength of the controls (e.g., fibers grown on the plant).

1.4. Discussion

Since the classical work of Beasley and Ting (1973), many variations of ovule culture have been reported; however, to my knowledge, this study is the first demonstrating the induced growth of submerged fibers in ovule culture. Furthermore, this is not a specialized phenomenon for a single strain and probably is representative of most cotton species since similar results were achieved with at least four strains (see Materials and Methods).

There is evidence that gibberellic acids and auxins are important for fiber differentiation (Beasley and Ting, 1973; 1974; De Langhe et al., 1978). Fiber primordial cells are first detected at about 16 hours preanthesis by the accumulation of phenolic substances and higher cytoplasmic density attributed to a higher number of ribosomes (Ramsey and Berlin, 1976a). Numerous ovule epidermal cells are differentiated into fiber initials on the morning of anthesis or later (Kosmidou, 1986). It has been suggested phenolic substances are related to auxin metabolism and consequently may be involved in the initiation of cotton fiber (Jasanwala et al., 1977). Such phenolic compounds could stimulate fiber initiation possibly by inhibiting IAA-oxidase, which would lead to the accumulation of IAA (Jasanwala et al., 1977). An increase of o-diphenol oxidase activity occurred during fiber initiation (Naithani et al., 1981). It has been suggested that auxin oxidation may play an important role in fiber initiation (Naithani et al., 1981). Exogenous GA_3 and/or auxin are required for unfertilized ovules cultured *in vitro* to produce fibers (Kosmidou, 1986). Gibberellic acids and auxin separately or in combinations could significantly affect the nucleolar development and the corresponding fiber length (Kosmidou, 1986). Auxins stimulate the synthesis of nucleolar materials and production of ribosomes, while GA_3 is involved in the synthesis of nucleolar material and inhibition of nucleolar vaculation (De Langhe et al., 1978). It has shown that auxins and GA_3 act synergistically in vitro on the fiber capacity for primary wall synthesis and elongation during the early elongation stages (Beasley and Ting, 1973, 1974, Kosmidou, 1986). It has been suggested that isolated cotton ovules at 2 DPA are deficient in the production of

optimum levels of gibberellic acids, and near optimum in the production of IAA (Beasley and Ting, 1973; 1974; Beasley et al., 1974; Dhindsa et al., 1976). Our study also implies that both GA_3 and IAA have roles in induction of submerged fiber growth changes from various ages (-2, -1, 0, 1, and 2 DPA) of ovules.

Although the results indicate that the plant hormone levels and the age of ovules used in starting the cultures have a major and repeatable impact on the production of submerged fiber growth, the possibility cannot be excluded that other factors may be involved. For example, the pH of the medium may be an important factor. We have used pH 4.0 (before autoclaving) rather than 5.0 (before autoclaving) as indicated in Beasley and Ting's method (1971; 1974), which has been referred by others (Trolinder et al, 1987; Haigler et al, 1991; Seagull, 1992; Davidonis, 1994; Triplett and Timpa, 1995; Hof and Saha, 1998). In another relevant study, a rather high pH 6 was used (Triplett, 1998). It is possible that high pH inhibits the submerged fiber growth. The pH value of the culture medium after autoclaving was tested because it may have been different from that before autoclaving (Table 1.6). The pH value of the media jumps from 4.0 before autoclaving to 4.5 after autoclaving, and from 5.0 before autoclaving to about 5.3 after autoclaving (Table 1.6). In this study, the pH value of the culture media was about 4.5 after autoclaving because it was adjusted to about 4.0 before autoclaving. However, the induction rate of the submerged fiber growth in the medium (IAA 10μM+GA_3 0.5μM, 0DPA at induction) at pH 5.5 (after autoclaving) was 80%, which is close to the induction rate (88%) at pH 4.5 (after autoclaving). Therefore, pH value of the culture medium may not play a role in inducing submerged fiber growth.

The well-known Beasley and Ting's medium (1973) contains 5 μM IAA and 0.5 μM GA_3. The same combination used in this study induced submerged fiber growth (Figures 1.1 and 1.2). Why, for about three decades, had no one studied submerged fibers until this study? There are at least two reasons. First, most of research groups used ovules of 2 DPA to initiate culture (Dhindsa et al., 1976; Beasley et al., 1979; Kloth, 1989; Triplett and Timpa, 1995; Triplett, 1998; Triplett and Johnson, 1999). The induction rate of submerged fiber growth at this age is very low (about 15% in this study). Therefore it

(submerged fiber growth) may not have caught the attention of the researchers. Secondly, although two research groups have mentioned "submerged fibers" (Maltby et al., 1979; Carpita and Delmer, 1980), and one research group even showed them in pictures (Birnbaum et al., 1974), they may not have considered there would be differences between submerged fibers and air-grown fibers.

The fact that 0 DPA ovules have more potential to produce submerged fibers than other ages of ovules, suggests that submerged epidermal cells of ovules at 0 DPA are more adaptable to the water stress and acid conditions (the culture medium is at pH 4.5) than both the younger and older ovules. Fiber initials from ovules at 0 DPA are more fully developed than those from ovules at –1 and –2 DPA (Kosmidou, 1986). The younger the unfertilized ovules are, the poorer the response to IAA and GA_3 to produce fibers (Kosmidou, 1986; Graves and Stewart, 1988). Fibers from ovules at 1 or 2 DPA have been in the elongation stage, unlike those from 0 DPA ovules that are in the initiation stage. Therefore fibers that have started elongating before being transferred into the culture medium may have been more sensitive to the liquid medium or less able to adapt to the liquid medium in comparison with fibers in initiation state.

This study has demonstrated that cultured cotton ovules produce air-grown fibers as well as submerged fibers at the same time and from the same ovule. The two types of fibers are quite different in terms of fiber length and secondary cell wall deposition (Table 1.2, Figures 1.3D-E). However it is difficult to distinguish isolated submerged fibers from isolated air-grown fibers visually. The standard method (Gipson and Joham, 1969) to measure fiber length is not convenient for submerged fiber culture. In that method, the locules from a boll are placed in a beaker of boiling water for 2 to 3 min (for bolls 20 days old or older, 2.5N HCl is used) to dissolve simple sugar and allow the seed with attached fiber to float free. The seed is then floated out on the convex side of a watchglass. The fiber is straightened with a stream of water. The fiber length (cm) is measured with a ruler. If this method is used, there is a need to cut the submerged portion from the air-grown portion. Also the accuracy of the measurement with this method is low, because it is impossible to determine the base and the tip for each individual fiber.

As an alternative, glycerol was used to loosen and straighten the fiber and avoid damage and breakage. The average length from measurements of the representative individual fibers was obtained with this alternative method.

The SEM study showed that submerged fibers near the air surface are normally longer and denser than the other submerged areas far from the air surface (Figure 1.5 B and F). Thus, the availability of carbon dioxide seems to affect the submerged fiber growth. It has been suggested that ambient CO_2 concentration is involved in regulating the turgor in the fiber since malate is one of products of dark CO_2 fixation (Dhindsa et al., 1975; Basra and Malik, 1983). Improvement of fiber growth was achieved through modifying the culture condition by including culturing the ovules in a 5% CO_2 atmosphere (Marden and Stewart, 1984). The submerged fibers could not obtain as much carbon dioxide as air-grown fibers because the concentration of the above gas is very low in the culture medium. Therefore, growth of submerged fibers has to rely on the aerial portion of the ovule. It is possible that air-grown fiber growth may support submerged fiber growth. To investigate whether or not there is a linear relationship between submerged fiber growth and air-grown fiber growth, the average weight per ovule of submerged fibers and air-grown fibers from the same ovary was determined (Table 1.7). The correlation coefficient (R^2) was 0.0034. Therefore submerged fiber growth is not correlated with air-grown fiber growth.

One potential shortcoming of submerged fibers is the shorter length, around half of that from fibers produced *in vivo* because the longer fiber is desired for textile industry. Possibly, programmed cell death might be promoted in submerged fibers, and this could be used as a new tool to study programmed cell death in plant cells. Secondary wall synthesis is initiated earlier in the submerged fiber (12-16 DPA) in comparison to the air-grown fiber (20-24 DPA). Thus shorter fiber length of the submerged fiber might be due to earlier cessation of fiber elongation during the primary wall growth phase. Shortage of availability of CO_2 and osmotic pressure may cause submerged fibers to stop elongation earlier.

Another novel feature of submerged fiber growth is the appearance of helical secondary wall thickenings (Figures 1.4 D and J). This feature not only is obvious under polarization microscopy, but also easily detected with bright field light microscopy (Figure 1.6). This pattern is very reminiscent of secondary wall thickenings in xylem from woody plants (Knebel and Schnepf, 1991). Such discrete and regular thickenings suggest that cytoskeletal control over the deposition of cellulose (Seagull, 1985; 1990; 1992) may be regulated in some way by hormonal levels. In *Zinnia*, the mesophyll cells can be induced to undergo differentiation into xylem elements, largely through hormonal induction (Fukuda and Komamine, 1980; Roberts and Haigler, 1990). Perhaps similar mechanisms are functioning here in the development of a terminally differentiated cotton fiber from the epidermal surface of the ovule. The frequency of the helical secondary cell wall thickenings was investigated with submerged fibers grown in the media containing different combinations of IAA and GA_3 (Table 1.8). It seems that increased levels of IAA or/and GA_3 caused an increase of the frequency of the helical secondary cell wall thickenings in submerged fibers (Table 1.8). Gould and Seagull (2002) showed that application of exogenous gibberellic acids increased reversal frequency significantly. Auxin depletion also caused altered organization the secondary cell wall in submerged fibers (Chapter 3).

Although submerged cotton fibers had smaller crystallite size, they were stronger than fibers grown *in vivo*. Hsieh et al. (2000) showed that the forces required to break single fiber increased with fiber development, and the overall crystallinity and crystallite sizes increased with fiber development for both *G. hirsutum* and *G. barbedense*. However, a positive relationship between single fiber strength and crystallite size was not found in submerged cotton fibers (Tables 1.4 and 1.5). Submerged fibers have the smallest cellulose crystallite size, but also have the strongest breaking force in comparison with air-grown fibers and fibers *in vivo*. In fibers with the greatest cellulose crystallite size, the breaking force of the fibers in vivo ranks second compared with submerged fibers and air-grown fibers. Also, air-grown fibers had slightly greater crystallite size, but their tensile strengths were much smaller in comparison with

submerged fibers. Possibly, this is due to the fact that submerged fibers begin secondary cell wall deposition earlier than air-grown fibers (Table 1.1), and have thicker secondary cell walls (Table 1.2). This trait of the submerged fiber is more like that of fuzz fibers. Fuzz fibers are shorter but stronger than lint fibers because they have thicker secondary cell walls than lint fibers (Berlin, 1986). Fuzz fibers initiate 4-10 days later than lint fibers (Lang, 1938; Beasley, 1979), but they begin secondary wall deposition about the same time as lint fibers (Beasley, 1979). This suggests that secondary wall deposition in fuzz fibers is more rapid than that in lint fibers. The greater wall thickness could result from either (a) packing density of the cellulose; (b) actual increased localized deposition zones; (c) more rapid secondary wall deposition which may be achieved by more densely distributed terminal complexes (TCs) in the plasma membrane; or (d) longer period of the secondary wall deposition. The packing density of the cellulose is under investigation (by Dr. Alfred D. French, SRRC, ARS, USDA). The growth environment of fibers grown *in vivo* is in the intermediate humidity state in comparison with submerged fibers and that of air-grown fibers (dry). On the other hand, water molecules, to some extent, may cause the smaller crystallites of cellulose, because hydrogen ions of the water molecule may interfere with hydrogen bonding between cellulose microfibrils (O'Sullivan, 1997). It has been shown that the breaking forces of dried fibers from Texas Marker 1 were about 75% of their hydrated counterparts (Hsieh et al., 2000). Therefore, the growth condition in a hydrated state for submerged fibers might play an important role in determination of the fiber strength and crystallite size.

1.5. Conclusions

Induction of submerged cotton fiber growth is correlated with the age of ovule at induction and combinations of exogenous plant hormones. The highest induction rate resulted from the ovule culture at induction of 0 DPA rather than other induction stages, -2, -1, 1 and 2 DPA. The optimal combination of exogenous plant hormones to induce submerged fiber growth is 0.5 μM GA_3+ 5-10 μM IAA. Compared with air-grown fibers and fibers *in vivo*, submerged fibers have thicker secondary cell walls and smaller crystallite sizes, and they are shorter and stronger. Those features render the submerged fiber a unique system to study plant cell elongation, cell wall thickening and cellulose synthesis at the molecular level. The submerged ovule culture system can also be used to study effects of external agents on cell wall assembly.

Table 1.1 Comparison of cotton fiber diameter and cell wall thickness of different fiber types[a]

	Fiber diameter (μm)*			Cell wall thickness (μm)*	
	Average	% of CK	stdev.	Average	stdev.
Submerged fiber (26DPA)	20.92**	69.82	±3.13	2.27	±0.27
Air-grown fiber (26DPA)	22.31	74.46	±1.36	<1.00	N/A
Fiber grown *in vivo* (28DPA) (CK)	29.96	100.00	±3.87	<1.00	N/A

[a] 1 DPA Ovules were cultured in the medium containing 0.5 μM GA_3 + 15 μM IAA; Measurements were performed when submerged fibers and air-grown fibers were at 26 DPA and *in vivo* fibers were at 28 DPA. * 50 samples for each treatment; CK = control; N/A=not available. ** According to the T-test, the fiber diameters of submerged fibers were smaller than air-grown fibers and fibers *in vivo* at the confidence level of 99%.

Table 1.2 Comparison of cotton fiber diameter, cell wall thickness and fiber length at 41 DPA[a]

	Fiber diameter (μm)[b]		Cell wall thickness (μm)[b]		Fiber length (mm)[c]	
	average	stdev	average	stdev	average	stdev
Submerged fiber	20.55**	±2.21	4.81**	±1.00	18.1**	±2.99
% of control	77.61		151.26		50.63	
Air-grown fiber	20.79	±2.47	1.83	±0.79	23.9	±2.90
% of control	78.51		57.55		66.80	
Fiber *in vivo* (control)	26.48	±2.66	3.18	±0.61	35.8	±2.43
% of control	100.00		100.00		100.00	

[a]Ovules were cultured at 1 DPA in medium containing 0.5 μM GA and 15 μM IAA. Measurement was performed at 41 DPA. [b]50 samples for each treatment. [c]30 samples for each treatment. ** According to the T-test, the fiber diameter of submerged fibers was significantly smaller than that of fibers grown *in vivo*, at the 99% confidence level, but about same as that of air-grown fibers. The cell wall thickness of submerged fibers was significantly larger than both air-grown fibers and fibers *in vivo*; air-grown fibers were significantly smaller than fibers *in vivo* in terms of fiber diameter and cell wall thickness, all at the 99% confidence level. Submerged fibers were significantly shorter than both air-grown fibers and fibers *in vivo* at the 99% confidence level. Air-grown fibers were significantly shorter than fibers *in vivo* at the 99% confidence level.

Table 1.3 Effects of the age of cotton ovules at induction on fiber length (mm) [a]

	Submerged-fibers[b]			Air-grown fibers[b]		
	0 DPA	1 DPA	2 DPA	0 DPA	1 DPA	2 DPA
Range	14-18	14-17	15-17	15-21	19-26	23-31
Avg.	15.8	15.8	15.8	17.6**	22.0	26.3
Stdev.	±1.6	±1.3	±0.7	±2.3	±2.3	±2.8

[a]Measurements were performed at 38 DPA; The culture medium contained 0.5 μM GA_3 and 10 μM IAA. [b]30 samples for each treatment (see materials and methods). ** in terms of air-grown fiber length, 0 DPA<1DPA<2DPA at 99% confidence level according to the T-test.

Table 1.4 Crystal sizes of submerged cotton fibers

Sample	Reflection	Position (2θ)	d(Å)	I%	FWHM	Crystallite size (Å)	Crystallite size average (Å)
SUB	101	14.80	5.98	29.9	1.71	47	46
	10-1	16.77	5.28	25.1	1.78	45	
	002	22.82	3.89	100.0	1.82	45	
AIR	101	14.68	6.03	31.8	1.70	47	49
	10-1	16.74	5.29	20.9	1.58	51	
	002	22.82	3.89	100.0	1.71	48	
VIVO	101	14.81	5.98	55.8	1.57	51	53
	10-1	16.81	5.27	32.8	1.52	53	
	002	22.90	3.88	100.0	1.51	54	

Table 1.5 Single fiber strength of submerged cotton fibers.

Fiber sample	Breaking Force (grams)	% of control
Submerged fibers	4.38 (±1.60)	119.0
Air-grown fibers	2.85 (±1.32)	77.4
Fibers grown *in vivo* (control)	3.68 (±1.80)	100.0

Table 1.6 The pH value of the MS medium before and after autoclaving*.

PH before autoclaving	PH after autoclaving
3.01	2.96±0.005
3.51	3.56±0.007
4.02	4.51±0.089
4.53	4.92±0.055
5.01	5.28±0.037
5.55	5.46±0.016

* MS medium was prepared as described in materials and methods. The pH value was adjusted to 3.01, 3.51, 4.02, 4.53, 5.01, and 5.05 using HCl or KOH respectively before autoclaving. The pH value was measured after autoclaving. The tests were repeated three times.

