MOLECULAR BIOLOGY AND PHYSIOLOGY

Fiber Initiation in 18 Cultivars and Experimental Lines of Three *Gossypium* Species

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ABSTRACT

A new technique was developed to study the cotton fiber initiation process and fiber initial densities. The goal was to provide an additional tool to cotton breeders and geneticists interested in fiber improvement. The objectives were to assess whether fiber initiation patterns reported for some *Gossypium hirsutum* L. and *G. barbadense* L. cultivars extend to a more diverse range of cultivars/lines; and to test if there is a relationship between fiber initials density, lint percentage, and fiber characteristics. This study was performed with 17 cultivars and experimental lines of *G. hirsutum* (upland cotton) and *G. barbadense* (Pima cotton) and one *G. arboreum* L. accession. The ovules were extracted from flowers at the day of anthesis and 1 and 2 d thereafter, stained with a fluorescent dye, $DiOC_6(3)$ (3,3′-dihexyloxacarbocyanine iodide), and observed under a light microscope. Staining cotton fiber initials with $DiOC_6(3)$ enabled the evaluation of a greater number of samples than the more time-consuming scanning electron microscopy protocols to assess the fiber initiation process. There were significant differences in fiber initial densities among cultivars and lines but these did not cluster into "upland" and "Pima" types as had been expected. Upland and Pima fiber initiation occurred at anthesis, whereas Sea Island Pima initiation was delayed a day. A delay in fiber initiation was also confirmed in fuzzless mutant lines. Fiber initial densities were not found to be useful predictors of lint percentage.

Cotton is the most important source of natural fiber for the textile industry and in 2010/2011 the worldwide production was 25.0 million metric tons (Cotton Incorporated, 2010). Improvement in cotton fiber yield and quality would, therefore, be important for the global market. To improve fiber quality and yield further, it is essential to gain an understanding of factors that contribute to fiber initiation and development.

Two types of hairs or trichomes form on the epidermis of the cotton seeds: lint fibers, which constitute the most commercially valuable cotton product and fuzz fibers (also called linters) (Lang, 1938). On the ovule surface approximately one in four epidermal cells become fiber initials (cells that will become lint fibers). These cells start enlarging, ballooning above the epidermis at anthesis in Gossypium hirsutum L. cultivars (Stewart, 1975). Fiber initials continue to elongate for approximately 30 d in Pima cotton and 24 d in upland cotton (Hawkins and Serviss, 1930) by diffuse growth (Tiwari and Wilkins, 1995). Quisenberry and Kohel (1975) showed that the elongation period is affected by environmental as well as genetic factors. Schubert et al. (1973) concluded that there was considerable overlap between the elongation and thickening stages because in the Acala and Pima types he studied, 25% and 60%, respectively, of fiber cell wall thickening also occurred during the cell elongation period. Five to 10 d after anthesis, fuzz fibers start elongating (Lang, 1938; Stewart, 1975).

Cotton fiber initiation has been mostly studied in upland cotton, *G. hirsutum*, although some information exists on a few wild cottons (Applequist et al., 2001). In upland cotton, fiber initials start developing the day of anthesis (0 dpa - days post anthesis; Ryser, 1999; Stewart, 1975). Lang (1938) reported the lint and fiber development pattern in four lines of *G. hirsutum* and two *G. barbadense* L. It has also been reported that fiber mutants with the N_1 allele have delayed lint initiation (Lang, 1938; Lee et al., 2006; Turley et al., 2007; Zhang et al., 2007).

All studies evaluating densities and development of cotton fiber initials on the epidermal surface have used scanning electron microscope (SEM) imaging (Applequist et al., 2001; Berlin and Ramsey, 1970; Li et al., 2008; Stewart, 1975; Tiwari

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and Wilkins, 1995; Turley et al., 2007; Zhang et al., 2007), and they have provided valuable information about fiber initiation. However, more researchers have access to light microscopes than electron microscopes. There is also considerable labor, time, and expense involved in obtaining SEM ovule images; therefore a limited number of samples can be processed. Probably as a result of the labor and expense involved in SEM imaging, to date no broad survey of fiber initials encompassing numerous cultivars has been published. Consequently, it is unclear if the few *G. hirsutum* cultivars that have been well-studied (i.e, cv. Hancock; Stewart, 1975) provide a suitable model for other upland cottons or *G. barbadense* cultivars.

Cotton ovule development and fiber initiation, as all biological characteristics and processes, exhibit natural variation even within lines or cultivars and this variation cannot be captured by sampling a limited number of individuals; therefore, it would be advantageous to develop a technique that allows the processing of larger numbers of samples to investigate the fiber initiation process and fiber initial density on the ovule surface. When we know more about the variation in fiber density among cotton species, cultivars, and lines, a more informed conclusion can be reached about the usefulness of this characteristic in fiber improvement.

DiOC$_6$(3) (3,3′-dihexyloxacarbocyanine iodide) is a cationic lipophilic carbocyanine dye. This dye is well retained in cell membranes and has little transfer from cell to cell; it also diffuses laterally within the cells to stain them entirely and has a bright signal, emitting green fluorescence. It gives low background signal because it has little fluorescence in water and samples do not need to be fixed for visualization. DiOC$_6$(3) has been used to stain endoplasmic reticulum (Terasaki et al., 1984) as well as mitochondria in tobacco and nuclear membranes in carrot (Matzke and Matzke, 1986). More recently DiOC$_6$(3) was successfully used to stain fiber initials in upland cotton (Taliercio and Boykin, 2007). In this study we take advantage of some of the properties of DiOC$_6$(3) mentioned above to stain fiber initials to make them easily visible and therefore quantifiable in ovule images.

