Chemical variation for fiber cuticular wax levels in upland cotton (*Gossypium hirsutum* L.) evaluated under contrasting irrigation regimes

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Abstract

The fiber from upland cotton (*Gossypium hirsutum* L.) makes up approximately 90% of the global cotton produced each year. Fiber quality is important to textile mills for processing and factors into bulk cotton sales. Fiber quality can be affected by many environmental factors, including water deficit, which makes identifying major fiber characteristics an important focus for breeding programs. Cotton fibers are specialized trichomes that are primarily composed of cellulose but have a cuticle composed of free waxes and cutin. Total cuticular wax of cotton fiber has been shown to act as a lubricant during textile processing, but has also been negatively correlated with important quality traits. The objectives of this study were to identify and quantify the cuticular wax compounds of cotton fiber under water-limited (WL) and well-watered (WW) irrigation treatments and assess their relationship with fiber quality from seven upland cotton lines. Through the most detailed characterization of cotton fiber cuticular wax to date, 41 quantifiable compounds were identified including free fatty acids, primary alcohols, aldehydes, alkanes, and tentatively identified alkanediols. Of these 41 compounds and their sum (total waxes), the abundance for nine were significantly different ($\alpha = 0.05$) between WL and WW conditions. Total wax and 36 compounds were highly repeatable ($r \geq .60$), indicating they will respond positively to selection in

cotton breeding programs. Irrespective of irrigation regime, strong positive correlations (r_p 0.64 – 0.80) were found for fiber length and uniformity with primary alcohols, fatty acids, and aldehydes. These findings suggest that the biosynthetic pathways associated with these compounds are contributing to the phenotypic variability of these two important fiber quality traits and thus the biochemical pathways associated with cuticular fiber wax are candidates for metabolic engineering via molecular breeding approaches.

Keywords

cotton fiber, fiber quality, cuticular wax, genomics-assisted breeding

Abbreviations

BLUE, best linear unbiased estimate; Co-A, coenzyme-A; GC-MS, gas chromatography-mass spectrometry; ITSD, internal standard; TLC, thin layer chromatography; SD, standard deviation of the BLUEs; SE, standard error of the repeatability estimates

1. Introduction

Cotton (*Gossypium* sp.) is an important natural fiber source used by the textile industry worldwide. Upland cotton (*G. hirsutum* L.) accounts for more than 90% of the 25.1 million metric tons produced globally each year (2010-2014 average, FAO, 2016). The United States is the third largest producer of cotton with approximately 70% being exported to foreign markets (Cotton Inc., 2016; National Cotton Council, 2016). Nearly 90% of the U.S. exported cotton is purchased by textile mills to produce woven fabrics and yarn for apparel and home-goods (National Cotton Council, 2016).

When purchasing bulk cotton, textile mills demand desirable fiber quality characteristics to ensure efficient production while still delivering superior products to the consumer (Bradow et al., 1997; Bradow and Davidonis, 2000). Fiber quality is quantified by a high volume instrument (HVI), which measures fiber length, elongation, uniformity, fineness, maturity, and strength (Bradow and Davidonis, 2000; Cotton Inc., 2016). Two fiber traits that are particularly important for yarn spinning are length,

with increased length minimizing fiber bunching (Thibodeaux et al., 2008), and uniformity, which reduces yarn hairiness (Krifa and Ethridge, 2006). Fiber quality is affected by many factors including the growing environment (Green and Culp, 1990), plant density (Bednarz et al., 2006), planting date, boll position on the plant itself (Davidonis et al., 2004), drought stress (Dağdelen et al., 2009; Dabbert et al., 2017), and genetics (Bradow and Davidonis, 2000; Paterson et al., 2003). With so many factors affecting fiber characteristics, it has been difficult to identify the primary determinants of quality, and therefore fiber characterization efforts have been renewed to meet the demands of textile mills.

Cotton fibers are specialized trichomes primarily composed of cellulose (~90%) found within the secondary cell walls of the seed coat (Gamble, 2004; Hartzell-Lawson and Hsieh, 2000; Hock et al., 1941). The cuticle is a structure that forms the outermost layer of the cotton fiber, and constitutes most of its noncellulosic components. The mature cotton cuticle itself is primarily composed of two basic classes of lipids, the free waxes and cutin (Degani et al., 2002; Yatzu et al., 1983), with minor amounts of non-cellulosic polysaccharides, proteins, pectins, ash, salts, and sugars embedded within and/or chemically linked to the cuticle (Gamble, 2003; Hartzell-Lawson and Hsieh, 2000). Of these components, wax is generally the most abundant, with known lubricant properties that have made it a focus for fiber spinning and wetting studies (Gamble, 2004; Hartzell-Lawson and Hsieh, 2000). Despite its importance in textile production, few studies have reported the composition of fiber wax and how it can vary in response to external factors including abiotic stress such as water deficit and high heat (Church and Woodhead, 2006).

Inverse relationships have been found between total fiber wax and micronaire, an indirect estimate of fiber maturity and fineness, regardless of growing environment and cotton variety (Bradow and Davidonis, 2000; Gamble, 2003). Similar negative correlations between percent fiber wax and micronaire were found by Pan et al. (2010) who also reported negative correlations of total wax with lint percentage, fiber length, strength, and uniformity in both white and colored cotton. These correlative

findings led the authors to conclude that varieties with reduced total wax should be selected for use in cotton breeding programs. However, Cui et al. (2002) reported an increase in fiber breakage during carding, a textile process that aligns individual fibers, by one order of magnitude in dewaxed cotton samples, providing support that the waxes act as a lubricant to reduce friction during processing. Similar results were reported by Taylor et al. (1997) who found that fiber wax was positively correlated with fabric strength, indicating that natural waxes are acting as lubricants during fabric production. Despite the established relationship between fiber wax, textile processing, and fiber properties, none of these studies looked at the components of the cuticular waxes themselves.