Table 1.7 Fiber production from cotton ovule culture at 28 DPA[a].

Plate [b]	Average dry weight of the submerged fiber per ovule (mg)[c]	Average dry weight of the air-grown fiber per ovule (mg)[d]
1	1.761	4.023
2	2.077	4.912
3	4.734	4.596
4	2.619	3.413
5	2.964	10.051
6	0	6.628
7	8.486	2.634
8	2.759	1.304
9	0	1.428
10	1.833	1.216

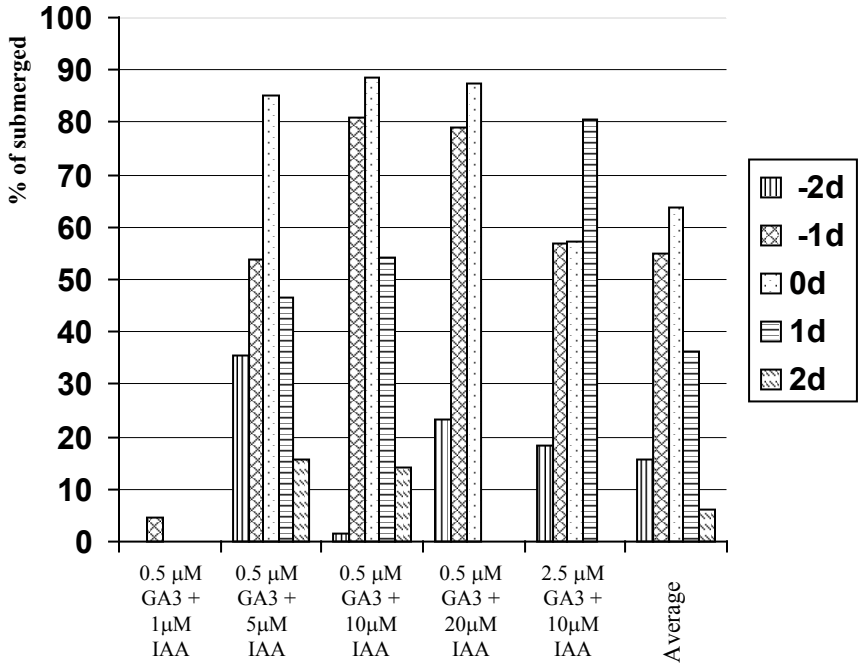
[a] To determine the existence of the linear relationship between submerged fiber growth and air-grown fiber growth. Ten plates were randomly selected.
[b] Each plate contained ovules from the same ovary.
[c] The dry weight of submerged fibers harvested from ovules in one plate was divided by the number of ovules producing submerged fibers.
[d] The dry weight of air-grown fibers harvested from ovules in one plate was divided by the number of ovules producing air-grown fibers.
R (Linear relationship)=-0.0580; R^2 (correlation coefficient)=0.0034. Thus there is no correlation shown between air-grown fiber growth and submerged fiber growth.

Table 1.8 Effects of hormonal levels on frequency of the helical secondary cell wall thickenings in submerged fibers*.

IAA (μM)	GA_3 (μM)	Number of fibers with helical thickenings	Total fibers observed	Frequency of helical thickenings (%)
5	0.5	39	160	24.4
10	2.5	44	106	41.5
10	0.5	70	220	33.3

* Cultures were derived from 0 DPA ovules. The analysis was made at 28 DPA.

Figure 1.2 The percentage of ovules producing submerged cotton fibers responding to various combinations of IAA and GA_3 as well as the ages of ovules at induction. Here, % of submerged was determined by the ratio of ovules producing submerged fibers to the total number of ovules in culture. Each group of columns indicates the response of ovules to specified hormone combinations, where each pattern of a column stands for the age of ovules at induction as indicated in the Figure. Average = the percentage of ovules producing submerged fibers averaged by the age of ovules at induction.



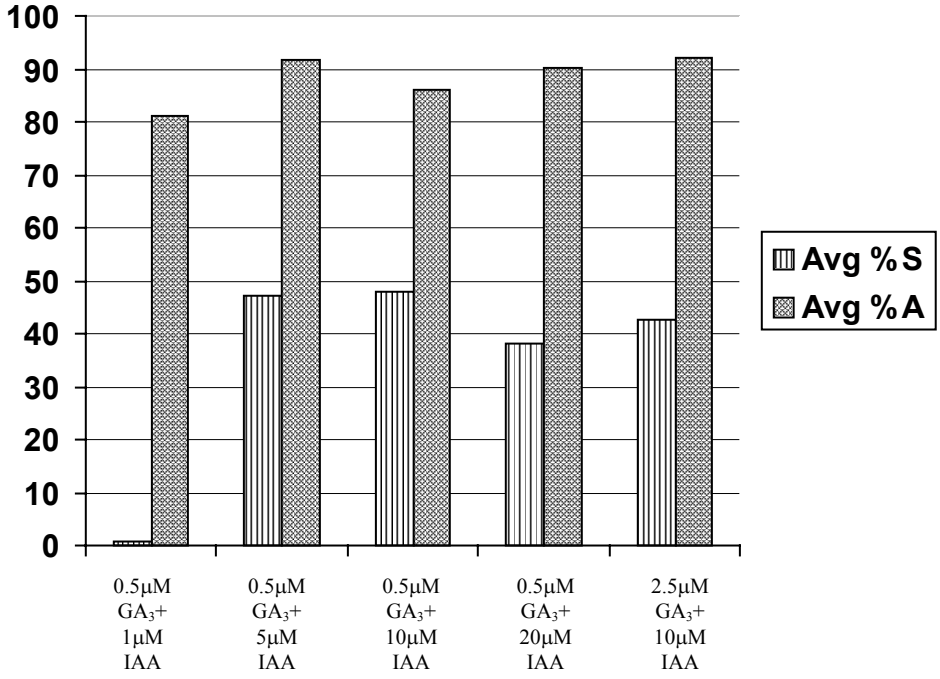


Figure 1.3 Comparison of the percentage of submerged fiber growth and air-grown fiber growth resulting from various hormonal combinations. % S or %A= the ratio of the number of ovules producing either submerged or air-grown fibers to the total number of ovules in culture. Avg % S / % A = average effects of certain hormone combination on % S or % A responding to 5 ages (-2, -1, 0, 1, 2 DPA) of ovules at induction.

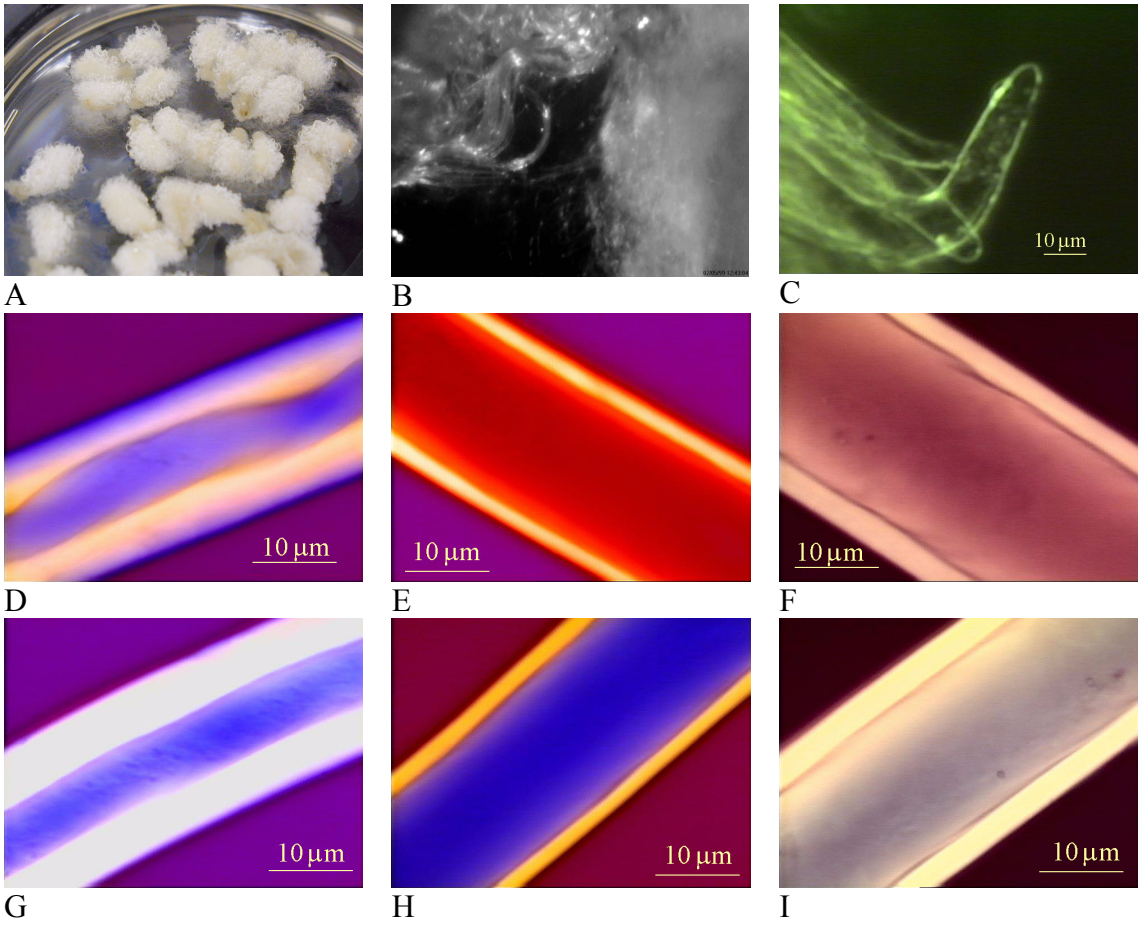


Figure 1.4 (A-I) Photo gallery of submerged, air-grown, and *in vivo*-grown cotton fibers.
(A), Cotton ovule culture at 28 DPA, and initiated from 0 DPA ovules in a medium containing 0.5 µM GA_3 and 10 µM IAA;
(B), Cotton ovule culture at 14 DPA, and initiated from 0 DPA ovules in a medium containing 0.5 µM GA_3 and 15 µM IAA. Note the presence of shiny, coiled air-grown fibers, left, and submerged fibers, right;
(C), Details of coiled fibers observed in air-grown fibers (cultured from 2 DPA ovule) at 3 days of culture. Optics=Bright field microscopy using a 550nm interference filter;
(D and G), Submerged fibers at 41 DPA, (polarization microscopy using first order red compensator at extinction). Note thicker secondary cell wall and smaller fiber diameters;
(E and H), Air-grown fibers at 41 DPA, (polarization microscopy using first order red compensator at extinction) Note thinner secondary cell walls;
(F and I), Fibers grown *in vivo* at 41DPA showing typical thick and uniform secondary walls, (polarization microscopy using first order red compensator at extinction)

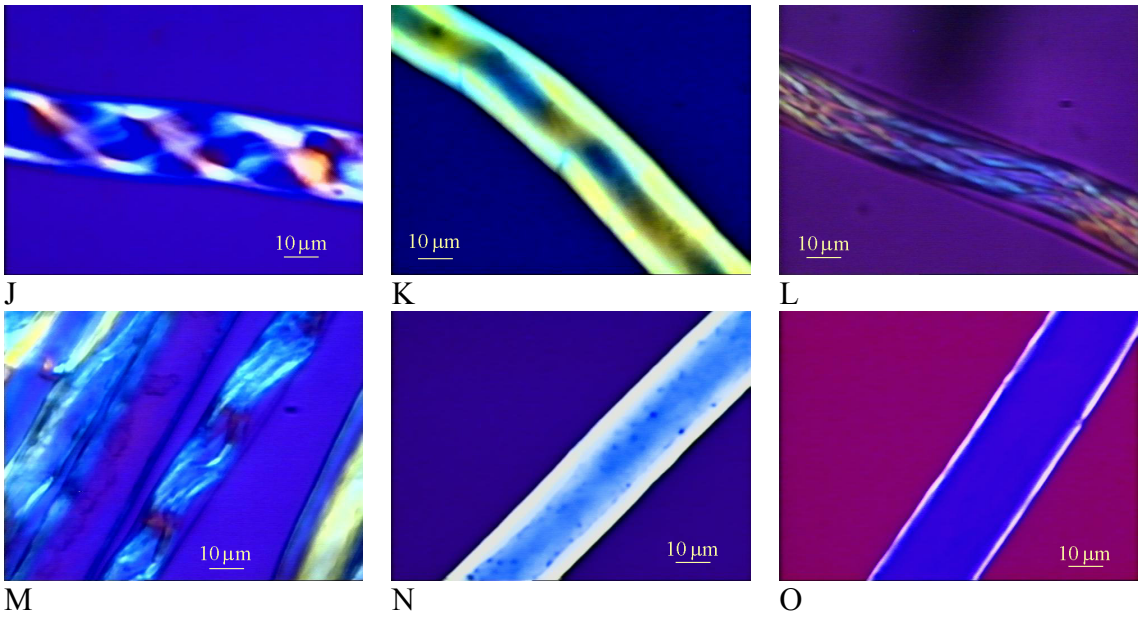


Figure 1.4 (J-O) Photo gallery of submerged, air-grown, and *in vivo*-grown cotton fibers.

J and K), Helical patters of secondary walls produced by submerged fibers at 41 DPA, (polarization microscopy using first order red compensator at extinction);

L and M), Longitudinally oriented bundles of secondary wall microfibrils from submerged fibers at 51 DPA. The fiber in L has a typical reversal as indicated by a change in the orientation of birefringence (polarization microscopy using first order red compensator at extinction);

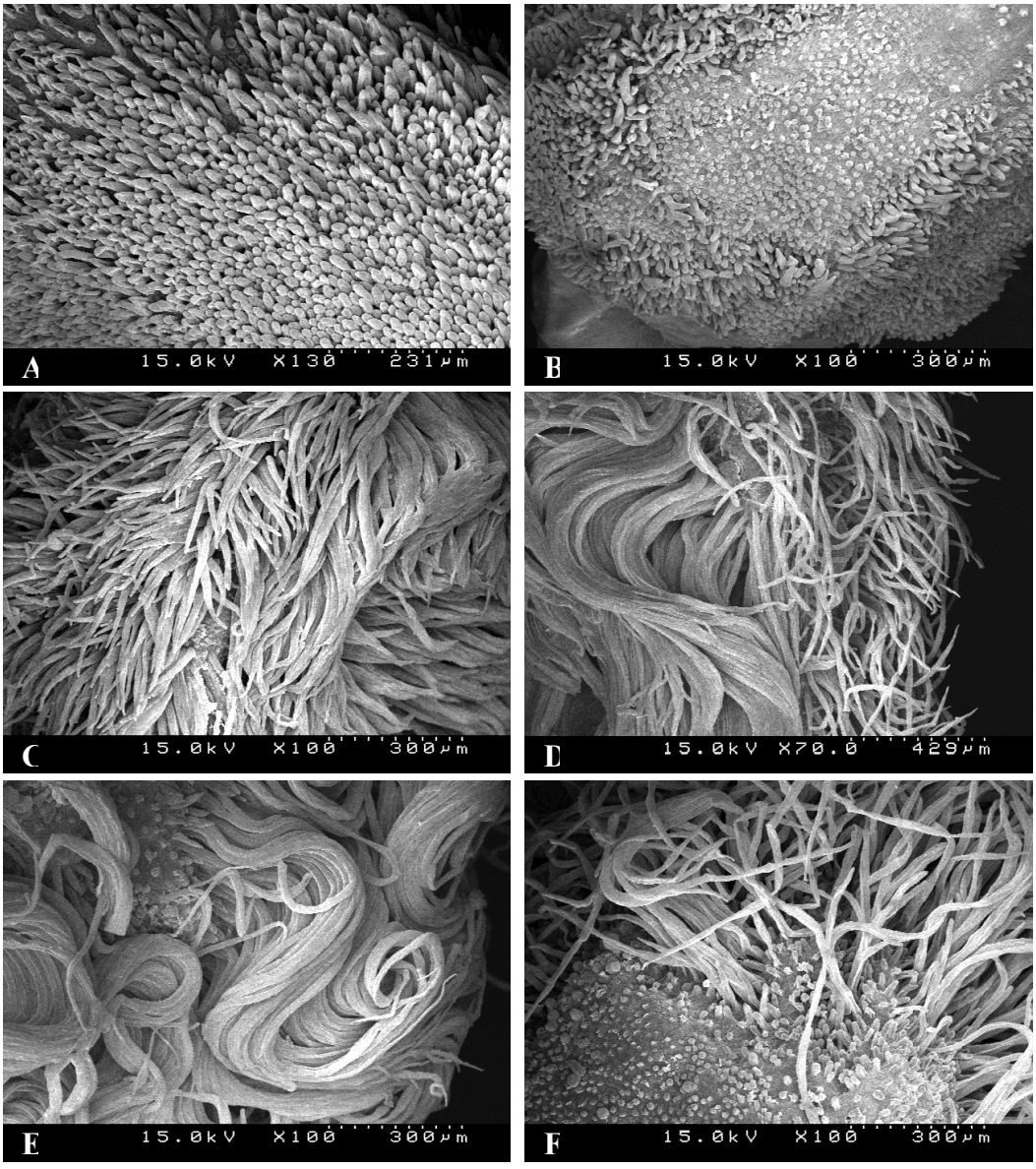
N), Atypical cytoplasmic particles adjacent to the thick secondary wall of a submerged fiber at 38 DPA, (polarization microscopy using first order red compensator at extinction);

O) Smooth and relatively thin secondary wall of a typical air-grown fiber at 38DPA, (polarization microscopy using first order red compensator at extinction).