The goals of this study were to provide an additional tool to plant geneticists and breeders working on improvement of fiber characteristics and who have no access to electron microscopes or need to process numerous samples, and to gain insight into the biology of fiber initiation in a wider range of cultivars and mutants than have been studied in the past. This new technique is not intended for the detailed study of individual fiber cells but to enable the processing of numerous samples with less time and cost than SEM imaging and to allow more researchers to perform this type of study, resulting in faster collection of information on fiber initials in numerous cultivars and lines. With these goals in mind, three objectives were selected for this study: 1) to demonstrate that DiOC$_6$(3) staining can be used to study the fiber initiation process and to quantify fiber initial densities in several samples of numerous cotton cultivars or lines; 2) to assess whether or not the fiber initiation patterns reported for some *G. hirsutum* and *G. barbadense* cultivars extend to a more diverse range of cultivars and fiber mutant lines; and 3) to test if there are relationships between density of fiber initials and lint percentage or fiber characteristics.

MATERIALS AND METHODS

Eighteen Gossypium cultivars and experimental lines were examined in this study to quantify fiber initial density and pattern of initiation (Table 1). Upland type cottons were 'SG 747' (Delta and Pine Land Co., Memphis, TN; now part of Monsanto), 'DP 5690' (Delta and Pine Land Co., PVP No. 9100116), 'ST 4793R' (Stoneville Pedigreed Seed Co., now Bayer CropSciences, Research Triangle Park, NC), and 'PM H1220' (Paymaster Technologies, Inc., Aiken, TX). Acala types included 'Acala 1517' (New Mexico Agricultural Station, Las Cruces, NM) and 'Acala Maxxa' (California Planting Cotton Seed Distributors [CPCSD], Shafter, CA), Pima types were 'Pima S7' (CPCSD) and 'PhytoGen 810' (a Pima cultivar from PhytoGen Seed Co. Indianapolis, IN). Sea Island types were 'Monserratt' (PI 528388, GB 228), 'Sea Island Seaberry' (PI 528396, GB 239), and 'Barbados' (PI 528389, GB 229); G. arboreum L. A2-100 (PI 529728) was also included. Another upland cultivar, FM 832 (Bayer CropSciences, Research Triangle Park, NC), was used in the 2006 sampling but not in 2007.

Table 1. Cotton cultivars and mutant lines used in this study. Their main characteristics and species assignments are specified.

Cultivar/Line	Yield	Fiber length	Fineness	Notes
Upland Types (*G. hirsutum*)	high	medium	medium	
SG747				
DP 5690				
ST 4793				
H1220				
Acala Types	high	medium-long	medium	
MAXXA				
Acala 1517				
Pima Types (*G. barbadense*)	medium	long	medium-high	
PIMA S7				
Phytogen 810				
Sea Island Types (*G. barbadense*)	medium-low	long	medium-high	
Monserrat				
Barbados				
Sea Island Seaberry				
Fiber mutants (*G. hirsutum*)				
Red plant w/fiber	medium	medium	medium	**red colored *G. hirsutum* mutants with and without fiber**
Red plant w/o fiber	none	none	none	
SOFC S.L. 1-7-1 b -fiberless-	none	none	none	
Pilose	medium	short	very low	**mutant with short, coarse and abundant fibers**
SOFC CGP56N - 11%	extremely low	medium-long	medium	**extremely low yield isoline**
SOFC CGP56F - 37%	medium	medium-long	medium	**average yield isoline**
G. arboreum	high	short	medium	**diploid species**
Accession A2-100				

Experimental lines used in this study were "pilose" with the T_1 allele (PI 528521; Percival, 1987); this line produces abundant seed fiber and is also characterized by high pubescence on the stems, leaves, and bolls (fruits) (Simpson, 1947). S.L. 1-7-1 b is a semi-red fiberless genetic stock of uncertain origin from the Stoneville collection. A pair of red plant lines of unknown origin found in the Stoneville seed collection also was used in the study. They have identical morphological characteristics except that one line has normal fiber (designated as red plant w/fiber) and one is fiberless (no lint, no fuzz) and is designated as red plant w/o fiber. It is likely that both fiberless lines are selections from the genetic stock S.L. 1-7-1 (a fiberless red plant, PI 528807; Percival, 1987). If that is the case, the genotype is $N_1N_1fl_1fl_1n_3n_3$ (Turley and Kloth, 2008). The red plant w/fiber is probably a backcross progeny from the cross

between S.L. 1-7-1 and a plant with fiber (R. Kloth, personal communication).

CGP56N 11%, and CGP56F 37% (37% lint) are NILs –nearly isogenic lines- developed by Rick Turley and are BC5 of DP 5690 (PVP 91001116) and the fiberless line 243 (Turley and Kloth, 2002; PI 616493). CGP56N 11% is homozygous for N_1, it is fuzzless and produces 11% lint whereas CGP56F 37% is fuzzy with 37% lint.

Sample Collection and Processing for Fluorescent Imaging. Flowers were collected the day of anthesis (0 dpa), 1 dpa, and 2 dpa at 13:00 h, brought to the laboratory and placed at 4°C until all were processed. The ovary was isolated from the rest of the floral organs and dissected to extract intact ovules from the 3 to 5 locules (depending on the cultivar/line). Using a dissecting microscope ovules were cut into halves along the chalazal crest. After each individual sample was processed, the half ovules were placed

in 1.5 ml microcentrifuge tubes filled with phosphate buffered saline (0.01M PBS, pH 7.2) and again placed at 4ºC. After processing all the samples, the PBS was replaced with a 12.5 µg/ml $DiOC_6(3)$ solution in 0.01M PBS buffer [$DiOC_6(3)$ (3,3′-dihexyloxacarbocyanine iodide; Sigma; stock solution: 0.5 mg/ml in ethanol]. The samples were inverted several times during the 30 min incubation at room temperature in the dark ($DiOC_6(3)$ is light sensitive). After incubation, samples were kept in the same microcentrifuge tubes and rinsed three times with phosphate buffered saline. They were then arranged on glass slides with the external side of the flanks facing up. Glycerol:PBS solution (50:50) was added and cover slips placed over the samples. These temporary slides last for at least 3 h if they are kept in the dark. As mentioned in the introduction, this dye has little transfer from cell to cell but it diffuses within the cells to stain them entirely emitting a bright, green fluorescence signal.

