THE EFFECT OF ABIOTIC STRESS ON COTTON FIBRE

By

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Submitted in fulfilment of the requirements for the degree of

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List of abbreviations

ACP	Acyl Carrier Protein
ACRI	Australian Cotton Research Institute
AGA	Apiogalacturonan
AIR	Alcohol insoluble residue
AMS	Agricultural marketing service
ANOVA	Analysis of Variance
A_w	Wall area
BOM	Bureau of Meteorology
CCRI	Central Cotton Research Institute
CO_2	Carbon dioxide
CL	Cuticular layer
CP	Cuticle proper
CSIRO	Commonwealth Scientific Industrial Research Organisation
DAS	Days after sowing
DD	Day degrees
d.f	Degrees of freedom
DPA	Days post anthesis

DNA	Deoxyribonucleic acid
ECR	Enoyl-CoA reductase
Elg	Elongation
Exp	Experiment
FA's	Fatty acids
FAS	Fatty Acid Synthesis
FTIR	Fourier-transform infrared spectroscopy
GC-MS	Gas chromatography mass spectroscopy
GDD	Growing degree days
G. Hirsutum	Gossypium hirsutum
H	Fineness
HG	Homogalactonuran
HPLC	High-performance thin-layer chromatography
HVI	High Volume Instrument
LACS	Long-chain acyl-coenzyme A synthase
L.S.D	Least significant difference
Max	Maximum
Mg	Milligrams

Mic	Micronaire
Min	Minimum
MR	Maturity ratio
mtex	Millitex
N	No
N/S	Not significant
P	Perimeter
Pers. Comm.	Personal Communication
Plus b	Yellowness
N.S.W	New South Wales
Rd	Reflectance
Rep	Repetition
RG-I	Rhamnogalacturonan I
RG-II	Rhamnogalacturonan II
RH	Relative humidity
RT	Room temperature
SEM	Scanning electron microscopy
U	Length uniformity

UI	Uniformity Index
USA	United States of America
USDA	United States Department of Agriculture
VLCFA	Very long chain fatty acid
Wd	Width
Y	Yes
XGA	xylogalacturonan

Abstract

Cotton fibre is the most important natural textile fibre, but it requires intensive scouring to disrupt the hydrophobic cuticle to allow dye to penetrate. The standard fibre qualities and amount of waxy cuticle material on cotton fibre varies across genotype. Some research has been undertaken addressing the influence of environment and management on standard fibre qualities and wax content, however no clear effect of either has been shown on cotton fibre cuticular wax despite the influence these variables have been shown to have on other standard fibre qualities and the cuticular wax content of other aerial surfaces of cotton plants. Predicted changes in climate in future will influence the ambient temperature of growing regions, likely increasing the amount of heat stress on cotton plants and may also affect water availability leading to water deficit stress. The effects of heat and water stress during flowering and early to mid fibre development period was captured for two consecutive growing seasons in a field production scenario for five upland cotton genotypes that varied in their known tolerance to both heat and water stress. It was hypothesized that stress at this time would tend to influence fibre initiation phases that might affect fibre perimeter determination and fibre lengthening, as well as wax content which is known to be deposited typically before the secondary cell wall thickening phase of fibre development. For all genotypes, water deficit increased measured cross-sectional properties with an associated increase measured in micronaire for both experiments. Increases were also seen following heat stress for fineness and maturity ratio in the second season. For fibre length, either water deficit alone or a combination of water deficit and heat stress, reduced fibre length for all but two genotypes. The exceptions were the water stress tolerant genotype which did not respond to water stress alone, and the poor water stress tolerant genotype which did not respond to either stress. Heat stress alone appeared to play the dominant role in reducing

fibre length for the genotype included for its good water use efficiency. Either water deficit stress or a combination of both stress treatments, increased cotton fibre strength in one of the experiments, while only a combination of both stress types in the other experiment produced the same effect. Both heat and water deficit stress were shown to significantly influence fibre cuticular wax deposition, but the effect was genotype dependant with the greatest effect observed on the genotypes included for poor heat tolerance and poor water deficit tolerance. For these genotypes significant increases were measured in cuticular wax. In an attempt to replicate the effects of water stress seen the field experiment in a glasshouse setting, water deficit stress was applied to a white control genotype, a naturally coloured high wax green genotype, and the poor water stress tolerant genotype. The only effects of water stress on fibre properties were to increase fibre fineness and strength, and decrease length, for which a main effect of stress was measured. There were no significant effects on fibre cuticular wax content or other fibre cross-sectional properties. This was attributed to the possible differences in the severity of the stress between the two experiments. Following this, an investigation into the dyeability of fabrics made from three different naturally coloured upland cottons with varying wax content was undertaken. One common white cotton, and two un-common coloured cottons, one brown and one green, were used for experiments. It was hypothesised that following dyeing, fabrics that were not scoured would have inferior colour fastness following a standard fabric wash test. The effect was expected to be more prominent for fabric made from the higher wax content coloured cottons. Fabrics made from these cottons were subjected to either traditional NaOH caustic scouring or hot ETOH scouring which more specifically targets the waxes, before being dyed and washed. It was found that while NaOH scouring resulted in greater dye uptake on the brown fabric, the ethanol scouring resulted in greater dye uptake on the naturally high wax green fabric. The