Figure 1.5 SEM analysis of cultured cotton ovules.

A) Air-grown fibers at 2DPA.
B) Submerged fibers at 2 DPA.
C) Submerged fibers (left) and air-grown fibers (right) at 4 DPA.
D) Air-grown fibers (Left) and submerged fibers (right) at 5 DPA.
E) Air-grown fibers at 6 DPA.
F) Submerged fibers at 6 DPA.

A-C, E and F, culture started at 0 DPA; D, culture started at –1DPA.



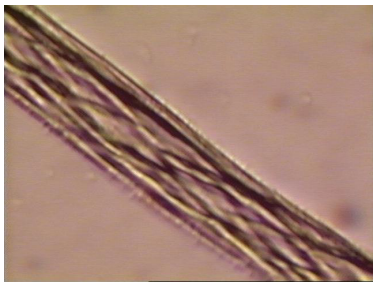


Figure 1.6 A submerged cotton fiber at 38 DPA viewed with light microscopy

Appendix 1 Culture medium for cotton ovules (Beasley and Ting, 1973) *

Component	Concentration (mg/l)
KH_2PO_4	272.180
H_3BO_3	6.183
$Na_2MoO_4 \cdot 2H_2O$	0.242
$CaCl_2 \cdot 2H_2O$	441.060
KI	0.830
$CoCl_2 \cdot 6H_2O$	0.024
$MgSO_4 \cdot 7H_2O$	493.000
$MnSO_4 \cdot H_2O$	16.902
$ZnSO_4 \cdot 7H_2O$	8.627
$CuSO_4 \cdot 5H_2O$	0.025
KNO_3	5055.500
$FeSO_4 \cdot 7H_2O$	8.341
Na_2EDTA	11.167
Nicotinic acid	0.492
Pyridoxine · HCl	0.822
Thiamine · HCl	1.349
Myo-inositol	180.160
D-Glucose	18016.000
D-Fructose	3603.200

* Beasley and Ting (1973) found that the greatest fiber production resulted when the culture medium above with addition of 5 µM IAA and 0.5 µM GA_3. pH was adjusted to 5.0 prior to autoclaving.

Appendix 2 Composition of MS salts mixture (Sigma, Catalog No. M-5524)

Components	Final concentration (mg/l)
Ammonium Nitrate	1650.0
Boric Acid	6.2
Calcium Chloride (Anhydrous)	332.2
Cobalt Chloride · $6H_2O$	0.025
Cupric Sulfate · $5H_2O$	0.025
Na_2 - EDTA	37.26
Ferrous Sulfate · $7H_2O$	27.8
Magnesium Sulfate	180.7
Manganese Sulfate · H_2O	16.9
Molybdic Acid (Sodium salt) · $2H_2O$	0.25
Potassium Iodide	0.83
Potassium Nitrate	1900.0
Potassium Phosphate Monobasic	170.0
Zinc Sulfate · $7H_2O$	8.6

Appendix 3 MS vitamin mixture (Sigma, Catalog No. M-3390)

Components	Final concentration (mg/l)
Glycine	2.0
Myo-Inositol	100.0
Nicotinic Acid	0.5
Pyridoxine · HCl	0.5
Thiamine · HCl	0.1

Chapter 2 Effects of Congo Red on submerged cotton fiber growth

Summary

Congo Red is a natural dye that has a high affinity for the biopolymer cellulose. Using submerged cotton ovule cultures, the effects of Congo Red on cell wall formation of cotton fibers was studied. The addition of Congo Red to the culture medium had an influence only on submerged cotton fibers and not on air-grown cotton fibers, indicating that Congo Red molecules were not translocated from cell to cell. Fiber initials grown in the presence of Congo Red failed to develop further and were retarded in elongation. When Congo Red was applied in the early primary wall stage, fiber cell elongation was inhibited, but amyloplast production was induced. When Congo Red was applied in late primary wall or early secondary wall stage (about 14-16 DPA), the effects were less severe, but a significant increase in birefringence of secondary cell walls was observed. In both conditions of treatment with Congo Red in the primary wall and the secondary wall stages, a "nodulation" occurred on the wall surface. Presumably, Congo Red-bound microfibrils cause wall loosening. As a result, the wall material accumulated externally which lead to the "nodulation". This assumption was supported by TEM observation of sectioned material. Neither cellobiohydrolases CBH I or CBH II had affinity for the external wall materials, implying that there was no cellulose present or binding sites for CBH had been occupied by Congo Red. X-ray diffraction data showed that Congo Red did not alter the allomorph of cellulose generated, but did decrease the crystallinity of cellulose in submerged cotton fibers. It is concluded that Congo Red altered cell wall formation in submerged cotton fibers by interrupting cellulose synthesis. Cotton fibers appear to have an alternative pathway of starch synthesis to utilize the available carbon source when cellulose synthesis was interrupted.

2.1. Introduction

Availability of fiber growth *in vitro* allows the effects of a variety of agents to be studied by addition to the culture media. Herbicides have been applied to study cellulose synthesis in cotton fibers (Delmer et al., 1987; Francey et al., 1989; Triplett and Johnson, 1999; Vaughn and Turley, 2001). Radioactive isotopes were used to study carbon assimilation and turnover in cotton fibers ((Francey et al., 1989; Carpita and Delmer 1980; 1981). Extensive studies have focused on regulation of exogenous phytohormones on fiber growth *in vitro* (Beasley and Ting, 1973; 1974; Dhindsa et al., 1976; Dhindsa and Ting, 1976; Hsu and Stewart, 1976; Stewart and Hsu, 1977; Dhindsa, 1978; De Langhe et al., 1978; Beasley and Eaks, 1979; Davidonis, 1989; 1992; Nayya et al., 1989; Gokani et al., 1998; Momtaz, 1998; Gialvalis and Seagull, 2001). To investigate roles of cytoskeletal components, microtubule and microfilament reagents were employed to cotton ovule cultures (Seagull, 1986; 1989; 1990; 1992). In such experiments, it is necessary that agents added to the media be taken up readily by cotton ovules.

An attempt was made to investigate how a cellulose-binding dye, Congo Red, interferes with cell wall formation in cotton fibers from ovule cultures. Upon adding Congo Red to the culture medium, it was found that the dye was not translocated from cell to cell. It stained only fibers in contact with the culture medium. This finding indicates that only submerged cotton fibers can be tested in this experimental design.

Congo Red is a naturally occurring dye. Its ability to stain cotton was discovered in 1884 by Bottiger (http://www.straw.com/sig/dyehist.html). Congo Red also has a high affinity for chitin (Salmon, 1997) and amyloid fibrils (Chiti et al., 1999). Herth (1980) discovered that Congo Red inhibits chitin microfibril assembly in a unicellular alga and provided evidence that microfibril assembly and crystallization are separate processes. Extensive studies have been carried out with various fungal cells, and consistent results indicate that Congo Red causes the alteration of wall synthesis and morphogenesis by interacting with chitin or (1→ 3)-β-D-glucan (Bartnicki-Garcia, 1994; Kopecká and Gabriel, 1992; Nodet et al., 1990; Rongcero and Durán, 1985; Pancaldi et al., 1984; Vannini et al., 1983). In the presence of Congo Red, the fibrous wall regeneration on the

surface of moss protoplasts was inhibited, characterized by short atypical fibers and the presence of amorphous wall materials (Burgess and Linstead, 1982). Colvin and Witter (1983) have demonstrated inhibition of cellulose microfibril formation in *Acetobacter xylinum* by Congo Red.

This study illustrates the effects of Congo Red on initiation of submerged cotton fibers and fiber elongation. Additionally, altered formation of fiber cell walls and a shift of carbon metabolism in submerged fibers treated with Congo Red is documented.

2.2. Materials and Methods

Cotton ovule culture

To obtain submerged cotton fibers, cotton ovules were cultured as described previously (Feng and Brown, 2000). Briefly, cotton ovaries were harvested at 0-2 DPA and sterilized in 70% ethanol for 7 min, then washed 3-5 times with sterilized distilled water. Cotton ovules were aseptically removed from the ovaries and transferred to liquid culture medium. The cultures were grown in dark at 28 ^{0}C, in a liquid medium containing MS basal salt medium supplemented with MS vitamin mixture, 10 μM IAA, 0.5 μM GA_3, 20 mM sucrose and 80 mM glucose.

Congo Red treatment

Filter-sterilized, 1.25 mM Congo Red stock solution was added to the culture medium to final concentrations of 0.1, 0.5, 1, 1.25, 2.5, 5 and 25 μM. Various growth stages (1 DPA to 20 DPA) were treated with Congo Red.

Light microscopy

Submerged fibers, either attached to ovules or excised, were mounted on slides in culture medium. Microscopic observations were carried out with a Zeiss microscope equipped for bright field, dark field, Nomarski Interference, phase contrast, polarization, and fluorescence. Images were captured via a Pixera camera or an Optronix CCD camera coupled to a Panasonic Video disc recorder.

SEM

Cotton ovules were fixed in 3% glutaraldehyde buffered with 1x culture medium for 2h at room temperature, then washed with the culture medium for 3x10min. The samples were washed with 0.1 M cacodylate buffer (pH 7.2) for 3x10 min before fixation in 2% osmium in 0.1 M cacodylate buffer for 2h at room temperature. After washing in distilled water for 3x10min, the samples were dehydrated in a 30%, 50%, 70%, and 100% ethanol series before critical point drying (Samdri-790, Tousimis Research Corp.). Dried samples were coated with gold (30800, Ladd Research Industries, Inc.), and then viewed

under a scanning electron microscope (Hitachi S-4500 field emission scanning electron microscope).

TEM

Cotton ovules were fixed in 4% glutaraldehyde in 1x culture medium for 2h at room temperature. After washing with culture medium and 0.1 M cacodylate buffer (pH 7.2), the samples were soaked in 2% osmium in 0.1 M cacodylate buffer for 2h, then washed with 0.1 M cacodylate buffer and distilled water. Dehydration conditions were 70% ethanol for 2x15min, 100% ethanol 2x 15 min, and 100% acetone 2x 15min. The samples were infiltrated in acetone/Epon812: 2/1 overnight, acetone/Epon812: 1/2 for 24h, and full Epon 812 for 24h with one change of fresh Epon. Embedded tissue was polymerized for 14 h at 60 ^{0}C.

Ultra thin sections were prepared with a thermal advance microtome (700121, Reichert, Austria) and mounted on a copper grid. After staining with 2% uranyl acetate and lead citrate, the samples were observed with a Philips 420 transmission electron microscope. Images were captured with a GATAN (Bioscan or Gatan 622) camera.

CBH gold labeling

Ultra thin sections of cotton fibers on copper grids were incubated with CBH I /CBH II for 3 min at room temperature, then rinsed with d H_2O. The samples were stained with 2% uranyl acetate for 10 min, rinsed with d H H_2O, followed with lead citrate staining for 10 min. After the samples were rinsed in 0.02 N NaOH, then in dH_2O, they were air-dried. Observations were made with TEM as described above.

X-ray diffraction

Various kinds of mature cotton fibers (older than 50 DPA) including fibers grown *in vivo*, air-grown fibers, and submerged fibers (with or without Congo Red treatment) were purified according to the methods previously described (Imai et al., 1999). Fibers were excised from the ovules, extracted in 1% NaOH for 2d at 70 ^{0}C, washed and neutralized with distilled water, then extracted in 0.1% sodium chlorite in acetate buffer (pH 4.9) at 70 ^{0}C. After washing in distilled water, the samples were further extracted in 2.5 N HCl for 2d at 70 ^{0}C, followed with washing in distilled water and centrifugation.

Purified, neutralized samples were air-dried. X-ray diffraction patterns were obtained with two different instruments. The film pattern was obtained with a Philips PW 1729 X-ray generator and a Debye-Scherrer camera. The digital pattern was obtained with a Philips PW 1720 X-ray generator and a vertical goniometer camera. The digital data was processed with Jade software.

Detection of amyloplasts

Four different methods were used to detect amyloplasts in submerged cotton fibers treated with Congo Red. 1), iodine staining: cultured fibers or sections were stained with Lugol's iodine solution containing 1% (w/v) iodine and 2% (w/v) potassium iodide, then observed under the light microscope. 2), DAPI staining: fiber sections were stained with 2 μg/ml DAPI working solution, and then viewed with the fluorescence microscope. 3), fibers or sections were directly viewed with polarizing light. 4), fiber sections were directly viewed with TEM.

2.3 Results

In the *in vitro* system, cotton ovules are floating in the liquid culture medium during incubation. Both air-grown and submerged portions of the ovules produce cotton fibers. When the filtered, sterilized Congo Red was applied to the liquid culture medium, it caused only submerged cotton fibers to be stained red, whereas the air-grown cotton fibers seemed to be unaffected (Figure 2.1). This implies that Congo Red is not translocated between cells of the ovule and does not enter the vascular system in the ovules. The molecular weight of Congo Red is 696.7, about twice that of GA_3, but it seemed unable to move from cell to cell. GA_3, however, is assumed to be translocated between cells based on the effects of exogenous GA_3 on the fiber production via cotton ovule culture (Beasley and Ting, 1973; 1974).

Not all epidermal cells on the submerged portion of the ovules were stained with Congo Red. Only cells active during or after fiber initiation took up the dye (Figure 2.2). Fiber initiation is stimulated by fertilization. Fertilization occurs around the time of anthesis and usually completed 48 h post-anthesis (Beasley, 1984). The ovules at 1d preanthesis, (when fiber initiation is assumed not to have begun) were treated with Congo Red in the culture medium. No stained fiber cells were observed (4-5 ovules were investigated). The above culture (1d preanthesis) was allowed to grow for 1d before treatment with Congo Red. Very few fiber cells were stained (Figure 2.3). In comparison, ovule cultures starting at 0 DPA (on the day of anthesis, at 4-6 hours post-fertilization) were incubated with Congo Red where many epidermal cells took up the dye, indicating that many fiber cells have initiated (Figure 2.2).

The severity of effects on the development of the submerged cotton fibers is dependent on the concentrations of Congo Red applied as well as the growth stage of the cotton fiber in which Congo Red was employed. Various concentrations (0.1, 0.5, 1.25, 2.5, 5 and 25 μM) of Congo Red treatments were administrated in the experiment.

Not all of the fiber cells were initiated at the same time. Over the duration of Congo Red treatment, the effectiveness of the dye was reduced because of dye

precipitation and absorption by the fiber cells. Consequently, the fiber cells that initiated later encountered relatively low levels of Congo Red. Therefore, they were less influenced by the dye than the fiber cells that developed earlier (Figure 2.4). As observed, the degree of staining of fiber cells was proportional to the concentration of Congo Red applied (Figure 2.2). Lower concentrations of Congo Red yielded a greater percentage of healthy cells. A Congo Red concentration of 2.5 μM caused the majority of affected fiber cells to die when applied during fiber initiation (Figure 2.5). However, some fiber cells remained viable when 0.5 μM Congo Red applied (Figure 2.4). The ovules incubated continually in the medium supplemented with 0.1 μM Congo Red appeared to grow normally, with only a minority of fiber cells affected.

Fiber initiation is characterized by a protruding growth of the surface wall of the epidermal cell and is also accompanied by cell wall synthesis. In initiating fiber cells, Congo Red was evenly distributed on the protruding walls (Figure 2.2-5). However, in fiber cells that had completed initiation, the dye binds more extensively to the tip or tapered regions (Figure 2.6). This discrete staining pattern, along with the shape of the cells, can be used to determine the fiber developmental stage. It is likely that, during fiber initiation, the epidermal cells can also undergo dramatic changes in the cellulose assembly phase. In this case, newly formed glucan chains may have been accessible to binding by Congo Red. Generally, fiber cells in earlier stages were more sensitive to Congo Red than those that developed later. Detailed responses of fiber cells to Congo Red at the different stages will be discussed in the following sections.

Effects of Congo Red during fiber initiation

Overall, Congo Red inhibited fiber initiation. As discussed earlier, Congo Red was distributed evenly on the walls of fiber cells during initiation. Most of these cells appeared to be dead, or dying and collapsed, thus failing to further develop even after incubation in the absence of Congo Red (Figure 2.5). However, the dye was more concentrated in the apical regions of fiber walls that were fully initiated or in early elongation phase (Figure 2.6). Congo Red prevented the transition from initiation to

elongation in fully initiated fiber cells. Most of these cells were alive but failed to form a tapered-tip, as evidenced by dye accumulation around the dome-like apical region (Figure 2.7-8). These experiments suggest that fiber initiation is a very critical stage in development, one that appears to be very sensitive to cellulose-binding agents such as Congo Red. Also, it implies that, during the transition state from initiation to elongation, the fiber cells undergo primarily tip growth rather than diffuse growth.