Images were obtained with 20X magnification from the flanks of the ovules in the center of the area just below the chalazal cap (Fig. 1a and b). Images were obtained with a Zeiss Axioskop 2 plus microscope (Zeiss, Thornwood, NY) using the FITC/EGFP filter (41012) (Zeiss) and captured with an AxioCam (Zeiss) and the AxioVision 4.4 software (Zeiss). For each cultivar or experimental line, five flowers from five different plants were analyzed (a total of five flowers/cultivar or line, one flower from each plant). From these five flowers, two ovules on each prepared slide were selected for imaging. The number of fiber initials as shown by $DIOC_6(3)$ fluorescence was quantified in three 10,000 µm² areas (Fig. 1b). Quantification of fiber initials was done with ImageJ 1.38x software (Rasband, 2007). ANOVAs were used to test whether there were differences among plants within lines and cultivars and among cultivars and lines; pairwise comparisons were used as follow up tests when appropriate (SAS, Cary, NC, and SigmaPlot, Systat Software, Inc., Chicago, IL).
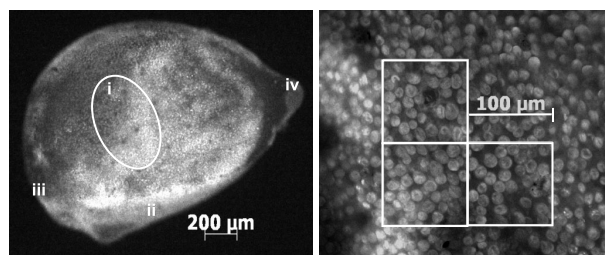

Figure 1. Fluorescent images of Acala Maxxa ovules at 0 dpa stained with $DiOC_6(3)$. (a) Ovule regions: i. general sampling area in the flank of the ovule, ii. chalazal crest, iii. chalazal end, iv. micropylar end. (b) The same ovule with sampling areas, fiber initials, and stomata.

A preliminary study was made on seven plants of ST 4793R to identify the best sampling strategy; for each plant, fiber initials were quantified in three ovules (from three different flowers, collected in three different dates) and for each ovule three subsamples (images) were taken. A survey of 10 cultivars/lines was performed in 2006 to assess the variability among cultivars/lines and the data used in a year-to-year comparison. Some cultivars and lines were found to have a delay in fiber development. Therefore, samples were taken at regular intervals to determine the best time for fiber initial quantification.

The *G. barbadense* Sea Island cotton types and DP 5690 were grown in the greenhouse and all other plants were field grown in Stoneville, MS. Greenhouse conditions were similar to the field but Sea Islands have an extended growing season compared to upland cotton and field harvest in Stoneville occurs when Sea Islands are normally beginning to bloom.

G. hirsutum lines and cultivars and *G. barbadense* Pima types were sampled from mid-July to mid-August; Sea Island cultivars were sampled from mid-August to mid-September. In all cases each plant was sampled only once. Although it would be preferable to grow all sampled plants in the same environment, some of the cultivars did not flower and produce bolls consistently in this area and had to be grown in the greenhouse to ensure the supply of flowers for ovule imaging. While acknowledging that the position of flowers on the plant and the position of the ovule within the capsule might affect lint characteristics, no attempt was made to control these factors as the results are intended to be whole plant estimates rather than to help elucidate the microvariations within cotton plants.

In this study, only the fiber initials on the flank of the ovules (Fig. 1a) were quantified (rather than several ovule regions). A previous study indicated that the choice of sampling area was justified; in five *G. hirsutum* cultivars Moore (1941) found significant differences in fiber population among six seed regions. However, the pattern shown in all cultivars was similar, the maximum number of fibers/area developed in the chalazal region of the ovule and fiber density decreased towards the micropyle and the raphe. He showed that from the six seed regions he quantified, only two showed differences among cultivars (Moore,1941); the imaging in this study was done on the approximate location of one of those two regions. The second highly variable sampling area in Moore's study is close to the micropyle and many of the cultivars/lines in this study lack lint in that area.

Lint Percent and Quality. To assess the relationships between fiber initials, lint (fiber) percentage, lint index, and fiber characteristics, the bolls of the same plants that were used in the imaging of fiber initials were harvested individually and quantified. Lint percentage is the percentage of the lint weight with respect to total weight (seed and lint). Lint index is the grams of lint per 100 seed. Lint per seed is often estimated by dividing the lint index value by 100. Fuzzy (undelinted) seed was used for calculation of weights per 100 seed. Fiber characteristics were assessed with an USTER AFIS[TM] instrument (Advanced Fiber Information System, Zellweger Uster, Uster, Switzerland). Measurements of the fiber quality presented here are: L(w), the mean length (mm) of the fiber based on fiber weight; SFC(w), short fiber (< 0.5 in) content in %; UQL, upper quartile length (mm) based on weight; L 2.5%, 2.5% span length (fiber length exceeded by only 2.5% of the fibers); Fine, mean fiber fineness (weight/unit length) in millitex (1 millitex = 1,000 m of fibers with a mass of 1 mg); and MR, maturity ratio = [mature fiber fraction (% of fibers with average circularity > 0.5) – immature fiber fraction (% of fibers with average circularity < 0.5)]/200 + 0.7 (Calhoun et al., 1997). Fiber initial densities, lint percentage, lint index, 100 seed weight, and fiber characteristics were analyzed with Pearson's product moment correlation coefficients (SigmaPlot, Systat Software, Inc., Chicago, IL).

RESULTS

Staining fresh cotton ovules with $DiOC_6(3)$ and imaging and quantification of the fluorescent fiber initials in numerous cultivars/lines were successful. $DiOC_6(3)$ enabled the clear visualization of fiber initials as well-defined, bright protrusions on the ovule surface (Fig. 1b), and quantification of the number of initials within the 10,000 μm^2 areas was easy and relatively fast. Non-elongating epidermal cells including stomata do not stain with $DiOC_6(3)$.