Much of the knowledge regarding the composition and synthesis of cuticular waxes comes from studies of plant leaves, stems, and fruits (Jetter et al., 2006; Parsons et al., 2013). In these organs, cuticular waxes are secreted by epidermal cells and help form a protective, hydrophobic barrier at the plant/environment interface (Jenks et al., 1994; Samuels et al., 2008). The waxes are typically composed of a complex mixture of very long-chain aliphatic molecules (C_{20} - C_{36}) including fatty acids, primary alcohols, wax esters, aldehydes, alkanes, secondary alcohols, ketones, as well as a variety of triterpenes (Yeats and Rose, 2013). The relative proportion of each class varies greatly between plant species, as well as between organs of the same species (Lee and Suh, 2015). The biosynthetic pathway for these major components has been fairly well characterized, and some of the genes encoding relevant enzymes have been identified (Yeats and Rose, 2013; Lee and Suh, 2015). Fatty acid biosynthesis occurs in the plastids of plant cells, and C_{16} and C_{18} fatty acids are exported from plastids to the cytosol where they are conjugated with Coenzyme-A (Co-A). These fatty acyl-CoAs are then elongated two carbon units at a time by elongase enzyme complexes located in the endoplasmic reticulum, resulting in acyl-CoAs that are up to 36+ carbons in length. The acyl-CoAs are then modified by two major pathways, either the acyl-reduction pathway, which produces primary alcohols that can further be reacted with a fatty acyl-CoA to produce wax esters, or modified by an alternate pathway that produces aldehydes, alkanes,

secondary alcohols, and ketones (being referred to henceforth as the decarbonylation pathway). Fatty alcohols, free fatty acids, and wax esters are expected to be identified from the cuticle of mature cotton fiber (Church and Woodhead, 2006).

Although natural waxes are known to be important for fiber processing in textile production, previous studies have shown negative correlations between total fiber wax and fiber quality traits (Bradow and Davidonis, 2000; Gamble, 2003; Pan et al., 2010). The identification of wax compounds associated with fiber quality could lead to a better understanding of how fiber quality traits, fiber wax, and textile processing are interrelated. Furthermore, a detailed description of the wax compounds coating cotton fiber would provide new insight to the biochemical pathways associated with their production, which could guide future efforts to modify fiber wax compounds and associated fiber quality traits using transgenic- and/or genomics-assisted breeding approaches. The objectives of this research were to (i) characterize the content and composition of cuticular waxes on mature upland cotton fibers (ii) determine how differential irrigation treatments affect cuticular wax compounds and fiber quality traits, and (iii) assess the relationship between these wax compounds and fiber quality traits evaluated under differential irrigation treatments.

2. Materials and Methods

2.1 Plant material

The fiber from seven upland cotton lines was evaluated for cuticular wax composition: DP 393 (PI 635100), DP 491 (PI 618609), FM 958 (PVP 200100208), NM24016 (PI 612327), STV 457 (PI 633625), STV 506 (PI 529523) and, TM-1 (PI 607172) which are known to vary for fiber quality and abiotic stress tolerance. Fiber samples were taken from these lines that were included as repeated checks from a previous experiment grown at the Maricopa Agricultural Center of the University of Arizona from 2010-12, described by Pauli et al. (2016). Briefly, experimental plots were one-row, 8.8 m in length, and

spaced 1.02 m apart with a plant density of 4.1 plants m⁻² and arranged in an $\alpha(0,1)$ lattice design with an average of three repeated checks per replicate per treatment per year. Plots were grown under either water-limited (WL) or well-watered (WW) treatments; the WL treatment started when 50% of plots were at first flower. Prior to mechanical harvest, 25 bolls were hand collected from each experimental plot and processed with a laboratory 10-saw gin. Lint percentage was calculated by weighing the ginned fiber and dividing by the total 25 boll sample weight. Fiber sub-samples (~10 g) were sent to Cotton Inc. (Cary, NC) for fiber quality assessment on the HVI (USTER® AFIS PRO, Charlotte, NC) including fiber uniformity (%), elongation (%), micronaire (unit), strength (kN m kg⁻¹), and length (upper half mean, mm). The remaining 25 boll samples were then stored in paper bags (Kraft, 52lb weight) in a humidity controlled chamber (20% relative humidity) at -20°C (Chasewood Co., Cypress, TX) before fiber wax extraction and analysis.

2.2 Extractions and analysis of cuticular waxes on cotton fibers

Fiber samples (156 in total) were removed from the -20°C freezer and allowed to warm to room temperature (22°C), to prevent excess fiber breakage, and organized in a completely randomized design for wax extraction. Fiber sub-samples were hand pulled wearing nitrile gloves and weighed to four significant figures on an analytical balance (Mettler-Toledo LLC., Columbus, OH) ensuring samples were between 0.1500 - 0.2000 g. Samples were individually placed in 20 mL glass scintillation vials and submerged in 10 mL of chloroform $\geq$ 99.5% purity (Sigma-Aldrich, St. Louis, MO). Three internal standards were added at extraction: 10 μ g of nonadecanoic acid, 10 μ g of tetracosane, and 20 μ g of pentacosanol (LGC Standards, Manchester, NH). Vials were capped and agitated for 30 s before the fiber was compressed with a spatula, then removed from the solvent with forceps. The wax extracts were heated to 60°C and reduced under nitrogen gas (N₂) until the volume could be transferred into a 2 mL glass vial. The scintillation vials were rinsed once with 2-3 mL of chloroform, the volume transferred again, then evaporated to dryness under N₂. For each wax sample, 100 μ L of N,O-bis(trimethylsilyl)

trifluoroacetamide (BSTFA) (Sigma-Aldrich, St. Louis, MO) and 100 μL of chloroform was added for a total volume of 200 μL , then vials were capped and processed as described below. Chloroform was used as the extraction solvent based on a pilot study comparing extraction efficiency and consistency with hexane using ~ 0.1 g samples from one 2010 WW condition TM-1 and NM24016 sample. Three technical replicates were performed on each sample with each solvent for comparison (data not shown).

Gas chromatography-mass spectrometry (GC-MS) analysis was performed using an Agilent 7890A gas chromatograph with a 5975C quadrupole mass spectrometry detector equipped with a HP-Ultra 1 capillary column (12 m length, 200 μm inner diameter, 0.33 μm film thickness) (Agilent Technologies, Santa Clara, CA) and helium as the carrier gas at 1 mL per min. Each vial was heated to 80°C for 35 min, then mixed five times for 6 s at 2000 rpm using an automated mixing/incubation platform on the GC-MS prior to a 1 μL injection. The column temperature was programmed with an initial temperature of 50°C, then increased 20°C/min to 260°C, held for 8 min, then increased at a rate of 25°C/min to a final column temperature of 325°C where it was held for 13.9 min giving a total run time of 35 min. The inlet and detector temperatures remained at 300°C. The molecular identities of individual wax compounds were determined by characteristic quadrupole electron impact ionization and a combination of relative retention times and mass fragmentation spectra. Several compounds were identified by comparison to the NIST MS Search Program version 2.0 (NIST, Gaithersburg, MD); however, those compounds missing from the library were compared to previously published spectra or were tentatively assigned based on their ion chromatograms. The quantification of each compound was determined by target ion peak areas (QIon) relative to the corresponding internal standards. Total wax was calculated by summing across all quantified compounds for each sample and reported as $\mu\text{g/g}$ of fiber.