Effects of Congo Red on the fibers during the elongation stage

During fiber elongation, Congo Red binding was ubiquitous in the fiber cell walls, but comparatively more dye accumulated in the apical or tip regions (Figure 2.9). This implies that cellulose located in the fiber tip region may be in an amorphous or less crystalline state than cellulose in the basal region of the fibers. The submerged fiber cells grown in the presence of 2.5 μM Congo Red for 4 d starting at 2 DPA, were transferred to Congo Red-free medium for another 4 d. The presence of dye persisted in the tip regions of these cells (Figure 2.10). This indicates that, during elongation, the fiber cells undergo primarily diffuse growth rather than tip growth. Incorporation of Congo Red in cellulose microfibrils enhanced visibility of the primary cell wall. Enhanced cellulose microfibril bands, as well as some globule structures on the walls, were distributed evenly along the fiber cell elongation axis after the culture was chased with the Congo Red-free medium (Figure 2.11). This also provides evidence that diffuse growth is present in the elongating fibers.

Application of Congo Red in the primary wall stage of cotton ovule cultures inhibited the fiber cell elongation (Figure 2.10, 12). Most of the affected submerged fiber cells at 9 DPA were shorter than 0.5 mm, in comparison with around 7-9 mm for the submerged cotton fibers at the same stage grown in the absence of Congo Red.

An interesting phenomenon was the appearance of “nodules” on the surface of the submerged fibers (Figure 2.13). These spotty structures were composed of cell wall materials based on SEM (Figure 2.14) and TEM analysis (Figure 2.15). This is evidence

that Congo Red caused wall loosening whereby the wall materials would begin to accumulate externally.

Effects of Congo Red on secondary cell wall formation

To study the effects of Congo Red on secondary cell wall formation, Congo Red was applied to the culture at 14-16 DPA, which is the late primary or early secondary wall stage. The presence of "nodules" was also observed in this study (Figure 2.16), suggesting each annulet might correspond to a very active cellulose synthesis site.

I observed possible localized Congo Red accumulation in submerged cotton fibers at 14 DPA (Figure 2.17). At this stage, fiber cells *in vivo* normally undergo elongation. However, our previous results suggested that submerged cotton fibers may cease elongation earlier than air-grown fibers and fibers *in vivo*. Since localized Congo Red may indicate localized cellulose synthesis (including pectin and hemicellulose assembly), I presume that secondary cell wall deposition started at this stage.

Enhanced birefringence of cellulose microfibrils made in the presence of Congo Red was also observed (Figure 2.18), probably due to an anisotropic absorption of the dye into the cellulose. This enabled observation of secondary cell walls of submerged fibers in earlier stages (at early 20^{th} DPA) than possible without Congo Red treatment. Therefore, monitoring secondary cell wall deposition is possible with Congo Red.

TEM study on alteration of cell wall formation induced by Congo Red

The ultra-structure of submerged cotton fibers further revealed that the outer growth of the cell wall induced by Congo Red treatment was consistent with the results observed with light microscopy and SEM. The outgrowth of wall materials resulted in the nodules which accumulated locally and were surrounded by cuticle (Figure 2.15). Wall materials in the nodules seemed to be loosely packed and randomly ordered. Some of these materials may have been lost during the process of dehydration (Figure 2.15).

The cell wall materials outside of the normal cell walls were neither labeled with CBH (cellobiohydrolase) I gold nor CBH II gold (Figure 2.19). The normal cell wall

portions produced in the presence of Congo Red were evenly labeled with both CBH I gold and CBH II gold (Figure 2.19), as were the cell walls without Congo Red treatment. The lack of CBH gold labeling in the wall materials outside the normal cell wall may indicate that there is no cellulose present, or that Congo Red masks binding sites for CBH enzymes.

Fasciation of the cellulose microfibrils was observed in cell walls treated with Congo Red (Figure 2.20). During the incubation in the presence of the Congo Red, cellulose microfibrils of the fiber cell wall might have attached to Congo Red resulting in thicker cellulose microfibrils. Fasciated cellulose microfibrils are shorter and less ordered than those produced under the normal conditions.

X-ray analysis of Congo Red treated cotton fibers

X-ray diffraction analysis was carried out to determine the crystallinity of cellulose in submerged cotton fibers treated with Congo Red (Figure 2.20). The diffraction pattern bands of 6.1Å and 5.3Å from submerged fibers treated with Congo Red appeared to have less intensity than those from submerged fibers without Congo Red treatment, air-grown fibers and fibers *in vivo*. This indicates that Congo Red treatment may alter the degree of crystallinity of the cellulose. Also, Congo Red seemed to broaden the diffraction pattern band of 3.9Å, suggesting that binding of Congo Red to cellulose may affect the periodic order of the glucose units recognized by this reflection. A sharp band around 2.7 Å may be suggestive of the dye stacking characteristics when excess Congo Red is used. The above data suggest that Congo Red binds to newly formed glucan chains, thus preventing their lateral aggregation into microfibrils. The decreased crystallinity of cellulose also supports regional isotropy of cell wall growth in submerged cotton fibers.

The digital X-ray diffraction data showed that the crystal size of cellulose was smaller in submerged cotton fibers treated with Congo Red than those in submerged cotton fibers without Congo Red treatment, air-grown fibers and fibers *in vivo* (Table 2.1). The crystal size is about 3.83 nm for Congo Red treated submerged cotton fibers,

4.57 nm for submerged cotton fibers without Congo Red treatment, 4.87 nm for air-grown fibers and 5.27 nm for fibers grown *in vivo* respectively (Table 2.1).

Amyloplast synthesis induced by Congo Red

It is unusual that amyloplasts were found to be synthesized in Congo Red treated submerged cotton fibers. Amyloplasts are common in the cells from endosperm tissue. Four different methods were used to detect amyloplasts. Iodine staining is a very common method to stain starch. Whole mount of fiber (Figure 2.22-23C) or fiber sections (Figure 2.26A) treated with Congo Red showed the presence of amyloplasts when stained with Lugol's Iodine solution. Amyloplasts displayed birefringence when viewed with polarizing light (Figure 2.23B). During starch synthesis or production of amyloplasts, plastid DNA synthesis is also very active. DAPI staining also revealed the existence of amyloplasts in submerged cotton fibers treated with Congo Red (Figure 2.24). TEM is a reliable method for identification of amyloplasts. Amyloplasts are membrane-bound organelles, containing starch granules, ribosomes and DNA materials (Figure 2.25, 27-28).

Amyloplasts occurred more frequently in submerged fibers when Congo Red treatment was administrated in the early elongation stage than the late developmental stage. There were up to 20-30 amyloplasts within 100 μm of fiber length of the whole mount fiber sample (Figure 2.22), and 1-4 amyloplasts in the cross section of each fiber (Figure 2.26A) when Congo Red was applied in 1-2 DPA. There were no amyloplasts observed in cross sections (1.5 μm thick) of submerged fibers (Figure 2.26B) or whole mount of fiber samples that were grown in the absence of Congo Red. In the submerged fiber cells treated with Congo Red at 15 DPA, there were 11 amyloplasts observed in 600 cells representing a 1.5 μm cross section.

Amyloplasts contained 1-6 starch granules, which were about 0.2-0.8 μm and highly labeled with CBH II gold (Figure 2.27). There was no concentric ring observed in the starch granules. It is known that CBH II has specific affinity to cellulose (β-1,4-

glucans) (Carrard and Linder, 1999). Thus, the apparent affinity of CBHII for starch (α-1,4- glucan) was inexplicable.

In order to test the affinity of CBH II for starch, starch powder and thin sections of cotton ovules (containing amyloplasts) without Congo Red treatment were prepared for CBH II labeling. Starch is common component of plant seeds, so cotton ovules at the primary wall stage (14 DPA) must contain some amyloplasts. Amyloplasts from cotton ovules without Congo Red treatment appeared to be similar to those from the cotton fiber cells treated with Congo Red; however, the starch granules had very low affinity for CBH II (Figure 2.28). This suggests that the uncharacteristic development of amyloplasts in submerged cotton fibers under Congo Red stress may be accompanied by the synthesis of β-1,4-glucans in these organelles. To further confirm that CBH II has no affinity for starch, a mixture of cellulose powder Sigmacell 100 (particle size, 100 μm, Sigma Co., USA) and starch powder was loaded on the EM grids for a CBH II labeling test. CBH II specifically bound to Sigmacell rather than starch powder (Figure 2.29). This result provides further evidence that CBH II labeling of amyloplasts from submerged fibers treated with Congo Red may demonstrate the presence of beta 1,4 glucans.

2.4. Discussion

This study demonstrates that Congo Red, a cellulose-binding agent alters cell wall formation in submerged cotton fibers. The Congo Red molecule is planar, rigid, and contains highly polar groups (Figure 2.30, Woodcock et al., 1995). A mechanism for the interaction between the dye molecule and cellulose has been proposed (Woodcock et al., 1995). The dye molecules preferentially bind parallel to the glucan chains. The two sulfonate groups in the dye molecule tend to form strong polar interactions with hydroxyl groups from the cellulose (Figure 2.30). The electrostatic interaction between the dye and cellulose microfibrils could provide a plausible explanation of how Congo Red enhances the birefringence of cellulose. The average dipole moment of the dye molecules lies parallel to the long axis of the cellulose microfibrils, so the dye-cellulose complex is strongly dichroic or birefringent. This is the principle that Congo Red was used to determine the cellulose fibril orientation using polarization confocal microscopy (Verbelen and Kerstens, 2000).

The dye, Congo Red tends to accumulate in the tip or apical regions of the elongating fiber cell (Figure 2.9). There are two possible reasons. First, there may be more active synthesis of cellulose in the tip regions than other regions during the elongation phase. More extensive incorporation of ^{3}H-glucose and ^{3}H-sucrose has been shown in the tip regions of the elongating cotton fiber (Ryser, 1977). Second, cellulose in the tip regions may be more amorphous or less crystalline, so Congo Red is easily accessible. It is proposed that there could be at least two intermediate outcomes that lead to alteration of the cell walls in submerged cotton fibers caused by Congo Red: decreased crystallinity of cellulose and disruption of cross-links between cellulose and other cell wall polymers. The mechanism of Congo Red's effect on cellulose synthesis in cotton fibers appears to be similar to that of chitin synthesis (Herth, 1980). The dye binds to nascent glucan chains, thus preventing their lateral aggregation into microfibrillar structures and leading to their random distribution (Vannini et al., 1983). The decreased crystallinity of cellulose caused by Congo Red treatment was also supported by the X-ray

diffraction data, which indicate a significantly decreased crystallite size of cellulose in submerged cotton fibers grown in the presence of Congo Red.

Cellulose is the major framework of plant cell walls. Therefore alteration of the cellulose formation tends to lead to changes of plant cell morphogenesis. This is demonstrated by the nodulation phenomenon and retarded cell elongation of submerged cotton fibers in the presence of Congo Red. In yeasts, Congo Red causes an anomalous morphology by interrupting crystallization of the chitin microfibrils (Pancaldi et al., 1985). Calcofluor White (also known as Tinopal LPW), a fluorescent brightening agent, disrupts assembly of cellulose microfibrillar sheets in the bacterium *Acetobacter xylinum* (Haigler et al., 1980). In *Oocystis aoiculata*, Calcofluor White also caused the cellulose microfibrils to be less organized (Roberts et al, 1982). In the presence of Congo Red, elongation of the fibrous wall at the surface of protoplasts of *Physcomitrella* was inhibited, resulting in shorter and atypical fibers with variable thickness (Burgess and Linstead, 1982). It is postulated that the formation of a "nodule" might be due to wall loosening which results in cell wall materials leaking outwardly. Wall loosening is required for plant cell expansion, and this process is believed to be regulated by auxin, which induces wall acidification (Hoson, 1993). Cell wall-modifying enzymes such as expansins, which are suspected to be responsible for breaking down the non-covalent bonds between cellulose and other wall polysaccharides, are more active under acidic conditions (McQueen-Mason et al., 1992). Once Congo Red molecules bind to cellulose microfibrils, they prevent bound cellulose microfibrils from interacting with hemicellulose, other cellulose microfibrils, or cell wall proteins. As a result, the primary cell walls become less rigid and are loosened. The fact that CBH gold failed to bind to the leaking wall materials, implies that either cellulose was absent in the leaking wall materials or Congo Red occupied all the binding sites of cellulose for CBH gold in the leaking materials.

It is significant that Congo Red inhibited cell wall elongation. Plant cell growth is believed to be driven by internal turgor pressure and restricted by the extension ability of the cell wall (Cosgrove, 1999). Here inhibition of the fiber cell elongation caused by

Congo Red is most likely due to the changes of the wall extensibility. The composition of the wall and the cross linking of the wall components determine the wall extensibility (Darley et al., 2001). Shpigel et al. (1998) showed that a bacterial cellulose-binding domain (CBD) enhanced root elongation of *Arabidopsis* at low concentrations but inhibited root elongation at high concentrations. The protein seemed to disrupt the structure of cellulose deposited in the wall resulting in effects on growth. As discussed earlier, the expansins facilitate cell expansion by weakening the bonding between cell wall polymers.

An alternative explanation for the altered cell growth of fibers is that Congo Red may block communication between the cytoskeleton and the cell wall matrix resulting in a failure of cytoskeletal components to coordinate cellulose microfibril deposition. Seagull (1993) proposed that the orientation of cortical microtubules directs the orientation of cellulose microfibrils deposition in the cotton fiber walls. Seagull (1998) also showed that microfilaments play a role in fiber initiation. However there is a controversial hypothesis that alignment of microfibrils can occur independently of microtubules (Baskin, 2001). In my experiments, fiber initiation was inhibited by Congo Red indicating that fiber initiation may take place independently of microfilaments. In addition, establishment of the growth polarity did not appear to be directed by the cytoskeletal components. It is possible that alteration of cellulose structure caused a failure of cellulose microfibrils to respond to cytoskeletal components. Plant cells seem to lack the cytoskeleton-integrin-ECM signaling complex, but structural adhesions between the plasma membranes and cell walls play important roles in a wide range of developmental, reproductive and pathogenic processes (Brownlee, 2002). Although plant cell wall synthesis is regulated by the cytoplasmic signal transduction, the former gives feedback to the later in order to allow the organism to respond environmental changes. How the fiber cells respond to Congo Red effects is unclear, but there must be some signaling components in the cell wall that are responsible for the fiber development.

It is interesting to find production of amyloplasts in submerged cotton fibers grown in the presence of Congo Red. Amyloplasts descend from leucoplasts, which are

colorless and not regarded as progenitors of other plastids. They contain substantial starch granules and are found in roots and storage tissues such as cotyledons, endosperm and tubers. In cultured BY-2 tobacco cells, amyloplast development is related to the level of auxin and cytokinin (Miyazawa et al., 1999; 2002). Depletion of auxin in the culture media induced production of amyloplasts. On the other hand, cytokinin promoted amyloplast formation induced by auxin depletion. Auxin is required for cell growth, i.e. the increase of cell volume, which also requires cellulose synthesis. Cell growth and cellulose synthesis can be inhibited under the conditions that deplete auxin. The effects of Congo Red applied in the very early developmental stages of fiber growth mimic those of auxin depletion by interrupting both cellulose synthesis and cell growth.

Therefore in both cotton ovule cultures and BY-2 tobacco suspension cultures, it appears that interruption of cellulose synthesis might cause cells to use available carbon sources to make a storage polysaccharide – starch. For cellulose synthesis, UDP-glucose serves as the precursor (Carpita and Delmer, 1981). UDP-glucose is synthesized from Glc6P (Haigler et al., 2001). ADP-glucose has been identified as an exogenous precursor for starch synthesis (Möhlmann et al., 1997; Neuhaus and Emes, 2000). Glucose 6-phosphate (Glc6P) is also identified as the most efficient precursor of newly synthesized starch (Möhlmann et al., 1997). Glc6P is synthesized from glucose. Therefore both starch synthesis and cellulose synthesis utilize the same carbon source - glucose.

Starch is composed of a mixture of 20 to 30 % amylose (with 0.1% branch points) and 70 to 80 % amylopectin (with about 4% branch points). Both consist of polymers of α-(1→ 4)-D-glucose. In amylose these are linked -(1→ 4)-, with the ring oxygen atoms all on the same side, whereas in amylopectin about one residue in every twenty or so is also linked -(1→ 6)- forming branch-points. The amylopectin molecules are oriented radially in the starch granule. The organization of the amylopectin molecules displays the amorphous and crystalline regions (or domains) generating the concentric layers that contribute to the "growth rings" that are visible by light microscopy (http://www.lsbu.ac.uk/water/hystac3.html; by Martin Chaplin). The crystallinity of starch gives rise to birefringence of the starch granules. The starch granules observed in

submerged cotton fibers lacked concentric rings, possibly due to cultures being kept at constant temperature.

However I cannot explain why CBH II had affinity for starch granules produced in submerged cotton fibers. It could be non-specific binding, but the control amyloplasts from endosperm showed no labeling with CBH II. Further, to confirm whether or not amyloplasts in submerged cotton fibers induced by Congo Red produce cellulose besides starch, CBH I labeling was studied. It turned out that the intensity of CBH I gold labeling to amyloplasts in submerged cotton fibers was very low, indicating the absence of cellulose in amyloplasts. To exclude possibility that CBH II might interact with other polymers such as proteins, 1% SDS was used to treat the sections before incubating with CBH II. The results showed that CBH II had a very low affinity for amyloplasts (Figure 2.31). Therefore, labeling of CBH II to amyloplast (without SDS treatment) may indicate non-specific binding rather than the presence of β-1,4-glucan in amyloplasts.