Preliminary sampling done on ST 4793R revealed that the most important source of variation was among plants ($p < 0.0001$); subsamples within ovules (images) and ovules from different flowers (ovaries) of the same plant were not found to be different ($p = 0.74$ and $p = 0.19$, respectively). It was concluded that several individual plants were necessary to characterize within cultivar/line variability more adequately, and five plants per cultivar or line

was the sample size used in this study. A second data set, with 18 cultivars and five plants for each cultivar/line was used to further analyze variation in fiber initial density. Fiber initial density was significantly different among cultivars (F = 4370, $p < 0.0001$, Fig. 2) and among plants in some of the cultivars/lines (F = 31, $p < 0.0001$, Fig. 2). The variability among plants of each cultivar was assessed individually for each line and cultivar, and half of the cultivars/lines presented significant variation among plants.
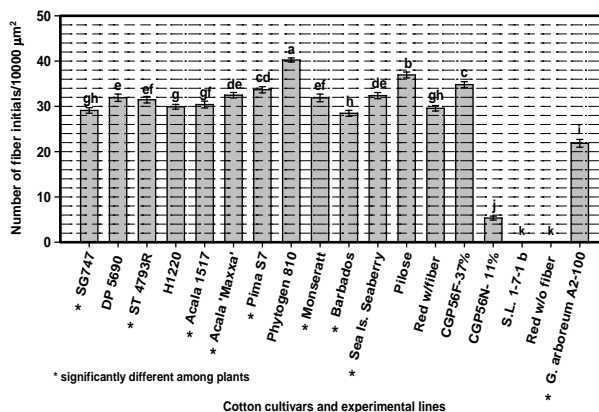

Figure 2. Fiber initial density in 18 cotton cultivars and experimental lines in 2007. Significant differences are indicated by different letters.

In 2007, PhytoGen 810 had the most fiber initials and *G. arboreum* had the least of those cultivars/lines that produce fiber (Fig. 2). Contrary to our expectations cultivars/lines did not cluster into upland, Pima, and Sea Island types based the number of initials/10,000 μm^2 (Fig. 2); instead representatives of these groups intermingled.

The ranking of the cultivars/lines based on fiber initial density was similar in 2006 and 2007 (Table 2, Fig. 2) except that Pima S7 performed better in 2006 than in 2007 and the reverse was true for CGP56F 37%. To observe year-to-year variation, nine of the cultivars/lines were sampled both in 2006 and 2007 (all of these were field grown). Of those only three showed significant differences, Acala 1517, PhytoGen 810, and CGP56F 37% (Fig. 3, cultivars/lines with variation among plants are marked with an asterisk; all were $p < 0.001$).

Fiberless lines did not show initials at any time on the smooth ovule surfaces where stomata are the only features (Fig. 4). On ovules with initials, guard cells can sometimes be seen directly and other times they can be located simply by the lack of initials in the stomatal regions (Figs. 1b and 5a). In the ovules from this study, fiber initiation started at the chalazal end of the ovules (Figs. 5a, b, and c).

Table 2. Mean (std. error) and ranges of fiber initial density (number of fibers in 10,000 µm²) at 0 dpa in the flanks of ovules in 2006. Significant differences are indicated by different letters.

Cultivar/line	Mean (st.error)	Range
PIMA S7	35.06 (1.91) a	19-48
Phytogen 810	33.50 (1.46) a	21-45
MAXXA	32.61 (1.38) a	20-35
ST 4793R	29.28 (1.02) b	23-38
SG747	29.17 (1.02) b	23-42
CGP56F-37%	28.25 (1.33) b c	23-38
FM 832	26.20 (1.15) c d	18-37
Acala 1517	25.83 (0.90) d	20-43
CGP56N- 11% [z]	4.67 (0.65) e	2-7
S.L. 1-7-1 b	0.00 (0.00) f	0-0

[z] **sampled at 1 dpa**

* significantly different between 2006 and 2007
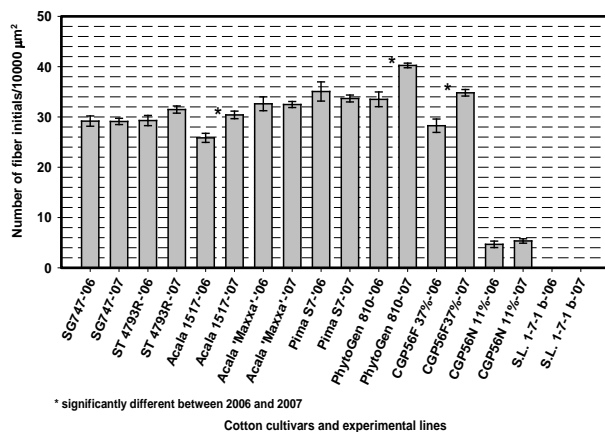
Cotton cultivars and experimental lines

Figure 3. Fiber initial density comparison for nine cotton cultivars and experimental lines in 2006 and 2007. Significant differences are indicated by different letters.
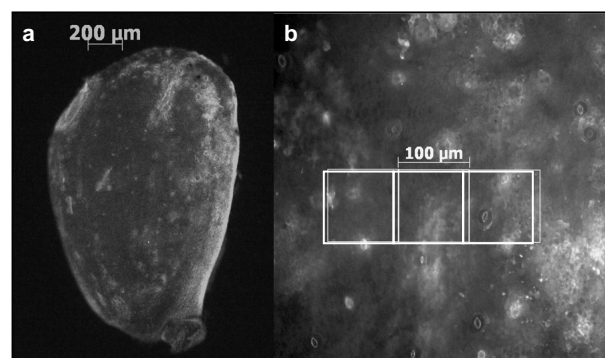

Figure 4. Ovule of a fiberless line at 0 dpa (red plant w/o fiber) stained with DiOC$_6$(3). (a) Smooth ovular surface dotted with stomata. (b) Higher magnification showing stomata.