2.3 Thin-layer chromatography

To verify that the fiber wax compounds extracted via the chloroform dip were derived primarily from the cuticle, the wax extracts of two samples (TM-1; 2011, WL and WW) were compared to total lipid extractions of the same samples using thin-layer chromatography (TLC). Total lipids were extracted using chloroform:methanol (1:2, v/v), as described by Bligh and Dyer (1959). Briefly, ~150 mg of cotton fiber was taken from the same 25 boll samples used for cuticular wax sampling and placed in a glass tube containing methanol:chloroform:water (2:1:0.45, v/v/v) then incubated at 4°C overnight. The solvents were partitioned into two phases after addition of 1M KCl dissolved in water and an equal volume of chloroform. Samples were then shaken, vortexed, and centrifuged so that phases could be extracted for further analyses. The bottom organic phase was transferred to a clean glass tube, dried under nitrogen gas, and re-suspended in chloroform. Alternatively, a modified Bligh and Dyer method was used wherein 100% isopropanol was used instead of methanol. In that case, samples were incubated first in hot isopropanol at 70°C for 30 min, then 100% chloroform and water were added, and then the rest of the procedure was carried out as described above. Total lipids and cuticular wax extracts were applied to a silica TLC plate (Merck KGaA, Darmstadt, Germany), along with lipid class standards (TAG-triacylglycerol, DAG – diacylglycerol, FFA – free fatty acid, WE – wax ester, PC – phosphatidylcholine, alkane and alcohol) and developed using hexane:diethyl ether:acetic acid (70:30:1, v/v/v) as the mobile phase. Lipids were stained using 0.05% primuline in 80% acetone and visualized under UV light.

2.4 Statistical analysis

To identify and remove significant outliers from the raw data, a mixed linear model for each wax compound from the fiber was fitted using ASReml-R version 3.0 (Gilmour et al. 2009). The full model (Model 1) used was as follows:

$$Y_{ijklmn} = \mu + \text{year}_i + \text{trt}_j + \text{genotype}_k + \text{year} \times \text{trt}_{ij} + \text{year} \times \text{genotype}_{ik} \\ + \text{trt} \times \text{genotype}_{jk} + \text{year} \times \text{trt} \times \text{genotype}_{ijk} + \text{rep}(\text{year} \times \text{trt})_{ijl} \quad \text{Model 1}$$

$$+ GC_col_m + GC_run_date(GC_col)_{mn} + \epsilon_{ijklmn},$$

in which Y_{ijklmn} is an individual observation; μ is the grand mean; $year_i$ is the effect of the i th year; trt_j is the effect of the j th irrigation treatment, which was either water-limited (WL) or well-watered (WW); $genotype_k$ is the effect of the k th genotype; $year \times trt_{ij}$ is the interaction effect between the i th year and j th irrigation treatment; $year \times genotype_{ik}$ is the interaction effect between the i th year and k th genotype; $trt \times genotype_{jk}$ is the interaction effect between the j th irrigation treatment and the k th genotype; $year \times trt \times genotype_{ijk}$ is the three way interaction effect between the i th year, j th irrigation treatment, and k th genotype; $rep(year \times trt)_{ijl}$ is the effect of l th replication within j th irrigation treatment level within the i th year; GC_col_m is the effect of the m th GC–MS column on which the fiber wax samples were run; $GC_run_date(GC_col)_{mn}$ is the effect of the n th day on which samples were run within each GC–MS column; and ϵ_{ijklmn} is the random error term following a normal distribution with mean 0 and variance σ^2 . The model terms $rep(year \times trt)_{ijl}$, GC_col_m , and $GC_run_date(GC_col)_{mn}$ were modeled as random effects with all other terms being treated as fixed effects. To identify significant outliers, Studentized deleted residuals (Neter et al. 1996) were used with degrees of freedom being calculated using the Kenward and Rogers approximation (Kenward and Roger 1997). The outlier removal process was carried out in an iterative fashion removing those data points from the raw data set.

Once all outliers were removed for each fiber wax compound trait, an iterative mixed linear model fitting procedure was conducted in ASReml-R version 3.0 using Model 1 as specified above. To remove all non-significant random terms from the full model, likelihood ratio tests were conducted with a significance threshold set at $\alpha = 0.05$ (Littell et al. 2006) generating a final, best fitted model for each fiber wax compound. This final model was used to generate best linear unbiased estimators (BLUEs) for each line within the respective irrigation treatments. Sequential tests of fixed effects were carried out

with degrees of freedom being calculated with the Kenward and Rogers approximation (Kenward and Roger 1997) in ASReml-R version 3.0.

For each wax compound of the fiber, repeatability (r) was calculated to express the proportion of the variance due to permanent, non-localized differences (i.e. not due to experimental error) between genotypes providing a measure of technical performance. Model 1 was reformulated so that all terms were modeled as random effects in order to estimate the respective variance components (Model 2). The variance component estimates from the full model were used to estimate r as follows:

$$r = \frac{\widehat{\sigma}_g^2}{\widehat{\sigma}_g^2 + \frac{\widehat{\sigma}_{gy}^2}{n_{year}} + \frac{\widehat{\sigma}_{gt}^2}{n_{trt}} + \frac{\widehat{\sigma}_{gyt}^2}{n_{year*trt}} + \frac{\widehat{\sigma}_\varepsilon^2}{n_{plot}}} \quad \text{Model 2}$$

where $\widehat{\sigma}_g^2$ is the estimated variance due to genotype, $\widehat{\sigma}_{gy}^2$ is the estimated variance associated with genotype-by-year variation, $\widehat{\sigma}_{gt}^2$ is the estimated variance associated with genotype-by-irrigation treatment variation, $\widehat{\sigma}_{gyt}^2$ is the estimated variance associated with the three way interaction between genotype, year, and irrigation treatment, and $\widehat{\sigma}_\varepsilon^2$ is the residual error variance. The variable n_{year} is the harmonic mean of the number of years in which each genotype was observed, n_{trt} is the harmonic mean of the number of irrigation treatments in which each genotype was observed, $n_{year*trt}$ is the harmonic mean of the number of year-irrigation treatment combinations in which each genotype was observed, and n_{plot} is the harmonic mean of the number of plots in which each genotype was observed. The denominator of Model 2 is equivalent to the phenotypic variance, $\widehat{\sigma}_p^2$. Standard errors of the estimated repeatability were approximated using the delta method (Lynch and Walsh 1998; Holland et al. 2003).