Conclusions

Submerged cotton culture provides an excellent system to study effects of agents like Congo Red, that cannot be translocated from cell to cell, on cotton fiber development. Addition of Congo Red in the culture media resulted in inhibition of cell growth and alteration of the cell wall formation. The crystallinity of cellulose also was decreased by Congo Red treatment. Production of amyloplasts induced by Congo Red may reflect an unknown mechanism to shift carbon metabolic pathways from cellulose synthesis to starch synthesis.

Table 2.1 Effects of Congo Red on the cellulose crystallinity of cotton fibers (older than 50 DPA).

Sample	Reflection	Position (2θ)	d(Å)	I%	FWHM	Crystallite size (Å)	Crystallite size average (Å)
CR_SUB	101	16.815	5.2681	17.2	2.106	38	38
	10-1	14.837	5.9657	19.9	2.085	39	
	002	22.694	3.9150	100.0	2.145	38	
SUB	101	14.800	5.9806	29.9	1.708	47	46
	10-1	16.772	5.2817	25.1	1.781	45	
	002	22.823	3.8932	100.0	1.820	45	
AIR	101	14.675	6.0315	31.8	1.704	47	49
	10-1	16.744	5.2903	20.9	1.584	51	
	002	22.823	3.8932	100.0	1.709	48	
VIVO	101	14.812	5.9760	55.8	1.572	51	53
	10-1	16.814	5.2685	32.8	1.515	53	
	002	22.904	3.8796	100.0	1.508	54	

Figure 2.1 A cotton ovule grown in the culture medium containing 5 μM Congo Red. Note only submerged cotton fibers were stained.

Figure 2.2 Cotton ovule epidermis of submerged portion incubated Congo Red. A), incubated with 0.1 μM Congo Red for 1d starting with 0 DPA; B) incubated with 2.5 μM Congo Red for 1d starting with 0 DPA. Note, only initiated fiber cells take up Congo Red. At this point, most of the fiber cells are in the initiation stage, and fiber elongation has not occurred. Also there were more fibers stained with Congo Red at the high level of Congo Red than the low level of Congo Red. Bar=100 μm.

Figure 2.3 Submerged ovule portion at 1 DPA. The ovule culture was started 1d before anthesis. After culture under normal growth conditions for 24 hours, the culture was treated with 2.5 μM Congo Red for 1 day. Note that very few fiber cells are stained with Congo Red, indicating that most of epidermal cells have not begun initiation at this stage. Bar= 100 μm.

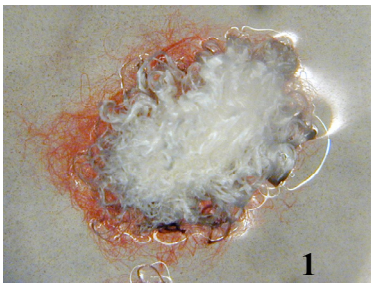
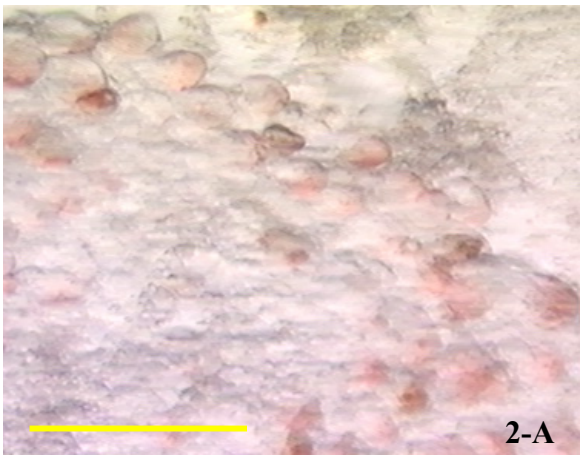
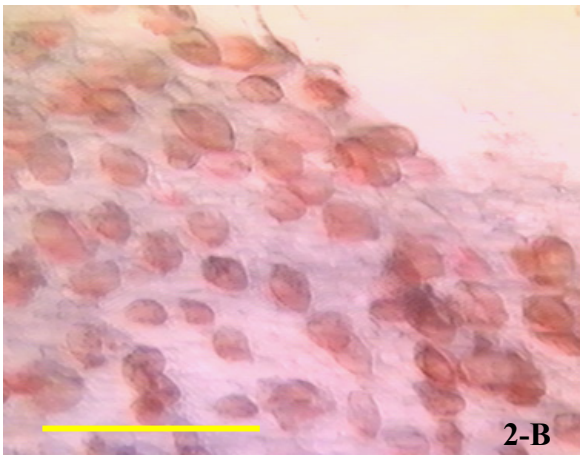
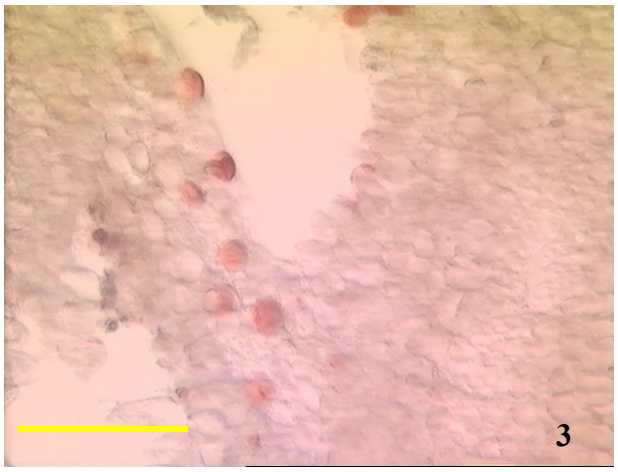


Figure 2.4 Submerged portion of cotton ovules that have been cultured, starting on the day of anthesis, continuing for 1d in the absence of Congo Red, then for 1d in the presence of 2.5 μM Congo Red, and finally chased with Congo Red-free medium for another 5d. Note most of fiber cells are short and have blunt fiber tips with concentrated Congo Red (black arrows); A fiber (red arrow) without apparent dye staining has tapered tip implying it initiated later. Bar= 100 μm.







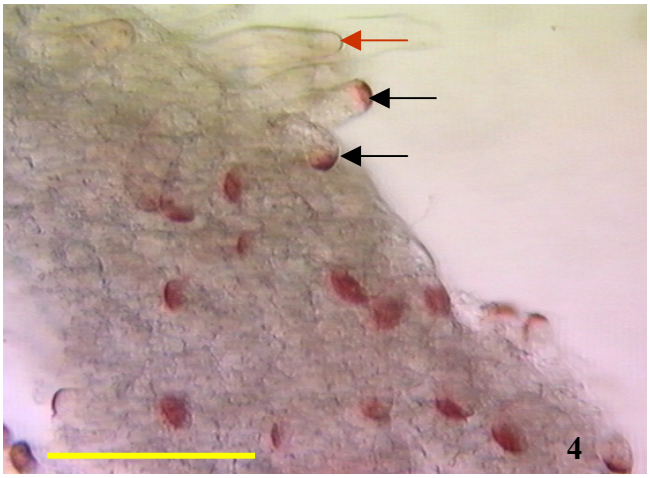


Figure 2.5 Cotton ovule epidermis of submerged portion incubated with 2.5 µM Congo Red for 1d starting at 0 DPA, then chased with the fresh medium for 5d. Notice that the majority of fiber cells stained by Congo Red failed to recover even in the fresh medium; Bar=100 µm

Figure 2.6 Submerged portion of cotton ovules that have been cultured in the absence of Congo Red for one day, starting at the time of anthesis, then incubated in the presence of 2.5 µM Congo Red for another day. Note, at this stage, most of the fiber cells have fully initiated and may have established growth polarity. Congo Red is more concentrated in the apical regions of these fiber cells (arrows). Bar=50 µm.

Figure 2.7 Submerged cotton ovule epidermis that was incubated for 1d in the absence of Congo Red starting at 0 DPA, then for 12 d in the presence of 0.5 µM Congo Red. Note that fiber elongation was greatly inhibited; Congo Red accumulated in the apical regions of the fiber cells. Bar=100 µm

Figure 2.8 Submerged cotton fibers of 6 DPA that were incubated in fresh medium for 1d and in the presence of 2.5 µM Congo Red for another 5d. Bar=50 µm.

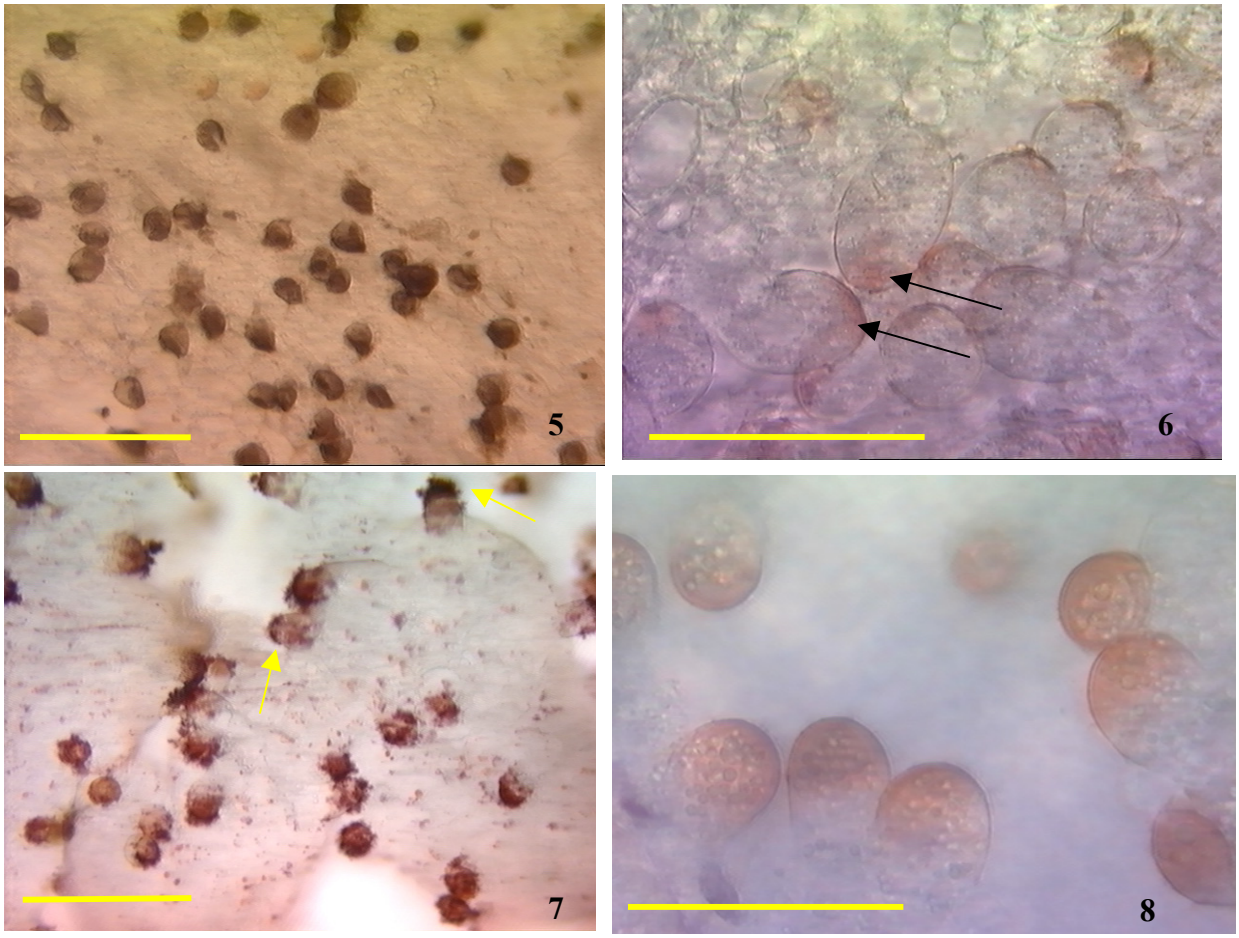
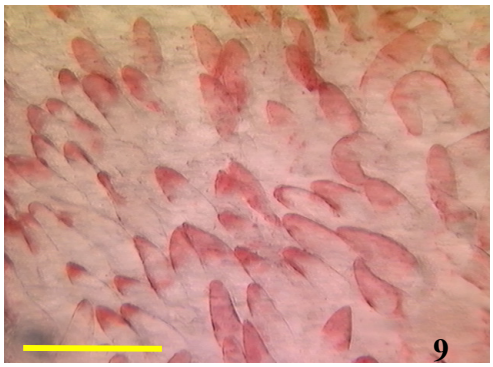
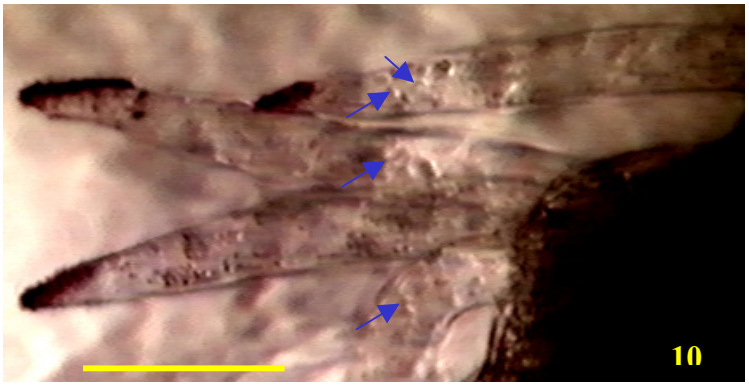


Figure 2.9 Submerged cotton fibers of 3 DPA grown in the presence of 2.5 μM Congo Red during the last day (the culture started at 1 DPA). Note the ubiquitous binding of dye in the fiber cell walls and higher concentration in the tapered apical areas. Bar=100 μm.

Figure 2.10 Submerged fibers at 10 DPA that were grown for 2d (0-2DPA) in fresh culture medium, 4d (2-6DPA) in the presence of 2.5 μM Congo Red, and 4d in the fresh culture medium. Note the accumulation of dye in the tip area indicating the absence of the tip growth. Several cellulose fibril bands are readily seen here (arrows). The extremely short length of fibers implies that the application of Congo Red in the early primary wall stage inhibited cell elongation. Bar=50 μm.

Figure 2.11 Congo Red (2.5 μM) treated, submerged fiber at 10 DPA. Top, viewed with brightfield light microscopy, and bottom, viewed by dark-field light microscopy. Notice the bands of cellulose microfibrils (arrows) and globular structure indicating the outgrowth of the cell wall materials. Bar=50 μm.





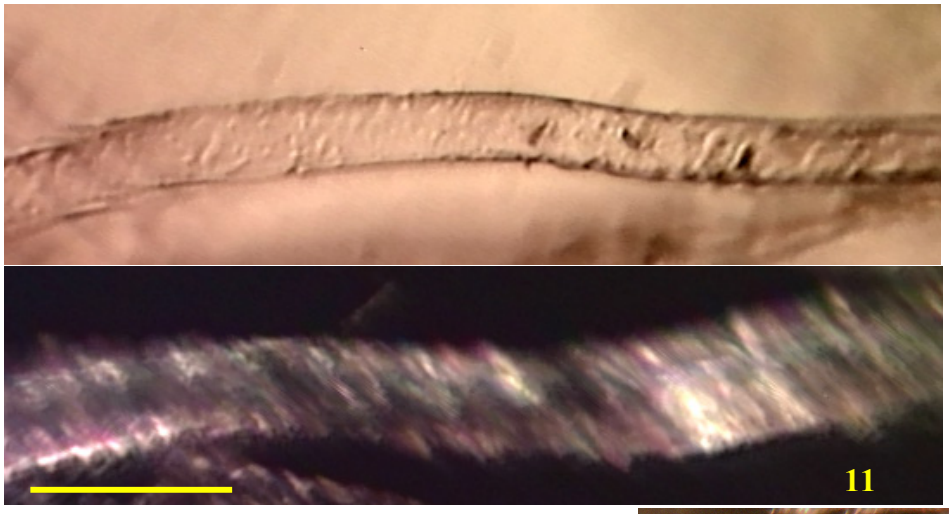


Figure 2.12 Dark field view of 23 DPA cotton fibers treated with 2.5 μM Congo Red for 4d starting at 1 DPA, then chased without the dye for an additional 4 d. Then Congo Red was added continually until 23 DPA. Note most fibers are very short

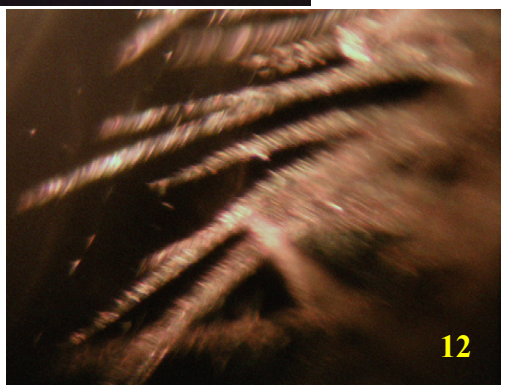


Figure 2.11 Congo Red (2.5 μM) treated, submerged fiber at 10 DPA. Top, viewed with brightfield light microscopy, and bottom, viewed by dark-field light microscopy. Notice the bands of cellulose microfibrils (arrows) and globular structure indicating the outgrowth of the cell wall materials. Bar=50 μm.