The Sea Island types and CGP56N 11% exhibited delayed fiber initiation and the final samples used to quantify fiber initials were taken 24 h after anthesis. During the day of anthesis at 13:00 h all the Sea Island ovules look similar to fiberless lines (Fig. 6a1, b1).
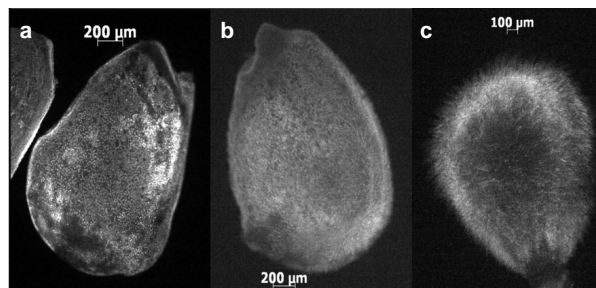

Figure 5. Fiber initiation on a *G. hirsutum* cultivar, ST 4793R. Fibers elongate first at the chalazal end of the ovule. (a) 0 dpa, (b) 1 dpa, (c) 2 dpa.
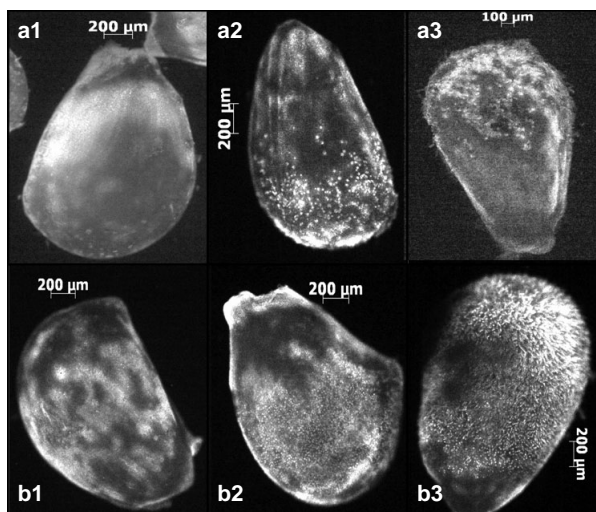

Figure 6. Delayed fiber initiation in *G.hirsutum* and G. barbadense*. (a) CGP56N 11%. (b) Sea Island Monseratt. (1) 0 dpa, (2) 1 dpa, (3) 2 dpa.

The area of the ovule surface covered by initials at the sampling date (0 dpa in normal cultivars/lines and 1 dpa in delayed ones) closely reflected the final coverage of seeds by lint. The ginned and unginned seed images demonstrate the final lint coverage (Figs. 7 and 8) For example, the CGP56N 11% line lacks fuzz entirely and Sea Islands produce a tuft of fuzz around the micropyle. The percentage of ovular surface occupied by fiber initials was variable among both *G. hirsutum* and *G. barbadense*. For example, at 0 dpa fiber initials cover approximately 85 to 90% of the ovular epidermis of *G. hirsutum* with some variation among cultivars. "Pilose" ovules are almost entirely covered (Fig. 7a); ST 4793R (Fig. 4a), Acala Maxxa (Fig. 1a), H1220, and SG 747 have approximately 90% of their ovular surface covered by initials, and in the red plant w/fiber, Acala 1517, and CGP56 37% (Fig. 7b) only about 85% of the ovule surface has initials. SEM images of *G. hirsutum* Hancock showed ovules with no initials near the micropyle (Stewart, 1975). Pima S7 ovules are covered by initials in approximately 75%

of their epidermis and the ginned seeds show bare tips (Fig. 8a). Sea Island Monseratt (Fig. 8b), and Sea Island Seaberry (Fig. 8c) ovules are covered only by initials on approximately 65% of their surface.
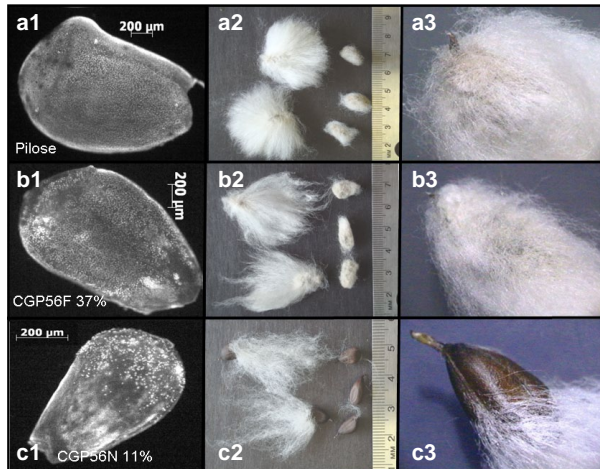

Figure 7. *G. hirsutum* **cultivars and lines; ovules at the time of fiber initiation and their corresponding seeds. (a) "Pilose", (b) CGP56F 37%, (c) CGP56N 11%. (1) DiOC$_6$(3) stained ovules at 0 dpa (1 dpa for CGP56N 11%). (2) Unginned and ginned seeds. (3) Detail of unginned seeds.**
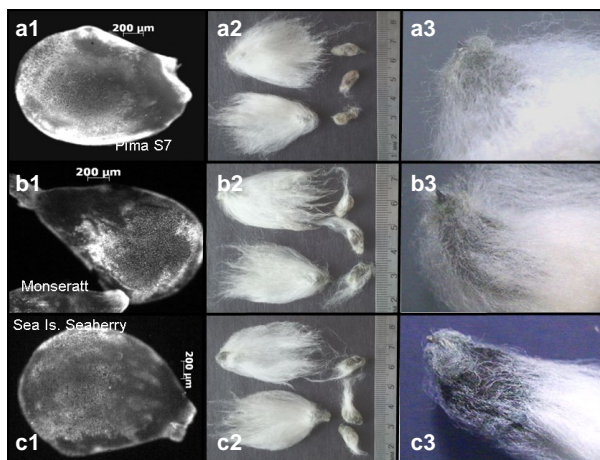

Figure 8. *G. barbadense* **cultivars; ovules at the time of fiber initiation and their corresponding seeds. (a) Pima S7, (b) Sea Island Monseratt, (c) Sea Island Seaberry. (1) DiOC$_6$(3) stained ovules at 0 (Pima S7) or 1 dpa (Sea Island Monseratt and Seaberry) . (2)Unginned and ginned seeds. (3) Detail of unginned seeds.**