The generated BLUEs for each irrigation treatment were also used to calculate Pearson's correlation coefficient (r_p , separate from repeatability) to assess the degree of association between the individual fiber wax compounds and fiber quality traits using the “Hmisc” package implemented within R

(R Core Team 2015). The Benjamini-Hochberg false discovery rate (FDR) correction was used to control for the multiple testing problem at an FDR of 5% as implemented with the function *p.adjust* in the R package “stats”. The FDR threshold for raw *P*-values in the WW irrigation regime was *P*-value < 0.02 and *P*-value < 0.01 for WL regime.

3. Results

3.1 Content and composition of cotton fiber wax

Characterization of cuticular wax for mature cotton fiber was assessed on 156 fiber samples from seven upland cotton cultivars grown under two irrigation regimes, with 46 unique compounds identified using chloroform as the extraction solvent. The main classes of compounds consisted of free fatty acids, primary alcohols, aldehydes, unbranched straight chain alkanes, and monoacylglycerols (MAGs) (Figure 1 and S. Figure 1). There were seven additional compounds tentatively identified as hydroxy aldehydes (TIC #29, #31), alkanediols (TIC #41, #42, #43), β -amyrenone (TIC #44), and octacosyl acetate (TIC #46) (S. Figure 1, S. Figure 2 a-e, S. Figure 3 a-c, and S. Table 1). Further examination of TICs #20, #41, #42 and #43 indicated compounds may be co-eluting and were putatively identified as C_{27} acid, C_{29} acid, C_{31} acid, and α - $C_{22:1}$ MAG (S. Table 1). Further experiments are needed for definitive structural identification of the co-eluting compounds, and since the compounds were not confirmed they were neither quantified nor reported further herein.

Eleven of the compounds identified included primary alcohols; three of the most abundant alcohols, regardless of irrigation treatment, were the C_{28} , C_{30} , and C_{32} primary alcohols (Table 1). These three alcohols accounted for more than 65% of the calculated total wax content for both irrigation treatments, which averaged 1261 $\mu\text{g/g}$ and 1295 $\mu\text{g/g}$ for WL and WW conditions, respectively. Free fatty acids were present as a homologous series ranging from C_{16} to C_{36} carbon chain length, with the most abundant being the C_{28} - C_{34} chain length homologues (Figure 1, Table 1). Primary alcohols showed a

similar relationship with highest abundance represented by the C₂₆-C₃₄ homologues. Aldehydes and alkanes were present in lower amounts and enriched in the C₂₆-C₂₈ homologues, or showed no prominent trends in chain length, respectively.

3.2 Thin-layer chromatography

The ginning process to separate seed from fiber unavoidably tears and breaks the fiber, allowing internal compounds to be exposed and potentially extracted with surface compounds. To confirm the compounds identified by GC-MS were cuticular and not internal, a cotton fiber total lipid extract (both surface and internal lipids) was compared to the surface wax extracts for two samples. Lipid classes were separated by thin-layer chromatography (TLC) (Figure 2), which revealed high amounts of phosphatidycholine (PC) in the total lipid extraction, indicative of intracellular lipids, while PC was present in only trace amounts in the surface lipid extracts. These data suggest that negligible quantities of internal compounds were extracted using the chloroform dip protocol for the surface (cuticular) wax lipids. Monoacylglycerols (MAGs) were detected in the surface wax extractions (Figure 1, Figure 2), which are not typically identified in aerial cuticular lipid extracts, but these were reported previously in the cuticular wax of *Arabidopsis* root tissues (Li et al., 2007).

Irrigation treatment effects on total fiber wax and composition

Compounds C₁₇ acid, C₂₁ acid, C₂₃ acid, C₂₅ acid, and β -C_{16:0} MAG were below 0.50 $\mu\text{g/g}$, a conservative threshold (data not shown), and removed prior to statistical analysis. The statistical analysis to determine if experimental factors affected the remaining 41 compounds, and total wax, indicated that all but five compounds were significantly different ($\alpha = 0.05$) among genotypes and all but seven compounds were significantly different ($\alpha = 0.05$) across years (S. Table 2). Conversely, only nine compounds were found to be significantly different ($\alpha = 0.05$) between irrigation treatments (S. Table 2), where quantities were generally lower under the WL treatment (Figure 1, S. Table 3). The 1,3-C₂₈

alkanediol showed the largest significant decrease (P -value <0.02) of $11.87 \mu\text{g/g}$ under WL conditions. The only compound that showed a significant increase (P -value = 0.02) under the WL treatment was C_{31} alkane at $0.52 \mu\text{g/g}$ (Figure 1, S. Table 2). Ten other compounds showed an increase in quantity under WL conditions but were not significant (Figure 1, S. Table 2).

Genotype had a statistically significant interaction ($\alpha = 0.05$) with year for 17 of the compounds, with six of them belonging to the free fatty acid group (S. Table 2). The year $\times$ genotype interaction was significant for the C_{26} , C_{28} , C_{30} aldehydes with P -values of 0.01 , 0.02 , and <0.01 , respectively. In addition, two of these aldehydes, C_{26} and C_{30} , had a significant irrigation treatment $\times$ year interaction (P -values <0.01). The interaction of treatment with year was most notable for the primary alcohol group, with six of the 11 compounds having a significant treatment $\times$ year interaction. Finally, genotype had a significant interaction with treatment for only five compounds; four of those compounds were primary alcohols and the fifth total waxes.

3.3 Repeatability of cuticular wax compounds of fiber

To partition the observed phenotypic variance due to experimental unit and technical variances, repeatability on an entry-mean basis was calculated for the 41 compounds and their sum, total waxes. Repeatability values ranged from 0.00 ($\alpha\text{-C}_{18:0}$ MAG) to 0.98 (C_{28} alcohol) across these 42 wax traits (Table 1). The repeatability estimates for the primary components in the acyl-reduction pathway (Figure 3) were all above 0.85 . The decarbonylation pathway components (e.g., aldehydes and alkanes; Figure 3) were also above 0.85 with the exception of C_{27} alkane (0.79). As indicated by the statistical analysis, the high repeatability estimates ($r > 0.60$) for all but five of the wax compounds (C_{25} alkane, C_{26} alkane, C_{28} alkane, C_{30} alkane, $\alpha\text{-C}_{18:0}$ MAG), suggest that environmental and technical factors had a relatively minor influence on these traits. Future studies with genetic mapping populations are needed to

estimate heritability for the genetic control on these traits, but the expectation is that these estimates would be moderately high for most of the wax traits.