Figure 2.12 Dark field view of 23 DPA cotton fibers treated with 2.5 μM Congo Red for 4d starting at 1 DPA, then chased without the dye for an additional 4 d. Then Congo Red was added continually until 23 DPA. Note most fibers are very short.

Figure 2.13 24 DPA fiber was treated with 2.5 μM Congo Red during 1-5 DPA and 9-24 DPA. The photo was taken under dark field illumination. Each spherical bundle might have been assembled by a cluster of TCs.

Figure 2.14 SEM observation of cotton fibers treated with 2.5 μM Congo Red. The fibers were grown in the culture medium without Congo Red for 2 d, then incubated for 4 d in the presence of Congo Red, and chased for an additional 4 d in the culture medium without Congo Red. The nodules initiated from the outer layer of the primary cell walls are consistent with the spherical bundles viewed under dark field and birefringent spots viewed by polarization microscopy. It is likely that nodules are susceptible to dehydration and breakage, due to loosely packed cell wall materials.

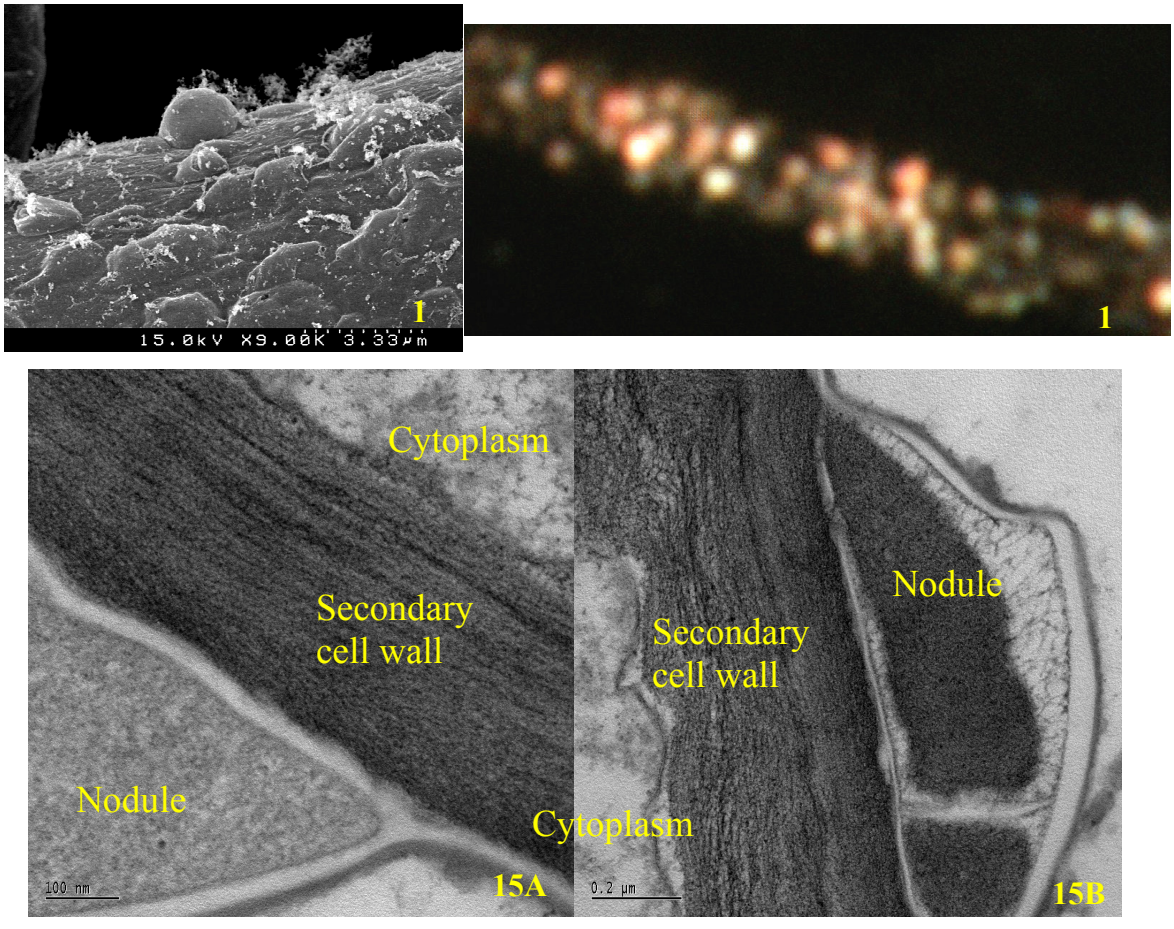
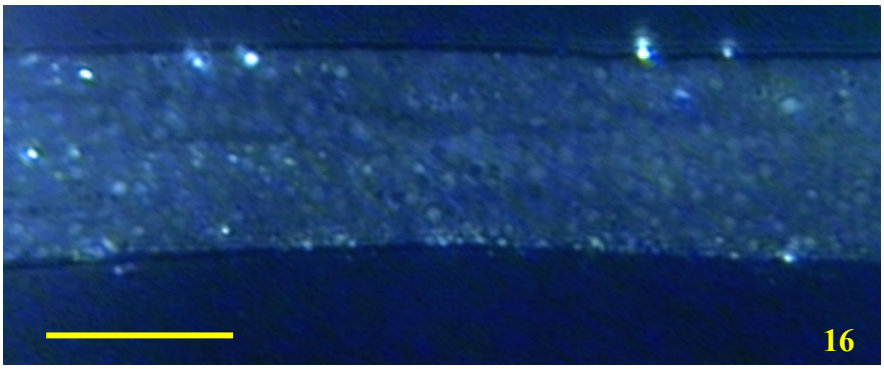
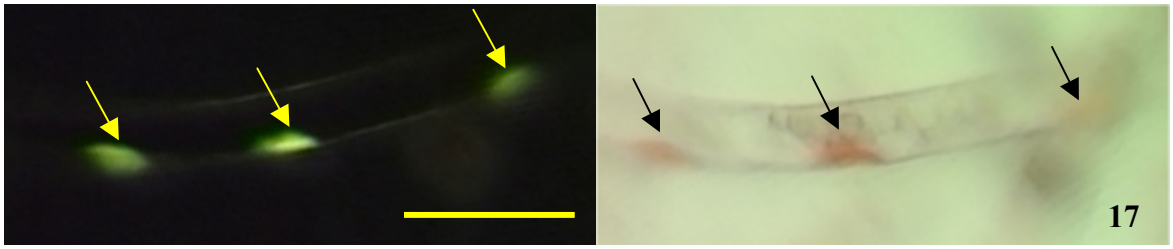


Figure 2.16 A 20 DPA submerged cotton fiber treated with 2.5 µM Congo Red during 16-20 DPA and viewed by polarization microscopy. Note the birefringent spots which appear to be localized concentrations of the dye or dye/cellulose complex. Bar=20 µm

Figure 2.17 Submerged fibers at 14 DPA that have been cultured in the absence of Congo Red for 12d, then in the presence of 2.5 µM Congo Red for 2d. Left, viewed with polarized light; right, viewed under brightfield conditions. Note that the localized Congo Red staining (arrows) might be an indication of cell wall helical thickenings. Bar=50 µm

Figure 2.18 Polarization microscopy of 27 DPA cotton fibers treated with 2.5 µM Congo Red for 5 d starting at 22 DPA. Binding of Congo Red to cellulose microfibrils appears to enhance the birefringence of the cellulose microfibrils. Bar=50 µm





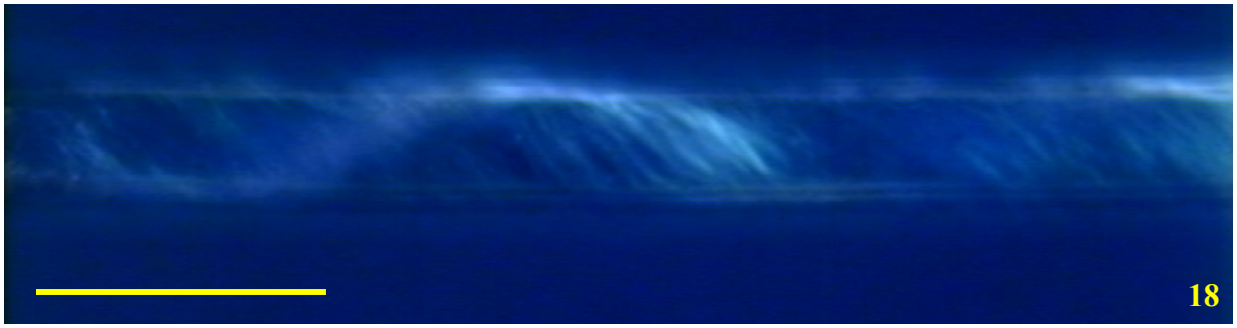


Figure 2.19 TEM analysis of effects of Congo Red on submerged cotton fiber growth. Thin sections of the submerged cotton fibers at 10 DPA grown in the fresh medium for 2d (0-2 DPA), in the presence of 2.5 μM Congo Red for 4d (2-6 DPA), and chased with the fresh medium for 4d (6-10 DPA). A), labeled with CBH I gold; B), labeled with CBH II gold. Note outgrowth of wall materials making the nodules (arrows), which accumulate locally surrounded by a cuticle, indicating formation of the secondary cell walls. Wall materials in the nodules might have been lost (D) during the process of dehydration, probably due to randomly ordered wall material. Both CBH I and CBH II gold bind to the fiber cell wall (CW), but no labeling occurs in the outgrowth materials of the fiber cells caused by Congo Red treatment .Bar=200nm.

Figure 2.20 Cellulose microfibrils of Congo Red treated cotton fibers viewed by TEM. Left and middle, ultrathin section of cell walls from 10 DPA fibers treated with Congo Red during 2-6 DPA. Right, negative staining of cellulose microcrystal preparations from mature *in vivo* fiber. Note cellulose microfibrils present in outer primary cell wall (left) might have attached Congo Red. On the other hand, absence of distinctive cellulose microfibrils in the inner primary cell wall might be due to chase incubation in the medium without Congo Red during 6-10 DPA. Cellulose microfibrils enhanced by Congo Red appear to be less ordered and less crystallized but even thicker than that from the secondary cell walls (right).

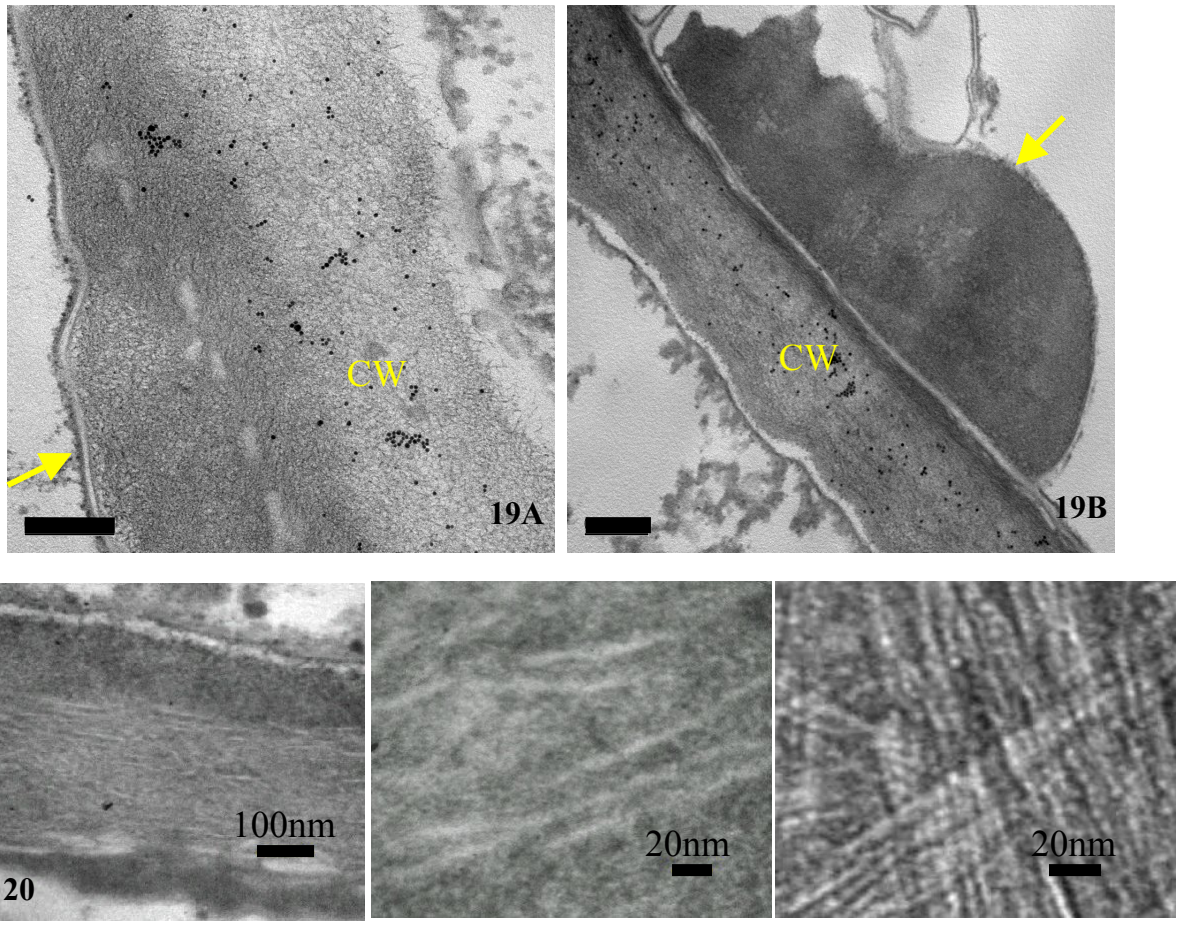
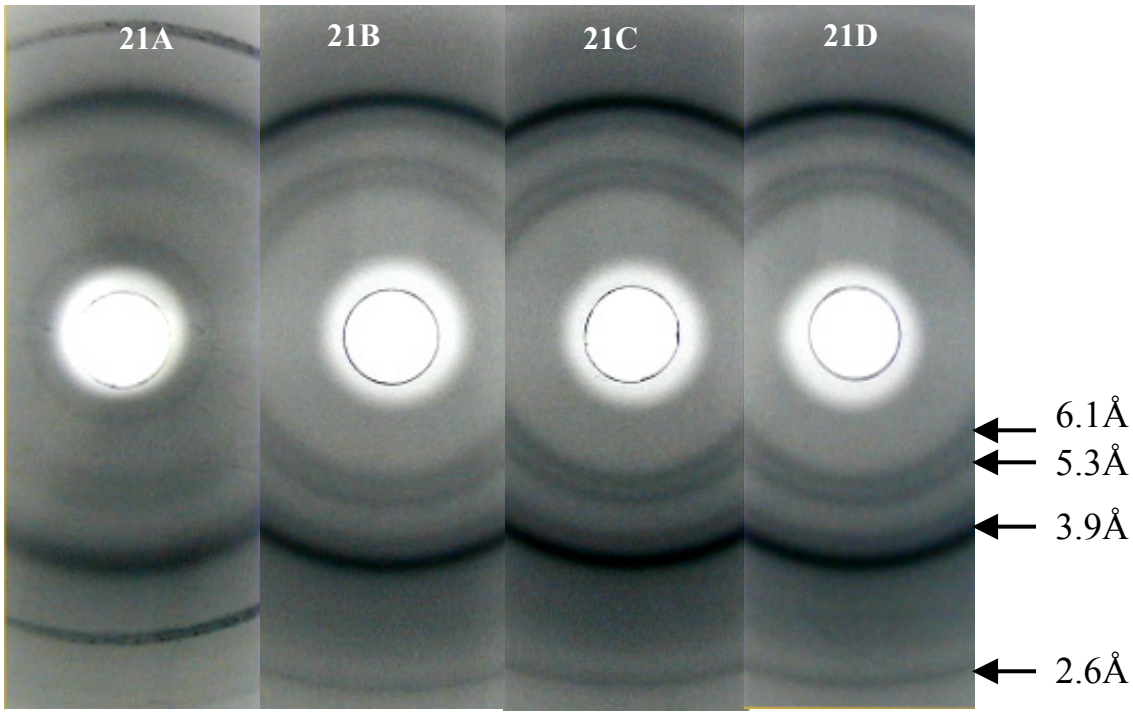


Figure 2.21 X-ray diffraction patterns of cellulose I in cotton fibers: a- submerged cotton fibers treated with Congo Red, b- submerged fibers, c- air-grown fibers, and d- fibers grown in vivo (b, c, and d, no Congo Red). Line broadening of the 6.1Å and 5.3Å reflections suggests that Congo Red can dynamically alter the degree of crystallinity of cellulose.