Correlations were calculated between the average density of fiber initials (number of initials/10,000 μm^2), lint percentage, lint index, 100 seed weight, and fiber characteristics quantified by the AFIS. Fiber characteristics are presented in Table 3 and lint or seed traits in Table 4. There was no significant correlation of fiber initial density with any fiber quality characteristics. When the CGP56N 11% line was included in the

correlation of density of initials and lint percentage, the Pearson's product moment correlation was 0.715 ($p < 0.001$). However, the removal of this line from the analysis causes the relationship to disappear (r = 0.024, $p = 0.848$). The latter analysis is more relevant biologically because it is obvious that lines with very few initials can never produce high lint percentage (Fig. 9). Lint index was only slightly better with a correlation of 0.749 ($p < 0.001$) including CGP56N 11% and 0.3515 ($p = 0.199$) when the line was excluded from the analysis. The correlation fiber initial density with 100 seed weight was (r = 0.49, $p = 0.52$) (Fig. 10).
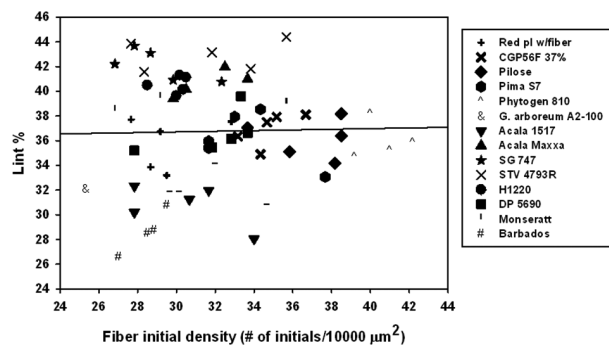

Figure 9. Lint percentage and fiber initial density in all linted lines except the CGP56N 11%. Values for individual plants shown.
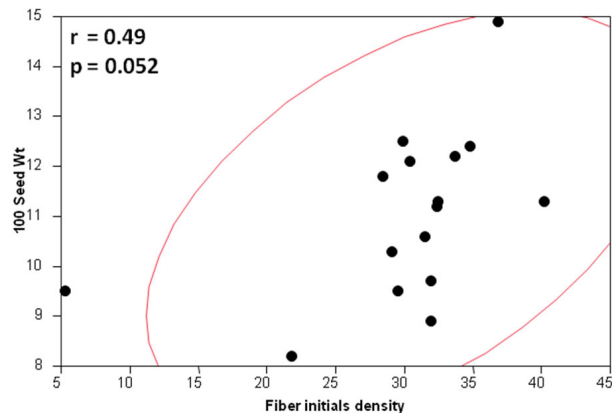

Figure 10. 100 seed index and fiber initial density for 16 cotton cultivars and experimental lines in 2007

DISCUSSION

The novel technique used in this study to quantify fiber initials in cotton ovules DiOC$_6$(3) staining of fresh ovule samples, proved effective in showing fiber initials and therefore allowing the quantification of fiber initial densities. In this study our goal was to evaluate the DiOC$_6$(3) staining method as a possible new tool for plant breeders and geneticists involved in fiber improvement that have no access to SEM or need to process numerous samples. Another

objective was to obtain information about the timing of fiber initiation and fiber initial density in several cotton lines and cultivars.

SEM imaging provides high quality images, but it is more expensive and the total sample processing time is longer. $DiOC_6(3)$ staining is less expensive and laborious and more samples can be evaluated with the same time expenditure. Five or six ovaries can be completely processed and imaged in one day, even when sample collection is started at midday. Regardless of the technique used, samples must be collected at a uniform and appropriate developmental stage and time. The $DiOC_6(3)$ stain technique adds one more tool that researchers can use to investigate cotton fiber initiation depending on their research goals, funding, and equipment available.

This study is a first step towards the characterization of fiber initial variation. The $DiOC_6(3)$ technique enabled the assessment of larger numbers of samples than any previously published studies; therefore, a more complete characterization of the variation within and among cultivars/lines was achieved. The approach in this study was to assess overall variation in fiber initial density at several levels of sampling. There were

highly significant differences in fiber density among cultivars (F = 4370, $p < 0.0001$, Fig. 2) and in some lines and cultivars among the plants sampled (F = 31, $p < 0.0001$); therefore some cultivars appear to have greater variability than others do although in all cases standard errors were small (Fig. 2). Different flowers of the same plant, different ovules within each ovary and different counting areas within the sampling area of the ovule were not significantly different. Future studies, therefore, should maximize sampling among cultivars and plants within cultivars rather than flowers within the same plant or several ovules from the same ovary or subsamples within ovule.

Environment seems to exert some influence on the number of initials as three of the eight cultivars with lint showed significant variation from 2006 to 2007 (Fig. 3) and because these are inbred lines, this variation might be due to environmental differences between the two seasons. In these three cases, Acala 1517, Phytogen 810, and CGP56 37%, ovules had higher fiber initial densities in 2007 than in 2006 (Fig. 3, Table 3). However, environmental factors were not controlled in this study so this is only speculation based on the simple comparison of fiber initial density in both years.

Table 3. Mean lint fiber (std. error) measurements for 16 cotton cultivars/lines. SL 1-7-1 b and red pl w/o fiber are not included in this table as they are fiberless.