3.4 Wax content and fiber quality trait correlations

The C₂₆ - C₃₀ homologues of the primary alcohols and aldehydes, and total waxes, were all positively correlated (Pearson's r_p , P -values < 0.01 for WL and < 0.02 for WW) with fiber uniformity under WL and WW conditions at a false discovery rate (FDR) of 5% (Table 2). These correlations are possibly being influenced by the NM24016 genotype that had higher values of the alcohol and aldehyde C₂₆ - C₃₀ homologues and fiber uniformity than the calculated mean for each treatment (data not shown). The C₁₆ acid was positively correlated with fiber uniformity, strength, and length under WL conditions ($r_p = 0.67$, P -value < 0.01; $r_p = 0.62$, P -value = 0.01; $r_p = 0.82$, P -value < 0.01), but was only significantly correlated with fiber length under WW conditions ($r_p = 0.84$, P -value < 0.01) (Table 2). The 1,3-C₃₀ alkanediol was also positively correlated with fiber length under WL ($r_p = 0.77$, P -value < 0.01) and WW ($r_p = 0.75$, P -value < 0.01) conditions at a FDR of 5% (S. Table 4).

Lint percentage and micronaire were both negatively correlated with total wax under both irrigation treatments (P -values ≤ 0.02) (Table 2). Micronaire was also negatively correlated with the C₂₆ - C₃₀ primary alcohols under WL and WW conditions (P -values < 0.01) (Table 2). Beta-sitosterol was the only compound found to be positively correlated with lint percentage under WL ($r_p = 0.65$, P -value < 0.01) and WW ($r_p = 0.60$, P -value = 0.01) conditions at a FDR of 5% (S. Table 4). Beta-amyrenone and the 1,3-C₃₀ alkanediol were the only two compounds found to be significantly correlated with fiber strength under WL ($r_p = 0.89$, P -value < 0.01; $r_p = 0.69$, P -value < 0.01) and WW ($r_p = 0.63$, P -value < 0.01; $r_p = 0.60$, P -value = 0.01) conditions respectively (S. Table 4).

Total waxes were correlated with the C₂₈, C₃₀, and C₃₂ primary alcohols and the 1,3-C₂₈ alkanediol, which had the highest concentrations among the compounds under WL and WW conditions

respectively (P -values < 0.01 for WL and < 0.02 for WW) at a FDR of 5%. (S. Table 5, S. Table 6). The correlations between the aldehyde and primary alcohol $C_{26} - C_{30}$ homologues were significant (P -values < 0.01 for WL and < 0.02 for WW) under both irrigation treatments. The correlation between these compounds of the two main pathways leading to alkanes and the other leading to primary alcohols and esters suggests they are similarly regulated (S. Table 5, S. Table 6). Furthermore, the C_{28} primary alcohol was significantly correlated (P -values ≤ 0.01) with 1,2- C_{28} alkanediol and 2-OH C_{28} aldehyde under both irrigation treatments, suggesting the C_{28} alcohol might serve as a precursor for the synthesis of the diol (S. Table 5, S. Table 6).

4. Discussion

This study provides the first detailed characterization of the chemical content and composition of the cuticular waxes on cotton fiber, and describes the relationships between fiber waxes, quality traits, and effects of irrigation treatment. Analysis using GC-MS revealed that cotton fiber cuticular waxes were composed primarily of very-long chain primary alcohols, which accounted for nearly 74% of the total waxes. Other major wax compounds included free fatty acids (4%), aldehydes (0.5%), and alkanes (1%). This distribution of wax compounds is similar to the cuticular wax of the adaxial and abaxial leaf sides of *Macaranga tanarius* (L.) Müll. Arg., which is composed of more than 70% primary alcohols, 14% fatty acids, 2% aldehydes, and trace amounts of alkanes (Guhling et al., 2005). The composition is also similar to barley (*Hordeum vulgare* L.) leaves, which is composed of 75% primary alcohols, 9% free fatty acids, 4% aldehydes, and 1% alkanes (Avato et al., 1982; Lee and Suh, 2015). The low abundance of alkanes in the cotton fiber composition is in contrast to reports of high amounts of both long and short chain alkanes in leaf, bract, and boll tissue of mature cotton (Bondada et al., 1996).

Unlike barley which also contained 11% wax esters, and is consistent with *Brassica napus* L. leaves (Lee and Suh, 2015; Tassone et al., 2016), there were essentially no detectable wax esters present

in the cuticular waxes of cotton fiber. Instead, cotton fiber cuticular waxes contained appreciable amounts of compounds tentatively identified as very-long-chain 1,2- and 1,3-alkanediols. These classes of compounds have been reported previously in cuticular waxes of *Cosmos bipinnatus* petals (Wen and Jetter, 2007; Buschhaus et al., 2013 a, b). While the biosynthetic route for production of alkanediols is unknown, one possibility is the hydroxylation of C₂₈ fatty acyl-CoA precursors at the C₂ or C₃ position, followed by reduction of the hydroxy-acyl-CoAs to form the alkanediols (Buschhaus et al., 2013 a). The C₂₈ primary alcohol was the most abundant compound in the cotton fibers and found to be significantly correlated with the 1,2-C₂₈ alkanediol. These findings give some evidence to support this alkanediol synthesis model by suggesting an over-abundance of the C₂₈ fatty acyl-CoA precursor allowing for hydroxylation.

Statistical analysis of cuticular waxes of cotton fiber showed that the irrigation treatment did not have a significant effect on total waxes. On average, the WL treatment had reduced amounts of cuticular wax across the fiber samples. Only the C₃₁ alkane had a significant increase under WL conditions. Previous studies in *Arabidopsis thaliana* (L.) Heynh., sesame (*Sesamum indicum* L.), and soybean [*Glycine max* (L.) Merr.] leaves have shown that cuticular wax significantly increases under water deficit; more specifically, the unbranched alkanes increase (Kosma et al., 2009; Kim et al., 2007a,b). Similar results of increased cuticular wax and long-chain alkanes were found for cotton leaves and bracts on greenhouse grown plants subjected to WW and WL treatments (Bondada et al., 1996). It is possible that when water deficit is perceived, cuticular wax resources are being allocated to the leaves and bracts to prevent water loss, rather than the fibers which are developed to protect the seed from biotic and abiotic stress and assist in seed dispersal (Wang et al., 2004). A field study with cotton grown under WL and WW conditions where leaf and fiber samples are collected for cuticular wax analysis is needed to test this hypothesis. Another alternative could be that the WL treatment imposed in this study was not severe enough to trigger a more extreme cuticular wax response on the fiber. The

significant increase in the C₃₁ alkane under the WL treatment suggests that there is potential for a response similar to that in leaves.