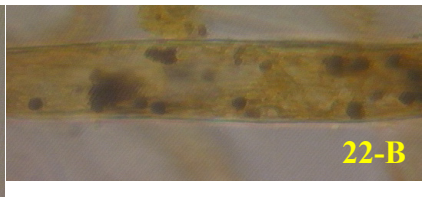
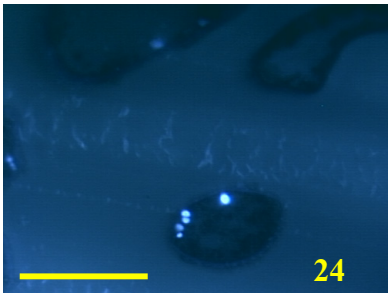
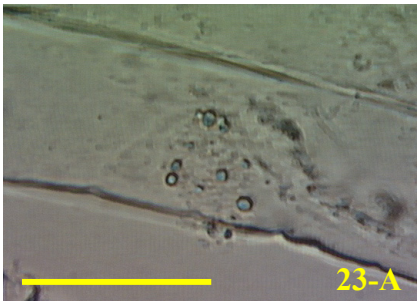
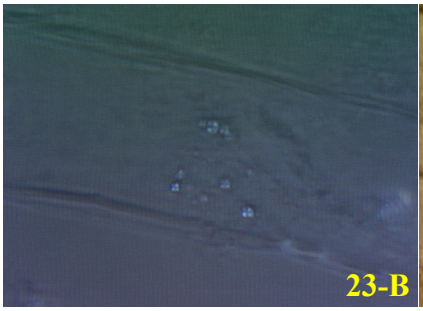
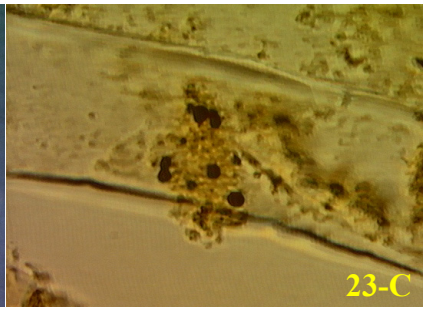


Figure 2.22 Iodine staining of submerged fibers at 14 DPA, culture started at 1 DPA, in the presence of Congo Red for 4 DPA, then followed with Congo Red free medium for 9 DPA. Note amyloplasts, stained dark purple, were present in both tip region (A) and basal region (B) of the fibers. Bar=20 μm.









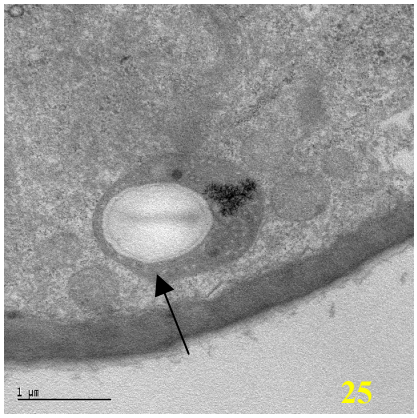


Figure 2.23 Amyloplasts in submerged cotton fibers treated with Congo Red. A), observed with regular light microscopy; B), viewed with polarizing light; C), viewed after stained with iodine. Bar=20 μm.

Figure 2.24 DAPI staining of cross sections of submerged fibers treated with Congo Red. Amyloplasts appeared bright blue viewed with fluorescent light. Bar=20 μm

Figure 2.25 Amyloplasts (arrow) viewed with TEM.

Figure 2.26 A, section of Congo Red treated cotton fiber cells (10DPA) stained with Lugol's Iodine solution. Note amyloplasts (black arrows) appeared to be purple granules after staining with iodine. Also, amyloplasts are frequently present in the submerged fiber cells grown in the presence of Congo Red. B, thick section of submerged cotton fibers (14DPA) stained with Lugol's Iodine solution showing a lack of amyloplasts in fiber cells not treated with Congo Red.

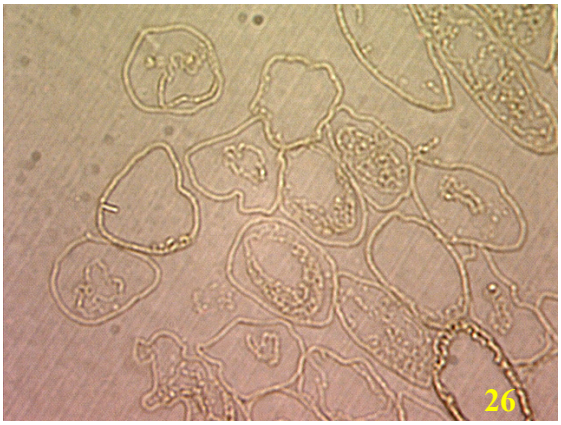
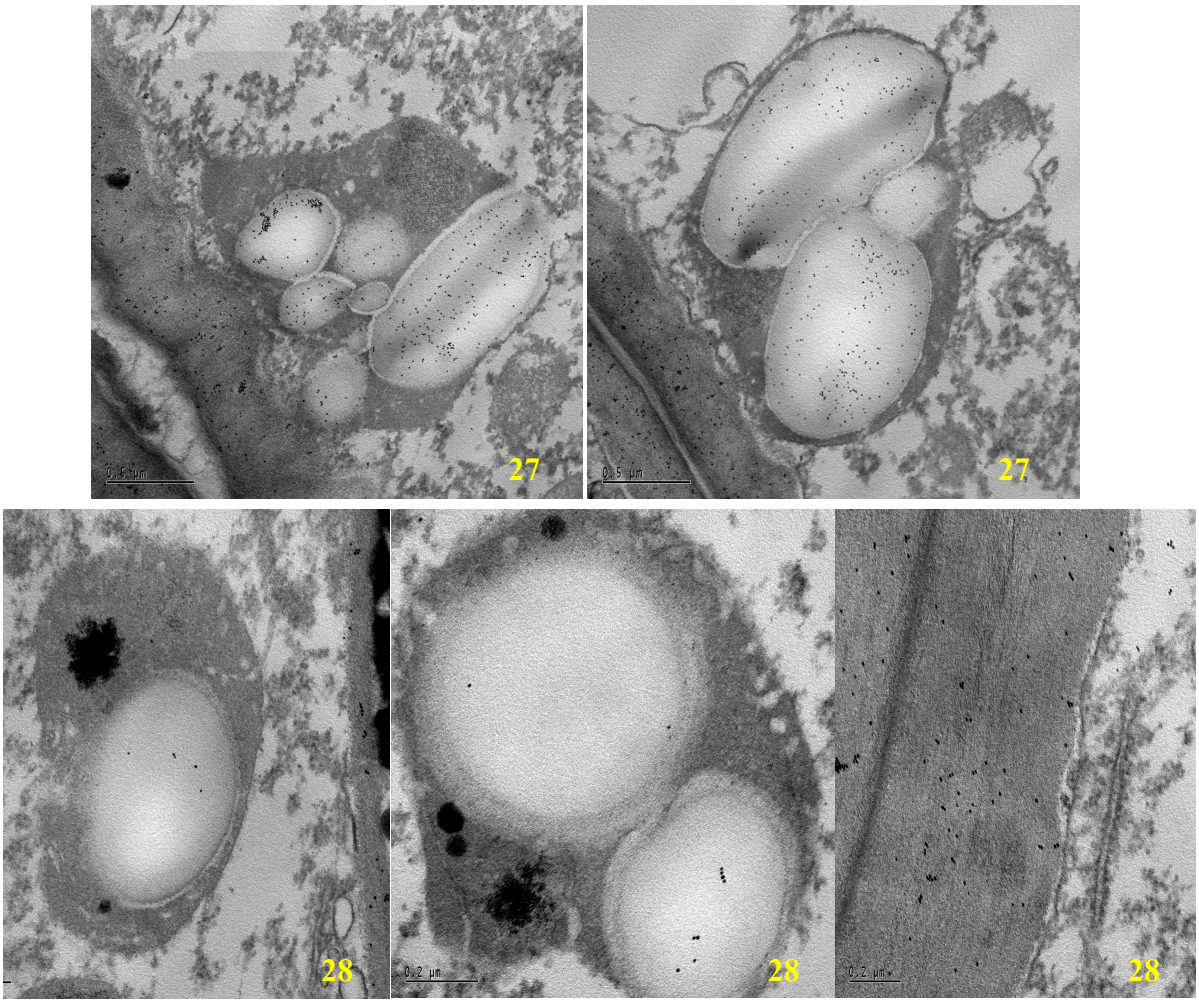


Figure 2.27 A-B, Ultrathin sections of Congo Red treated submerged cotton fibers (10DPA) labeled with CBH II gold. Note that labeling is present not only in fiber cell walls, but starch granules were also extensively labeled with CBH II gold.
Figure 2.28 Ultra thin sections of air grown portion of the cotton ovule (14 DPA) labeled with CBH II gold. Note starch granules of the amyloplasts from cotton ovules had low affinity to CBH II gold (A and B) whereas the fiber cell wall shows relatively dense labeling by CBH II gold (C).



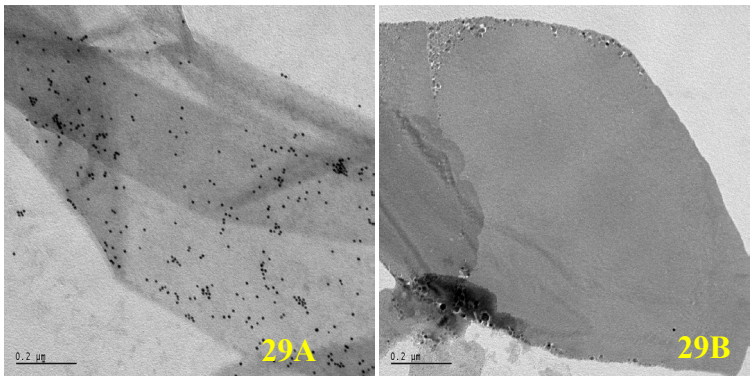
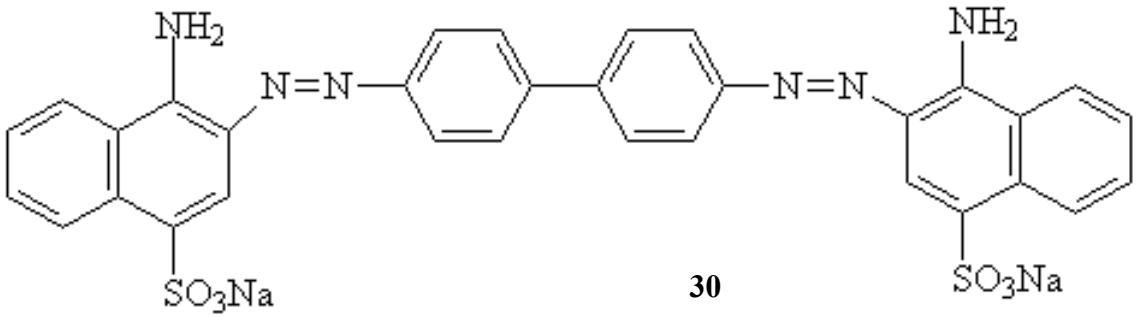


Figure 2.29 CBH II labeling test to cellulose powder (A) and starch powder (B). Note CBH II had extensive labeling to cellulose whereas it hardly bound to starch.



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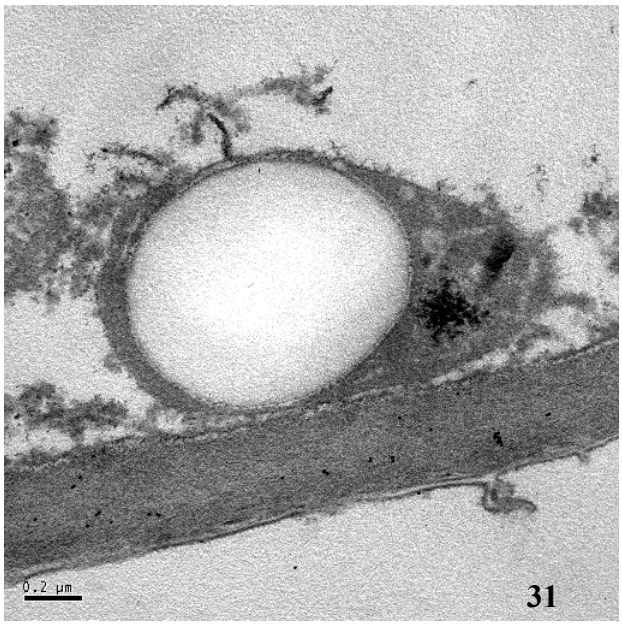


Figure 2.30 The structure of the molecule of Congo Red

Figure 2.31 CBH II labeling to ultrathin sections of Congo Red treated submerged cotton fibers (10DPA). The section was rinsed with 1% SDS to remove proteins prior to incubation with CBH II gold. Note that there was no evident CBH II binding to the amyloplast.

Chapter 3 Preliminary investigation of effects of auxin depletion on submerged cotton fiber growth

Summary

This study investigated whether or not auxin depletion in the culture medium will cause the submerged cotton fibers to produce amyloplasts. Auxin (indole-3-acetic acid) depletion in the early stage (1-2 DPA) of cotton ovule development resulted in limited submerged cotton fiber growth, and amyloplast analysis could not be carried out. When auxin depletion occurred at 7 DPA and 14 DPA, there were no amyloplasts found in the submerged cotton fibers, but cellulose microfibrils in the secondary cell wall were greatly disorganized. Possibly, indole-3-acetic acid might play an important role in regulating the arrays of microtubules which, in turn, may help to organize the patterns of cellulose deposition.

3.1. Introduction

In Chapter two, amyloplast production in submerged cotton fibers induced by Congo Red treatment was discussed. This abnormal phenomenon also occurred in BY-2 tobacco cell cultures under conditions of auxin depletion (Miyazawa et al., 1999; 2002). Around a century ago, auxin was discovered as the hormone involved in the bending of coleoptiles toward light (Taiz and Zeiger, 1998). Auxins affect many developmental processes such as promoting cell elongation, inducing cell division, promoting lateral root formation, and regulating apical dominance (Kende and Zeevaart, 1997; Taiz and Zieger, 1998). Auxins promote cell elongation by inducing proton extrusion into the cell wall, and wall acidification leads to an increase in wall extensibility (Taiz and Zeiger, 1998). Therefore under auxin depletion conditions, the wall extensibility will be limited, and further wall formation will be inhibited. In other words, auxins can also affect cellulose synthesis. Congo Red interferes with cellulose assembly in submerged cotton fibers by binding to nascent cellulose microfibrils, thus preventing lateral associations between microfibrils. Interruption of cellulose synthesis leads fiber cells to produce amyloplasts as shown in Chapter 2. To relate Congo Red treatment with auxin depletion, one needs to ask if auxin depletion can cause submerged cotton fibers to produce amyloplasts.

Preliminary results showed that no amyloplasts were produced in submerged cotton fibers grown in a medium without auxin; however, deposition of the secondary cell wall displayed unusual patterns under these treatment conditions. The altered patterns of the secondary cell wall thickening will be illustrated in this chapter, and possible explanations will be discussed.

3.2. Materials and Methods

Cotton ovule culture

Cotton ovules of 0 DPA or –1 DPA were grown in the culture medium as described previously (Feng and Brown, 2000). The cultured ovules were transferred to the culture media without auxin (IAA) at various stages: 1 or 2 d, 7 d, and 14 d after initiating culture.

Iodine staining

Iodine staining was carried out when the ovule cultures were about 24-28 DPA. The whole mount of submerged cotton fibers were stained with Lugol's Iodine solution containing 1% (w/v) iodine and 2% (w/v) potassium iodine. Observations were made under light microscopy to investigate the presence of amyloplasts.

Microscopic observation of secondary cell wall thickening

Submerged cotton fibers were excised from the cotton ovules at about 50 DPA. Excised fibers were mounted in the culture medium, and observed with the polarized light. Images were captured via an Optronix CCD camera.

The fiber samples were also prepared for transmission electron microscopy (TEM). Cotton ovules of interest were fixed in 4% glutaraldehyde in 1x culture medium for 2h at room temperature. After washing with culture medium and 0.1 M cacodylate buffer (pH 7.2), the samples were immersed in 2% osmium in 0.1 M cacodylate buffer for 2h, then washed with 0.1 M cacodylate buffer and distilled water. Dehydration conditions were 70% ethanol for 2x15min, 100% ethanol 2x 15 min, and 100% acetone 2x 15min. The samples were infiltrated in acetone/Epon812: 2/1 for overnight, in acetone/Epon812: 1/2 for 24h, and in full Epon 812 for 24h with one change of fresh Epon. Embedded tissue was polymerized for 14 h at 60 ^{0}C. Ultra thin sections were prepared with a thermal advance microtome and mounted on copper grids. After staining with 2% uranyl acetate and lead citrate, the sections were observed with a Philips 420

transmission electron microscope. Images were captured with a GATAN (Bioscan or Gatan 622) camera.

3.3. Results

Absence of amyloplasts

Cotton ovules that were cultured in the absence of IAA from 1 or 2 DPA had very limited fiber growth. Apparently, exogenous IAA is required for the young ovules to grow fibers. A majority of the submerged portion of ovules produced callus. Therefore, it was impossible to analyze amyloplast production in the submerged cotton fibers.

Submerged cotton fibers reach almost half of the fiber maximal length at 7 DPA, and complete fiber elongation at around 14 DPA. IAA depletion administrated at 7 DPA or 14 DPA had little effect on submerged fiber growth. Iodine staining was used to detect the presence of amyloplasts in submerged fibers grown in the absence of IAA. There were no amyloplasts observed in the submerged fibers that had been grown in the IAA-depleted media since 7 DPA or 14 DPA.