Type	Fiber initials density[z]	L(w)[y]	SFCw[x]	UQL[w]	L 2.5 %[v]	Fine[u]	MR[t]
SG747	29.1 (0.62)	25.8 (0.70)	5.3 (0.80)	30.1 (0.70)	35.7 (0.54)	194.2 (3.13)	0.97 (0.016)
DP 5690	31.9 (0.80)	25.9 (0.29)	6.6 (0.62)	31.1 (0.23)	38.4 (0.32)	154.1 (1.71)	0.90 (0.008)
STV 4793R	31.5 (0.71)	25.2 (0.12)	5.2 (0.39)	29.2 (0.14)	34.8 (0.24)	195.0 (4.42)	0.97 (0.011)
H1220	29.9 (0.53)	25.9 (0.20)	5.3 (0.18)	30.4 (0.29)	36.1 (0.26)	196.4 (2.90)	0.97 (0.009)
Acala 1517	30.4 (0.75)	23.8 (0.32)	5.0 (0.41)	27.4 (0.32)	34.5 (0.53)	181.0 (0.86)	1.00 (0.003)
MAXXA	32.5 (0.60)	25.5 (0.65)	5.5 (1.06)	29.8 (0.53)	36.1 (0.60)	176.6 (1.50)	0.95 (0.015)
PIMA S7	33.7 (0.69)	29.3 (0.49)	5.3 (0.55)	35.5 (0.42)	43.0 (0.51)	147.3 (2.70)	0.93 (0.008)
Phytogen 810	40.2 (0.46)	29.7 (0.35)	3.9 (0.40)	35.3 (0.41)	43.8 (0.55)	140.7 (1.91)	0.95 (0.009)
Monseratt	31.9 (0.84)	26.0 (0.44)	11.3 (0.69)	33.1 (0.55)	41.2 (0.69)	123.0 (1.73)	0.83 (0.006)
Barbados	28.5 (0.65)	29.5 (0.44)	8.7 (0.60)	37.6 (0.45)	46.6 (0.45)	133.0 (1.40)	0.87 (0.007)
Seaberry	32.4 (0.68)	26.8 (0.56)	11.1 (0.23)	34.4 (0.79)	42.8 (0.84)	129.0 (1.78)	0.87 (0.009)
Pilose	36.9 (0.65)	18.5 (0.43)	11.7 (1.56)	21.4 (0.36)	26.1 (0.34)	209.6 (2.04)	0.97 (0.009)
Red pl w/fiber	29.6 (0.61)	23.6 (0.57)	6.6 (1.09)	27.4 (0.50)	32.9 (0.52)	186.8 (3.15)	0.95 (0.008)
CGP56F - 37%	34.8 (0.66)	27.1 (0.38)	4.7 (0.62)	32.1 (0.36)	38.5 (0.42)	178.7 (1.33)	0.99 (0.005)
CGP56N- 11%	5.3 (0.44)	29.3 (0.50)	2.7 (0.17)	33.8 (0.57)	40.5 (0.51)	198.4 (1.29)	1.02 (0.004)
G. arboreum A2-100	21.8 (0.86)	19.4 (0.10)	12.0 (0.05)	22.8 (0.25)	29 (0.05)	189.0 (8.02)	0.94 (0.000)

[z] Fiber initials density, number of fiber initials in 10,000 μm^2

[y] L(w), the mean length (mm) of the fiber based on fiber weight

[x] SFC(w), short fiber (< 0.5 in) content in %

[w] UQL, upper quartile length (mm) based on weight

[v] L 2.5%, 2.5% span length (fiber length exceeded by only 2.5% of the fibers)

[u] Fine, mean fiber fineness (weight/unit length) in millitex (1 millitex = 1000m of fibers with a mass of 1 mg)

[t] MR, maturity ratio= [mature fiber fraction (% of fibers with average circularity >0.5) – immature fiber fraction (% of fibers with average circularity < 0.5)]/200 + 0.7

The general pattern of fiber initiation described by Stewart (1975) holds true for these cultivars/lines; before anthesis epidermal cells are flattened and polygonal and during initiation they balloon out and expand away from the ovule surface. Fibers at the chalazal end of the ovule are the first to develop and initiation progresses towards the micropyle (Joshi et al., 1967; Lang, 1938; Stewart, 1975). Contrary to what was reported for TM-1 (Texas Marker-1, Zhang et al., 2007) and *G. hirsutum* Hancock (Stewart, 1975), the cultivars in the present study did not appear to have longer fibers on the crest area at 1 dpa (Fig. 5); however, this aspect was not formally tested. Tiwari and Wilkins (1995) found both patterns, longer fibers in the chalazal end and longer fibers on the funicular crest, on the same plants of Acala SJ-2. Stewart (1975) noted the chalazal side develops faster so that fibers point towards the micropyle.

The timing of initiation, as indicated by $DiOC_6(3)$ staining, was found to be variable among the cultivars/lines investigated in this study. The fuzzless mutant line CGP56N 11%, with the N_1 allele, was found to be delayed in lint initiation (Fig. 6a1, a2, and a3); this agrees with previous reports for fuzzless mutants (Lang, 1938; Lee et al., 2006; Turley et al., 2007; Zhang et al., 2007). However, the NIL CGP56F (37% lint) did not show fiber initiation delay (Fig. 7b).

Sea Island types (*G. barbadense* Sea Island Barbados, Monseratt, and Seaberry) also showed developmental delay (Sea Island Monseratt, Fig. 6b1, b2, and b3) as described by Lang (1938) for another Sea Island. These Sea Island types are all long fiber (long-staple) cottons and the late start in fiber development does not appear to cause a shorter final fiber length. This might be due to *G. barbadense* fibers showing more overlap between the elongation and secondary wall formation stages (Schubert et al., 1973). However, other *G. barbadense* cultivars, Pima S7 and PhytoGen 810 did not demonstrate fiber initiation delay although they shared other morphological features such as a trilocular small ovary with a reduced number of seeds per boll in comparison to *G. hirsutum* and generally have long fibers. Lang (1938) noted a delay in lint initial development in a hybrid Sakel x Pima and it is possible that the fiber initial delay characteristic came from the *G. barbadense* "Sakel". All *G. barbadense* cultivars showed a tuft of fuzz at the micropylar end of the seed (Fig. 8). As previously reported by Lang (1938), this tuft of fuzz fibers developed several days after lint development and these initials do not appear in images obtained at 0 to 2 dpa.