The characterization of cotton fiber wax properties provides an opportunity to examine the relationships between fiber wax and quality traits, with the goal of identifying compounds that might be eventual targets for fiber quality improvement through molecular breeding approaches. Strong correlations under WL and WW treatments were found for primary alcohols and fatty acids with fiber uniformity and length, respectively, and micronaire which are important for yarn production. The biosynthetic pathway for the development of free fatty acids and primary alcohols is fairly well characterized in the acetyl-CoA and acyl-reduction pathways, providing potential candidate genes for transgenic breeding efforts focused on metabolic engineering of cotton fiber cuticles. Notably, several biosynthetic genes involved in the production of very-long chain fatty acids—precursors for the biosynthesis of waxes—have been shown to be actively expressed in elongating cotton fibers (Wanjie et al., 2005).

Unfortunately, negative correlations were also found between fatty acids, primary alcohols, and aldehydes with lint percentage. This indicates that selection for metabolic engineering of cotton fibers for increased uniformity, length and reduced micronaire may also reduce the lint produced per boll and ultimately decrease yield. Strong positive correlations were found between β -sitosterol, and lint percentage, as well as for β -amyrenone with fiber strength. Beta-sitosterol is a phytosterol while β -amyrenone is a triterpene; the biosynthetic pathways for phytosterols and triterpenes are not as well characterized, but share commonality in early synthesis with squalene synthase (Lee et al., 2004). Ideally the squalene synthase and the acyl-reduction pathways would not be linked, enabling dual improvement to lint percentage and fiber quality by increasing outputs from both pathways via metabolic engineering. Now that two draft *G. hirsutum* genome sequences are available (Li et al., 2015;

Zhang et al., 2015), cotton homologs for candidate genes can be identified and empirically tested for fiber quality improvement.

5. Conclusion

This study provides a detailed characterization of the chemical content and composition of cuticular wax of cotton fiber from seven upland cotton lines. Primary alcohols were found to be the most abundant compounds, accounting for more than 70%, in both WL and WW treatments. The significant correlations between the fiber quality traits of length, uniformity, and micronaire with certain free fatty acids, primary alcohols, and aldehydes may be useful for improvement of upland cotton using molecular breeding approaches. These same fatty acids, primary alcohols, and aldehydes had high repeatability estimates indicating they will be responsive to directional selection, and furthermore, the enzymes involved in these wax biosynthetic pathways could be plausible candidates for focused metabolic engineering. Efforts focused on identifying key rate-limiting enzymes in the acyl-reduction and decarbonylation pathways associated with cuticular wax synthesis could help cotton breeders improve fiber quality in parallel with breeding for other economically important traits.

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Figure 1. Quantification of the 46 compounds identified in the cuticular wax extraction separated by water-limited (WL) and well-watered (WW) irrigation treatments. Each bar represents the average of generated best linear unbiased estimators (BLUEs) across all 156 samples in $\mu\text{g/g}$ of fiber. Compounds identified to be significantly different between irrigation treatments are denoted with an *.

Figure 2. Thin-layer chromatography (TLC) of lipids extracted from two cotton samples, TM-1 (WL) and TM-1 (WW), using three different methods including hot isopropanol/chloroform (total lipid extract) methanol/chloroform (total lipid extract) and chloroform alone (cuticular wax).

Figure 3. Schematic showing the potential biosynthetic route for cotton fiber wax metabolites produced through the acyl-reduction and decarbonylation pathways. The thickness of lines denotes approximate metabolite abundance.

S. Figure 1. Representative total ion chromatogram (TIC) for compounds identified in the cuticular wax of mature upland cotton fiber.

S. Figure 2. The electron impact- mass spectra (EI-MS) with relative abundance of the tentatively identified unknown compounds listed by the peak identification from the TIC a.) #29 hydroxyaldehyde, b.) #44 β -amyrenone, c.) #31 hydroxyaldehyde, d.) #46 octacosyl acetate, e.) $\text{C}_{19:0}$ acid internal standard.

S. Figure 3. The automated mass spectral deconvolution and identification system (AMDIS) spectra percent abundance scaled to the tallest base peak of the tentatively identified unknown compounds listed by the peak identification from the TIC a.) #41 1,3 C_{28} alkanediol, b.) #42 1,2 C_{28} alkanediol, c.) #43 1,3 C_{30} alkanediol.

Table 1. Summary statistics of the best linear unbiased estimators (BLUEs) in µg/g of fiber and standard deviation (SD) for each compound, and their sum, identified in the cuticular wax of mature upland cotton fiber separated by irrigation treatment listed by the total ion chromatogram (TIC) number. The repeatability estimates are reported with their standard error (SE).