Alteration of cellulose microfibril organization in secondary cell walls

The secondary cell wall thickenings displayed altered patterns in submerged fibers grown in the absence of IAA (Figure 3.1). In the medium supplemented with IAA, the cellulose microfibril organization displayed smooth and regular patterns (Figure 3.1I). In the growth environment without IAA, the secondary cell wall thickenings appeared to be pits and patches, or irregular shapes of clusters (Figures 3.1A-H). It seemed that cellulose deposited locally that might surround the clusters of the cellulose synthase complex. The microfibril orientation appeared to lack any guidance from any cytoskeletal elements, and this was observed over the entire cell surface.

TEM analysis revealed that the cellulose microfibrils deposited locally seemed to be parallel with each other (Figure 3.2). This is consistent with the observations achieved with polarized light in which the cellulose microfibrils appeared to be in “patches” (Figures 3.1 A-H).

3.4. Discussion

The lack of amyloplasts in the submerged cotton fibers grown in the absence of IAA might be explained from several aspects. First, unlike BY-2 tobacco cell culture, whose hormonal source totally depends on the culture medium, submerged cotton fibers can obtain IAA from the cotton ovule in addition to the culture medium. Therefore auxin depletion might not be severe enough to prevent submerged cotton fibers from synthesizing cellulose. Secondly, submerged cotton fibers were more sensitive to IAA depletion in the early developmental stage (1-2 DPA), but limited fiber growth prevented us from analyzing amyloplasts. Ovule cultures were derived from ovules at 0 DPA. After 1-2 d of culture, the ovules cannot make sufficient auxin at this stage (Beasley and Ting, 1973; 1974; Beasley et al., 1974; Dhindsa et al., 1976) when transferred to the auxin-depleted medium.

Obviously, exogenous IAA was not required for secondary cell wall formation, because when IAA depletion was applied at 7 DPA and 14 DPA, submerged cotton fibers still produce significant secondary cell walls. However, endogenous IAA by itself could not meet the needs for the fiber cell to organize the cellulose microfibrils well. Additional IAA needed to be supplied to the medium to enable more 'normal' fiber development in this study.

The auxin content in cotton fibers has been studied (Jasdanwala et al., 1977; Naithani et al., 1981; 1982; Nayyar et al., 1989). The auxin level, which is assayed in the primary wall stage, represents the remaining auxin and does not reflect the level of auxin synthesis and consumption (Naithani et al., 1982). In upland cotton, the remaining auxin content goes up during the early elongation stage, peaks at around 10 DPA, and then decreases sharply till about 15 DPA (Naithani et al., 1982). It has been suggested that auxin oxidation might be related to cotton fiber development (Jasdanwala et al., 1977; Naithani et al., 1981). During the elongation stage, IAA oxidase, peroxidase (Jasdanwala et al., 1977), and o-diphenol oxidase (Naithani et al., 1981) activities were maintained at low levels, but these enzymes increased significantly during secondary thickening and maturation stages. Increases in enzymes mentioned above could lead to less accumulation

of endogenous IAA. Therefore, probably less endogenous IAA is required during the secondary cell wall synthesis stage than during the elongation stage. However, according to results from this study, the cultured ovules in the secondary wall stage still need a certain level of IAA to produce "normal" fibers. It appears that the cultured ovules during the secondary wall stage cannot produce sufficient IAA required for organization of the cellulose microfibrils in submerged fibers.

Gould and Seagull (2002) found that application of exogenous gibberellic acids increased the reversal frequency significantly. They suggested that gibberellic acids might regulate microtubule arrays. They also found that fibers treated with gibberellic acids and indole-3-acetic acid exhibited fewer Z-reversals than untreated fibers. However, to our knowledge, my study, for the first time, showed that indole-3-acetic acid is required for the organization of cellulose microfibrils in cotton fibers during the secondary cell wall deposition stage.

It is unknown how auxin may be involved in the deposition of secondary cell walls in cotton fibers, while the role of auxin in cell wall formation during cell expansion of woody dicot stem has been shown (Mellerowicz et al., 2001). The results from this work mimic those in cotton fibers treated with microtubule disrupting agents (Yatsu, 1983; Seagull, 1989; 1990). Disorganization or altered local deposition of cellulose microfibrils was exhibited when colchicine, a microtubule-disrupting agent, was applied to cotton locules (Yatsu, 1983) or ovules grown *in vitro* (Seagull, 1989). Therefore, it is suspected that indole-3-acetic acid might regulate arrays of microtubules.

In spite of the two factors resulting from this preliminary study, the lack of amyloplast production and the altered secondary cell wall organization in submerged fibers caused by auxin depletion do suggest interesting effects of IAA. Obviously, more evidence needs to be provided. Therefore, several experiments are proposed to be carried out in the future: 1) during the early fiber developmental stage, cultured ovules are treated with auxin depletion for a short term (12h-48h), and then followed with the regular medium. The purpose of this experiment would be to circumvent poor fiber growth with the long-term treatment of auxin depletion. Thus, there might be sufficient

fiber growth for amyloplasts analysis; 2) in addition to whole mount fibers to be used for iodine staining, fiber sections of young ovules will need to be prepared. In addition, these materials should analyzed using thin sections for TEM. These experiments could further confirm whether or not amyloplasts are induced in submerged fibers at early stage by auxin depletion; 3) air-grown fibers will be studied in the same way as above; and, 4) it will be important to investigate whether or not the arrays of microtubules are disturbed during auxin depletion treatment for better understanding of how auxin is involved in the organization of secondary cell walls in cotton fibers.

3.5. Conclusion

It has been shown that during auxin depletion there is no production of amyloplasts in submerged cotton fibers. Altered organization of cellulose microfibrils in the secondary cell wall of submerged cotton fibers through auxin depletion implies a role of auxin in the orientation of cellulose microfibrils.

Figure 3.1 (A-D) Submerged fibers viewed with polarizing light. A-D, submerged fibers grown in regular culture medium containing GA3 and IAA for 7 d starting with 0 DPA, then in the absence of IAA for 47 d; A, and C, viewed without a compensator; B and D, viewed with a compensator. Note that the secondary cell wall thickenings in the fibers grown in the condition of IAA depletion appeared to be pits or patches compared with the smooth pattern in the fibers grown in the IAA supplemented condition continuously. Bar=20 μm.

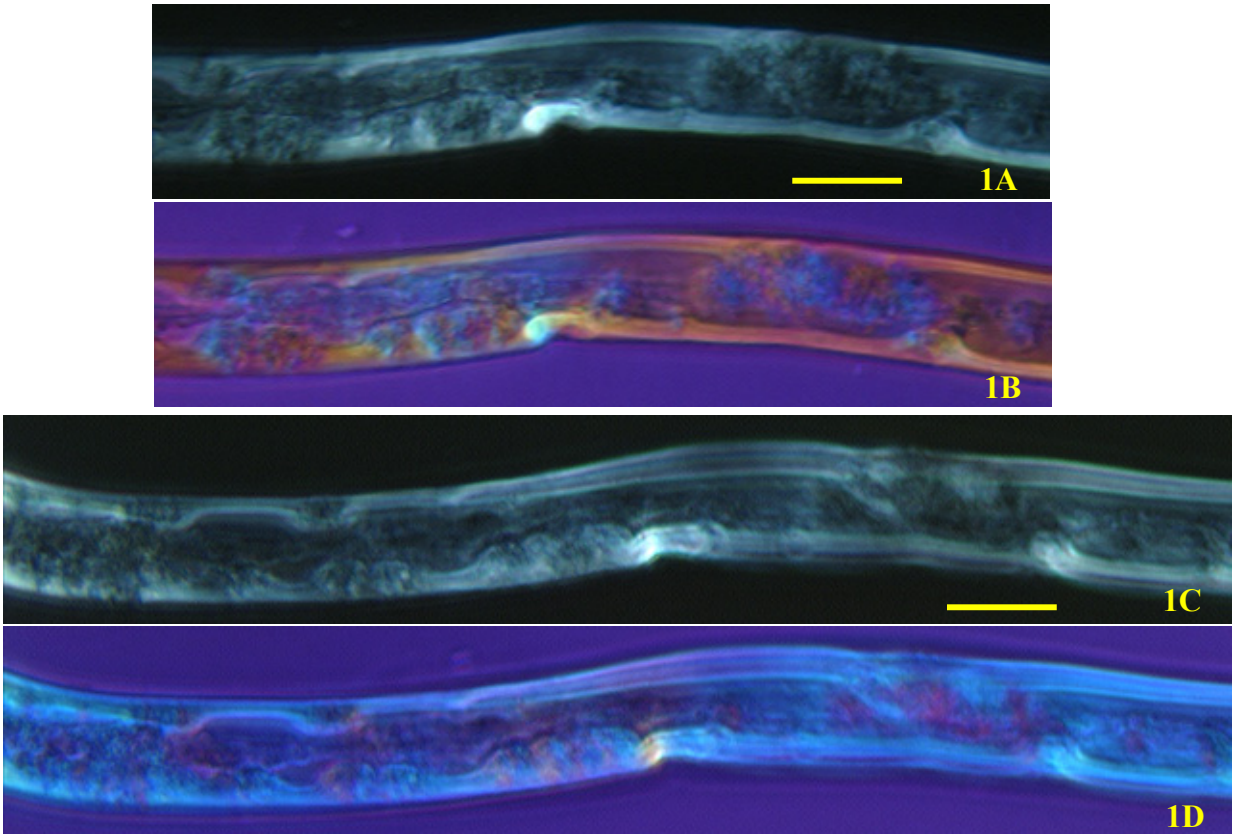


Figure 3.1 (E-I) Submerged fibers viewed with polarizing light. E-H, before being cultured in the IAA-depleted medium for 35 d, submerged fibers grown in the regular culture medium for 14 d starting with 0 DPA; I, a submerged fiber at 40 DPA grown in the regular culture medium continuously. E and G, viewed without a compensator; F, H, and I, viewed with a compensator. Note that the secondary cell wall thickenings in the fibers grown in the condition of IAA depletion appeared to be pits or patches compared with the smooth pattern in the fibers grown in the IAA supplemented condition continuously. Bar=20 μm.

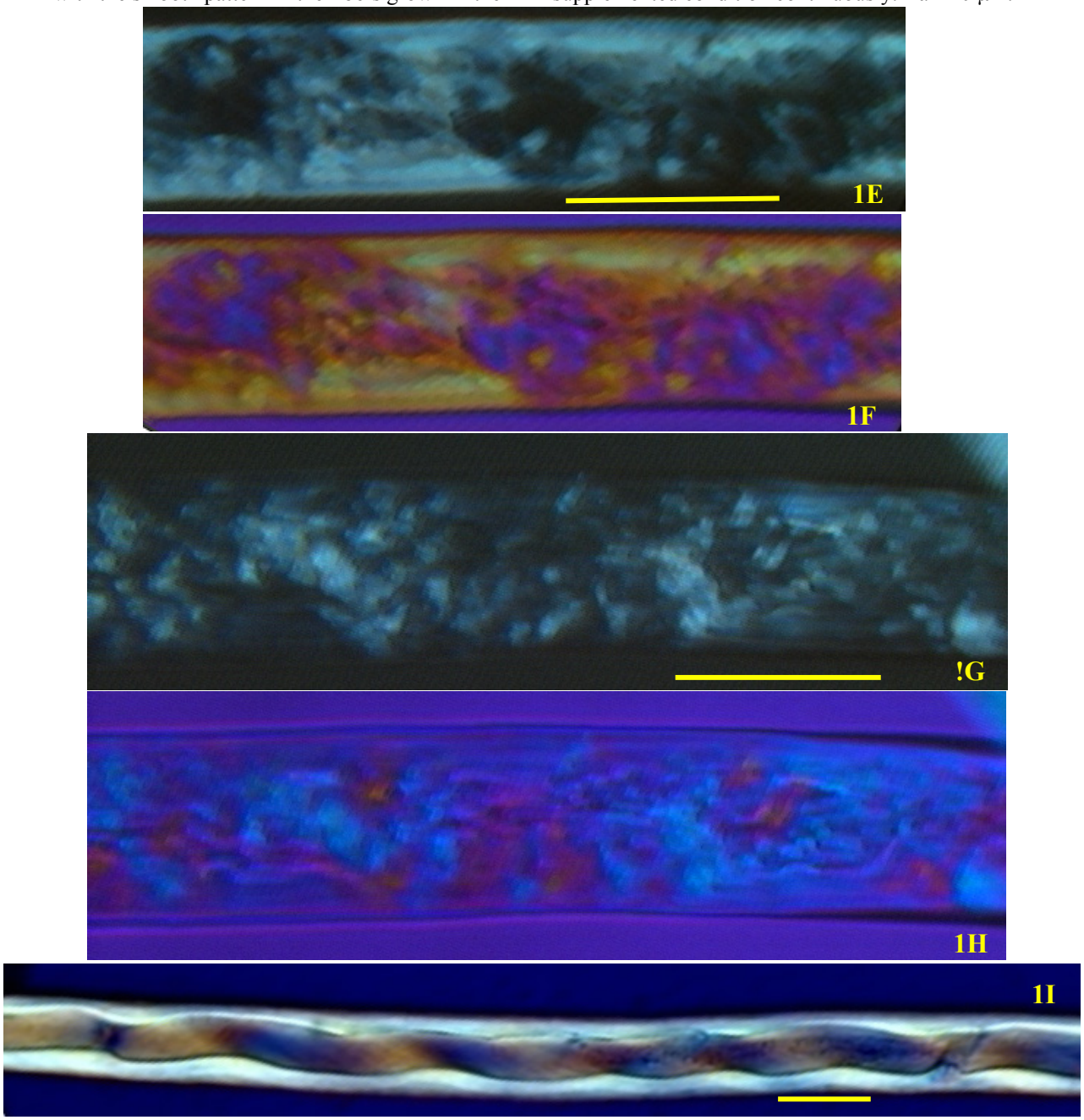
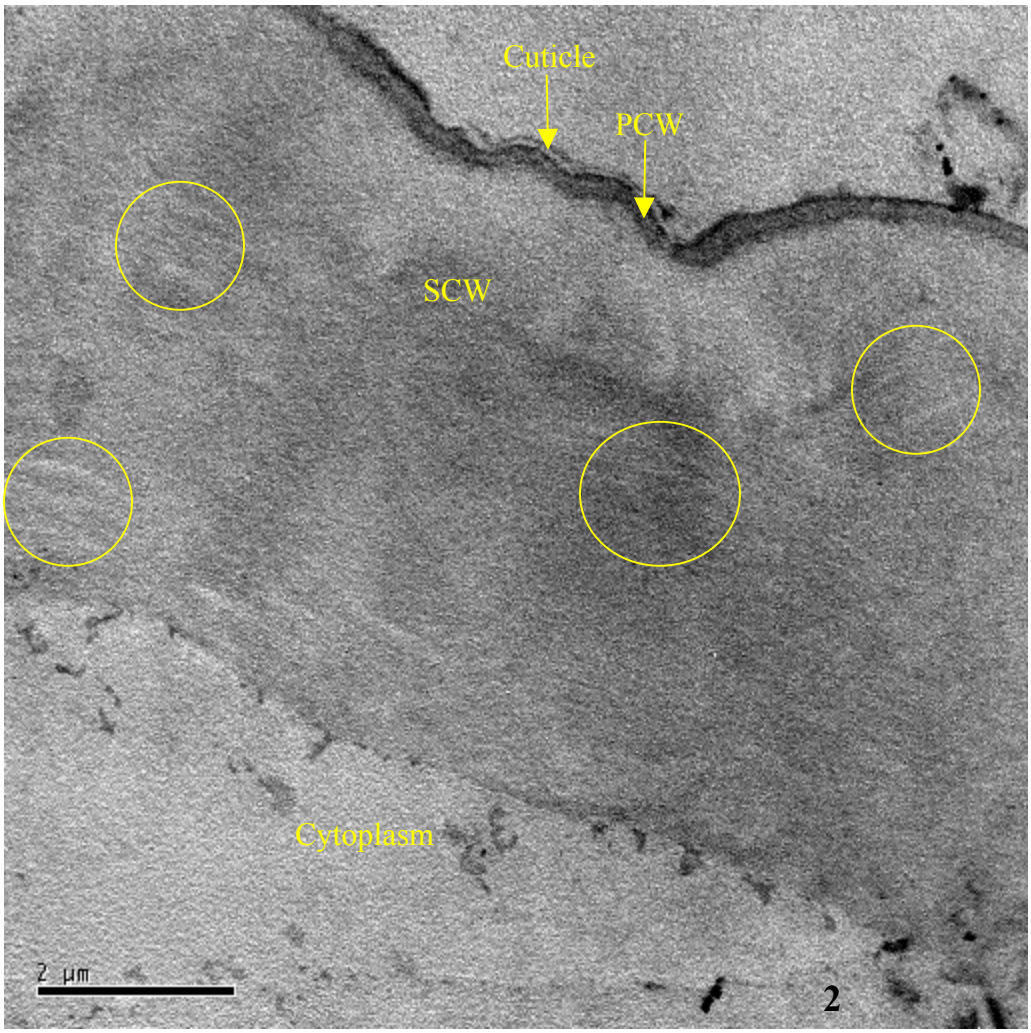


Figure 3.2 A cross section of a submerged cotton fiber that had been grown in regular medium for 15 d, then followed for 41 d in the IAA depleted medium. Locally deposited cellulose microfibrils (in yellow circles) appeared to be parallel orientation with each other.



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