It has been suggested that the number of fiber initials can be used as a predictor of lint percentage in cotton breeding (Li et al., 2008). In the present study, no relationship was found between these two variables. It is possible that fiber initial density may be a more useful predictor when comparing lines or cultivars that are closely related and share most morphological characteristics. In contrast to the present study, Li et al. (2008) used four ovule areas to calculate the average initial density instead of the one area used in this study. However, even in their study, the lint percentage and fiber initial densities correlations were not always significant even when using total ovule averages for their calculations. In cultivars or lines that are less closely related, several morphological features that play a role in determining lint percentage show great variation, for example, the area of the ovule epidermis covered by initials, or the diameter and length of the fiber. These variables are probably more influential in determining lint percentage than the number of initials per unit area of the ovule. For example, Phytogen 810 had the highest fiber initial density on the ovular flanks and produced the longest fibers (Figs. 2 and 3, Table 3), but initials did not cover the entire ovule, its fibers were fine, and the lint percentage ranked 11th out of the 16 fibered cottons in this study (Table 2). Another line with high fiber initial density, "pilose" (the second highest on Fig. 2), ranks 10th out of the 16 lines with fiber in lint percentage (Table 2). Pilose initials cover the entire ovule but fibers are typically short and coarse (Kloth, 1993; Lee, 1984; Fig. 7a1, a2, and a3). These two examples illustrate some of the difficulties of predicting lint percentage as several characteristics are involved besides fiber initial density.

As discussed by Whorley et al. (1974) lint percentage is not a biological entity, but the ratio of lint/seed and seed cotton/seed (seed cotton/seed = boll size /seeds per boll) so all the factors in this equation affect lint percentage. Bednarz et al. (2007) pointed out that selection for increased lint percentage in the last 30 yr has led to smaller-seeded cultivars. Lint index takes into account differences in seed size, but in this study the correlation with fiber initial density was only slightly better (Table 4). It has also been reported that increased lint percentage has been accompanied by reduced boll mass and seed mass and fiber strength and length (Miller and Rawlings, 1967; Stewart and Kerr, 1974). It appears that, sometimes, selecting for a

particular variable has led to unintended selection in other fiber characteristics. It is unlikely that fiber initial density alone can be used as an easy and early predictor of lint percentage or potential yield. However, it is possible that an equation can be made to predict potential yield using estimates based on the most important components contributing to the characteristic. Establishing this type of model could aid in directing selection of new cultivars by providing a better understanding of how selecting for one variable will affect others so as to avoid undesirable results.

Some of the fiberless seeds produced a few long fibers emerging from the funicular crest area. The same was observed in the XinWX and XZ142WX fiberless lines (Zhang et al., 2007). Therefore, these fiberless lines seem to retain the ability to make fibers and they may have mutations that affect regulatory rather than structural genes. On the other hand, Zhang et al. (2007) proposed multiple signaling pathways to explain this occurrence. This is also possible as in tetraploid *Gossypium* many of the genes are duplicated and have different expression patterns. The gene from one genome could control trichomes on most of the ovular surface and the other copy could control trichome formation only on the funicular crest area. This issue could be further investigated by transferring N_1 allele to a diploid species such as *G. arboreum* and examining the resulting seeds for the presence or absence of fibers at the funicular crest area.

CONCLUSIONS

The $DiOC_6(3)$ fiber initial dying technique presented here is a new tool that will allow more scientists to research the fiber initiation process in numerous cultivars and enable faster data collection. There are many aspects that remain unexplored in this field such as percentage of ovule surface covered with fiber initials, relationship between plant hirsutism and seed fiber, variation of fiber yield in seeds in different positions on the plants, and comparison of this variation among cultivars. A better understanding of these variables will make it possible to concentrate efforts on those that are the most relevant to fiber improvement both in yield and in quality.

Table 4. Mean lint and seed (std. error) measurements for 16 cotton cultivars/lines. SL 1-7-1 b and red pl w/o fiber are not included in this table as they are fiberless.

Type	Fiber initials density[z]	Lint Index[y]	100 Seed Wt (g)[x]	Lint %[w]
SG747	29.1 (0.62)	7.5 (0.22)	10.3 (0.23)	42.1 (0.58)
DP 5690	31.9 (0.80)	5.1 (0.13)	8.9 (0.15)	36.5 (0.72)
STV 4793R	31.5 (0.71)	8.0 (0.26)	10.6 (0.23)	43.0 (0.55)
H1220	29.9 (0.53)	8.5 (0.15)	12.5 (0.16)	40.5 (0.31)
Acala 1517	30.4 (0.75)	5.5 (0.18)	12.1 (0.32)	31.1 (0.70)
MAXXA	32.5 (0.60)	7.6 (0.32)	11.3 (0.29)	40.4 (0.46)
PIMA S7	33.7 (0.69)	7.1 (0.13)	12.2 (0.10)	36.8 (0.99)
Phytogen 810	40.2 (0.46)	6.4 (0.44)	11.3 (0.38)	36.1 (0.52)
Monseratt	31.9 (0.84)	6.1 (0.13)	9.7 (0.14)	38.5 (0.47)
Barbados	28.5 (0.65)	4.9 (0.30)	11.8 (0.21)	29.3 (1.80)
Seaberry	32.4 (0.68)	5.1 (0.07)	11.2 (0.05)	31.3 (1.09)
Pilose	36.9 (0.65)	8.4 (0.13)	14.9 (0.03)	36.2 (0.70)
Red pl w/fiber	29.6 (0.61)	5.3 (0.12)	9.5 (0.10)	35.8 (0.95)
CGP56F - 37%	34.8 (0.66)	7.2 (0.13)	12.4 (0.10)	36.8 (0.51)
CGP56N- 11%	5.3 (0.44)	1.2 (0.23)	9.5 (0.19)	11.5 (1.59)
G. arboreum A2-100	21.8 (0.86)	5.3 (0.25)	8.2 (0.03)	38.4 (6.35)

[z] **Number of fiber initials in 10,000 μm^2**

[y] **Grams of lint per 100 seed**

[x] **Weight in g of 100 fuzzy (not delinted) seed**

[w] **Weight of lint as a percentage of total cottonseed weight**

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DISCLAIMER

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