		Water-limited treatment				Well-watered treatment				Repeatability	
TIC No.	Compound	Mean	SD	Minimum	Maximum	Mean	SD	Minimum	Maximum	Estimate	SE
1	C ₁₆ acid	2.91	0.63	1.95	4.36	3.04	0.84	1.93	4.47	0.68	0.18
3	C ₁₈ acid	1.53	0.30	1.16	2.45	1.53	0.38	1.07	2.22	0.70	0.19
4	C ₂₀ acid	0.58	0.16	0.41	0.92	0.58	0.12	0.43	0.82	0.95	0.03
6	C ₂₂ acid	3.00	0.54	2.20	3.95	3.14	0.45	2.34	3.85	0.86	0.10
8	C ₂₄ acid	1.60	0.32	1.04	2.20	1.54	0.31	1.02	2.05	0.92	0.06
10	C ₂₆ acid	0.92	0.11	0.68	1.11	0.92	0.12	0.76	1.14	0.86	0.10
11	C ₂₈ acid	7.84	1.47	5.31	11.45	7.81	1.41	5.47	10.88	0.96	0.03
12	C ₃₀ acid	15.41	3.97	9.35	24.99	15.27	4.12	7.29	22.89	0.92	0.06
13	C ₃₂ acid	13.14	2.91	8.74	18.63	13.01	2.87	8.15	18.24	0.86	0.10
14	C ₃₄ acid	5.03	0.97	3.36	6.36	5.11	0.93	3.01	6.82	0.66	0.23
15	C ₃₆ acid	0.58	0.14	0.33	0.82	0.61	0.14	0.35	0.89	0.86	0.10
16	C ₂₂ alcohol	0.69	0.36	0.27	1.34	0.67	0.36	0.26	1.77	0.68	0.21
17	C ₂₄ alcohol	0.57	0.21	0.34	1.01	0.51	0.14	0.30	0.75	0.65	0.19
18	C ₂₆ alcohol	16.11	2.87	12.21	22.48	18.70	3.63	12.22	26.68	0.92	0.06
19	C ₂₇ alcohol	6.41	1.08	4.70	8.49	6.49	1.23	4.79	9.49	0.96	0.03
20	C ₂₈ alcohol	427.45	113.64	308.49	677.43	434.64	95.80	293.25	608.68	0.98	0.01
21	C ₂₉ alcohol	22.74	3.76	17.16	29.33	22.15	3.09	16.93	28.08	0.91	0.07
22	C ₃₀ alcohol	322.83	56.15	240.10	452.60	319.41	46.66	241.78	434.48	0.96	0.03
23	C ₃₁ alcohol	6.69	1.11	4.38	8.87	6.50	1.09	4.62	8.45	0.88	0.09
24	C ₃₂ alcohol	118.54	18.36	83.66	147.50	116.32	15.08	81.21	143.90	0.94	0.04
25	C ₃₄ alcohol	23.74	4.58	15.12	29.16	23.40	3.99	15.11	29.13	0.95	0.04
26	C ₃₆ alcohol	4.07	0.76	2.61	5.71	4.14	0.78	2.73	5.82	0.93	0.05
27	C ₂₆ aldehyde	0.58	0.25	0.32	1.37	0.58	0.18	0.39	0.91	0.96	0.03
28	C ₂₈ aldehyde	3.92	1.50	2.28	8.38	3.78	1.16	2.08	6.12	0.96	0.03
30	C ₃₀ aldehyde	3.08	1.15	1.66	6.51	3.00	0.87	1.94	5.12	0.89	0.08
32	C ₂₅ alkane	2.80	1.34	0.96	5.32	2.81	0.97	1.46	4.49	0.19	0.35
33	C ₂₆ alkane	3.09	1.24	1.48	5.38	3.07	0.90	1.82	4.65	0.13	0.34
34	C ₂₇ alkane	4.17	1.09	2.40	6.63	3.97	0.92	2.56	5.77	0.79	0.12
35	C ₂₈ alkane	2.11	0.67	1.28	3.79	2.02	0.43	1.41	2.79	0.42	0.35
36	C ₂₉ alkane	1.99	1.36	0.35	6.07	1.69	0.96	0.50	4.11	0.91	0.05
37	C ₃₀ alkane	1.53	0.28	1.02	2.22	1.52	0.22	1.16	1.90	0.30	0.38
38	C ₃₁ alkane	5.64	1.65	3.72	11.07	5.12	1.08	3.12	7.47	0.94	0.04
41	1,3-C ₂₈ alkanediol	153.15	24.92	118.54	207.22	165.03	23.73	128.64	200.33	0.61	0.23
42	1,2-C ₂₈ alkanediol	8.71	2.23	4.31	11.91	8.66	1.56	6.11	12.19	0.78	0.14
43	1,3-C ₃₀ alkanediol	8.42	2.06	5.16	13.94	9.26	2.21	6.67	15.69	0.77	0.17
40	α-C _{18:0} MAG	1.87	0.76	1.31	4.86	1.68	0.20	1.43	2.14	0.00	0.00
44	β-amyrenone	4.74	1.64	2.48	8.60	5.88	1.59	3.26	8.53	0.79	0.16
45	β-sitosterol	4.16	1.10	2.01	6.45	4.73	1.29	2.41	7.17	0.91	0.06
46	octacosyl acetate	5.91	1.52	3.68	9.35	6.11	1.53	2.98	9.72	0.95	0.04
29	2-OH C ₂₈ aldehyde	32.77	9.32	23.88	67.67	33.37	5.43	23.55	42.81	0.61	0.21
31	3-OH C ₂₈ aldehyde	21.21	3.46	15.16	26.35	22.62	3.14	17.15	28.68	0.89	0.08
	Total waxes	1261.00	217.11	929.15	1719.00	1295.00	194.39	968.57	1683.00	0.97	0.02

Table 2. Pearson correlation coefficients (above) and *P*-values (below) showing relationships between identified compounds in the acyl-reduction and decarbonylation pathways and fiber quality traits. Correlations were considered significant at 0.01 for WL and 0.02 for WW at a FDR of 5%.

	Free Acids											Primary Alcohols								Aldehydes			Alkanes				
Water-Limited (WL) Trait Correlations	C ₁₆	C ₁₈	C ₂₀	C ₂₂	C ₂₄	C ₂₆	C ₂₈	C ₃₀	C ₃₂	C ₃₄	C ₃₆	C ₂₂	C ₂₄	C ₂₆	C ₂₈	C ₃₀	C ₃₂	C ₃₄	C ₃₆	C ₂₆	C ₂₈	C ₃₀	C ₂₅	C ₂₇	C ₂₉	C ₃₁	Total wax
Lint percentage (%)	-0.61	-0.43	0.00	-0.05	-0.54	-0.64	-0.27	0.02	0.16	0.34	0.54	-0.20	-0.37	-0.77	-0.71	-0.51	-0.30	-0.04	0.26	-0.66	-0.62	-0.47	0.02	-0.37	-0.38	-0.31	-0.62
	0.01	0.07	1.00	0.84	0.02	<0.01	0.27	0.92	0.51	0.15	0.02	0.41	0.12	<0.01	<0.01	0.02	0.21	0.86	0.28	<0.01	0.01	0.04	0.95	0.12	0.11	0.20	<0.01
Fiber elongation (%)	-0.27	-0.12	-0.29	-0.40	-0.54	-0.40	-0.62	-0.38	-0.09	0.31	0.57	-0.47	-0.42	-0.26	-0.25	-0.06	0.24	0.51	0.64	-0.06	-0.06	-0.02	0.29	0.14	-0.08	-0.22	-0.15
	0.27	0.63	0.23	0.09	0.02	0.09	<0.01	0.11	0.71	0.19	0.01	0.04	0.08	0.29	0.31	0.80	0.32	0.02	<0.01	0.82	0.80	0.93	0.23	0.57	0.73	0.37	0.53
Fiber uniformity (%)	0.67	0.67	0.14	0.12	0.24	0.44	0.41	0.26	0.13	0.25	0.22	-0.31	-0.23	0.69	0.74	0.80	0.64	0.48	0.34	0.63	0.69	0.71	0.32	0.57	0.66	0.55	0.80
	<0.01	<0.01	0.57	0.63	0.33	0.06	0.08	0.28	0.61	0.31	0.38	0.20	0.34	<0.01	<0.01	<0.01	<0.01	0.04	0.15	<0.01	<0.01	<0.01	0.18	0.01	<0.01	0.02	<0.01
Fiber strength (kN m kg ⁻¹)	0.62	0.30	0.37	-0.02	-0.27	-0.02	0.25	0.10	-0.07	0.00	0.17	0.19	0.08	0.33	0.30	0.40	0.34	0.21	0.32	0.06	0.09	0.11	-0.36	-0.21	0.07	0.13	0.40
	0.01	0.21	0.12	0.94	0.26	0.93	0.31	0.68	0.77	0.99	0.48	0.44	0.75	0.17	0.21	0.09	0.16	0.38	0.18	0.80	0.71	0.64	0.13	0.40	0.79	0.60	0.09
Micronaire (unit)	-0.86	-0.64	-0.39	-0.28	-0.40	-0.52	-0.58	-0.24	0.04	0.19	0.30	-0.27	-0.29	-0.78	-0.75	-0.71	-0.44	-0.12	0.07	-0.53	-0.55	-0.51	0.14	-0.21	-0.46	-0.50	-0.76
	<0.01	<0.01	0.10	0.24	0.09	0.02	0.01	0.33	0.88	0.45	0.21	0.26	0.23	<0.01	<0.01	<0.01	0.06	0.64	0.78	0.02	0.02	0.03	0.55	0.39	0.05	0.03	<0.01
Fiber length (upper half mean, mm)	0.82	0.46	0.43	0.22	0.13	0.39	0.48	0.29	0.13	0.06	0.03	0.09	0.05	0.56	0.52	0.55	0.42	0.15	0.11	0.32	0.35	0.35	-0.16	0.06	0.27	0.32	0.60
	<0.01	0.05	0.06	0.36	0.59	0.10	0.04	0.23	0.58	0.79	0.91	0.71	0.83	0.01	0.02	0.02	0.07	0.54	0.67	0.18	0.14	0.14	0.52	0.81	0.27	0.18	0.01
	Free Acids											Primary Alcohols								Aldehydes			Alkanes				
Well-Watered (WW) Trait Correlations	C ₁₆	C ₁₈	C ₂₀	C ₂₂	C ₂₄	C ₂₆	C ₂₈	C ₃₀	C ₃₂	C ₃₄	C ₃₆	C ₂₂	C ₂₄	C ₂₆	C ₂₈	C ₃₀	C ₃₂	C ₃₄	C ₃₆	C ₂₆	C ₂₈	C ₃₀	C ₂₅	C ₂₇	C ₂₉	C ₃₁	Total wax
Lint percentage (%)	-0.53	-0.35	-0.13	-0.26	-0.53	-0.66	-0.29	-0.07	0.04	0.30	0.39	-0.18	-0.18	-0.82	-0.75	-0.49	-0.24	-0.03	0.16	-0.74	-0.77	-0.55	-0.32	-0.61	-0.49	-0.39	-0.64
	0.02	0.14	0.59	0.28	0.02	<0.01	0.23	0.77	0.88	0.21	0.10	0.47	0.47	<0.01	<0.01	0.03	0.33	0.92	0.51	<0.01	<0.01	0.02	0.18	0.01	0.03	0.10	<0.01
Fiber elongation (%)	-0.19	-0.21	-0.33	-0.46	-0.50	-0.26	-0.65	-0.47	-0.17	0.34	0.55	-0.39	-0.31	-0.25	-0.24	-0.21	0.23	0.56	0.57	-0.06	-0.13	-0.10	0.04	-0.12	-0.30	-0.45	-0.18
	0.43	0.39	0.16	0.05	0.03	0.27	<0.01	0.04	0.49	0.16	0.02	0.10	0.21	0.30	0.32	0.40	0.35	0.01	0.01	0.79	0.60	0.69	0.87	0.63	0.21	0.05	0.45
Fiber uniformity (%)	0.45	0.42	0.14	0.09	0.13	0.43	0.16	-0.02	-0.18	-0.12	-0.06	0.16	-0.11	0.72	0.71	0.64	0.39	0.28	0.10	0.64	0.70	0.70	0.43	0.66	0.58	0.33	0.66
	0.05	0.07	0.57	0.70	0.59	0.06	0.52	0.92	0.46	0.63	0.82	0.50	0.67	<0.01	<0.01	<0.01	0.10	0.24	0.68	<0.01	<0.01	<0.01	0.06	<0.01	0.01	0.16	<0.01
Fiber strength (kN m kg ⁻¹)	0.35	0.32	0.26	-0.11	-0.28	0.13	0.10	-0.06	-0.27	-0.07	0.12	0.53	0.31	0.16	0.04	0.26	0.11	0.05	0.13	-0.04	-0.05	0.15	-0.08	0.15	0.34	0.16	0.14
	0.14	0.19	0.29	0.66	0.25	0.59	0.67	0.80	0.26	0.76	0.63	0.02	0.22	0.51	0.87	0.28	0.66	0.83	0.59	0.86	0.83	0.54	0.74	0.54	0.16	0.51	0.58
Micronaire (unit)	-0.83	-0.80	-0.52	-0.47	-0.44	-0.77	-0.63	-0.43	-0.22	-0.14	-0.07	-0.16	-0.02	-0.72	-0.69	-0.79	-0.51	-0.25	-0.16	-0.60	-0.64	-0.71	-0.52	-0.80	-0.84	-0.74	-0.78
	<0.01	<0.01	0.02	0.04	0.06	<0.01	<0.01	0.07	0.36	0.57	0.77	0.52	0.92	<0.01	<0.01	<0.01	0.03	0.30	0.51	0.01	<0.01	<0.01	0.02	<0.01	<0.01	<0.01	<0.01
Fiber length (upper half mean, mm)	0.84	0.79	0.41	0.24	0.17	0.67	0.46	0.27	0.13	0.08	0.06	0.26	0.07	0.50	0.39	0.49	0.32	0.13	0.16	0.35	0.32	0.38	0.28	0.55	0.54	0.59	0.49
	<0.01	<0.01	0.08	0.32	0.48	<0.01	0.05	0.26	0.61	0.73	0.79	0.28	0.78	0.03	0.10	0.03	0.18	0.61	0.52	0.14	0.18	0.11	0.24	0.01	0.02	0.01	0.03

Figure 1 revised

Figure 2

Figure 3