NaOH scouring adequately disrupted the hydrophobic cuticle of all experimental fabrics resulting in a dye result that was colour fast following the application of a standard wash test. Further to this a novel assessment of cotton polysaccharide content by GC-MS of three fabrics was performed. Following scouring, dyeing and washing fabrics were analysed spectroscopically to assess the ability of traditional scouring to remove polysaccharides and to assess the influence these polysaccharides may have on fabric dyeability and colour fastness. It was shown that the non-cellulosic polysaccharide content was significantly greater in naturally coloured cotton, but it did not negatively affect dyeability, and could be adequately scoured using traditional NaOH scouring method. These results further highlight the importance of carefully managing cotton growing conditions and stress to reduce any impact on fibre quality and cuticular wax component that may negatively affect the dyeability of cotton fabrics.

Chapter 1. Introduction and literature review.

Cotton is the most important natural textile fibre with approximately 25 million tons produced worldwide annually across 75 growing countries amounting to approximately 12 billion dollars in total international trade (www.cottonaustralia.com.au).

Significant efforts are made to breed cotton genotypes and to manage cotton crops with better traditional fibre attributes such as strength, whiteness, length and micronaire. Before cotton textile fabrics are dyed they must undergo energy intensive elevated temperature caustic scouring to remove the waxy cuticle to allow dye to penetrate fibres. The standard fibre qualities and amount of waxy cuticle material on cotton fibre varies across genotype. While some research has been undertaken addressing the influence of environment and management on standard fibre qualities and wax content, no clear effect of either has been shown on cotton fibre cuticular wax despite the influence these variables have been shown to have on other standard fibre qualities and the cuticular wax content of other aerial surfaces of cotton plants. It would be a desirable future scenario where cotton breeders of commercial cultivars can have greater control over the fibre quality and cuticle wax composition of fibre. Similarly, in sight of potential changes to the propagation conditions and climate of the future, it is of value to have a greater understanding of the effects of management and environment on standard fibre quality and cotton fibre cuticle wax.

The objectiveof this research is to gain a better understanding of the interaction between various cotton genotypes and the conditions in which they are grown, and the subsequent influence these factors have on standard fibre qualities and wax levels on cotton fibre.

Additionally, the effect of various fibre and cuticular components on the dye fastness of cotton fabrics will be examined.

1.1 Cotton

Cotton is a member of the genus *Gossypium*, from the family *Malvaceae* within the order *Malvale* (Wakelyn et al., 2006). There are 52 cotton species but the majority are wild shrubs with no commercial importance (Wendel et al., 2010). There are four commercially grown species; two allopolyploid species *Gossypium hirsutum* and *Gossypium barbadense* (2n =56) which account for 98% of all commercially grown cotton, and two diploid species *Gossypium arboreum* and *Gossypium herbaceum* (2n = 26) which account for the remaining 2% (Wakelyn et al., 2006, Barozai and Husnain, 2014, Wendel et al., 2010). These species, unlike their wild counterparts, have been selectively bred for commercial purposes to produce long, fine white fibres (Wendel et al., 2010). There are 8 recognised diploid genome groups named A through G and K. Australian cotton belongs to the C, G and K genomes (Wendel et al., 2010).

Cotton is grown for fibre and seed. Cotton seed can be utilised as an animal feed and also to produce cotton seed oil (Wakelyn et al., 2006). Cotton fibres can be processed to produce fabric which may be used for the production of items such as clothing and furniture (Wakelyn et al., 2006).

1.1.1 Cotton fibre

Cotton fibre (Figure 1.1) development includes four stages: (1) Initiation; (2) Elongation; (3) Secondary wall thickening; and (4) maturation (Haigler, 2010) (Figure 1.2). The initiation of the fibre involves the eruption of the fibre from the seed epidermal surface at the beginning of anthesis (flowering). At this stage the fibre is a small globular structure with a bulbous tip.

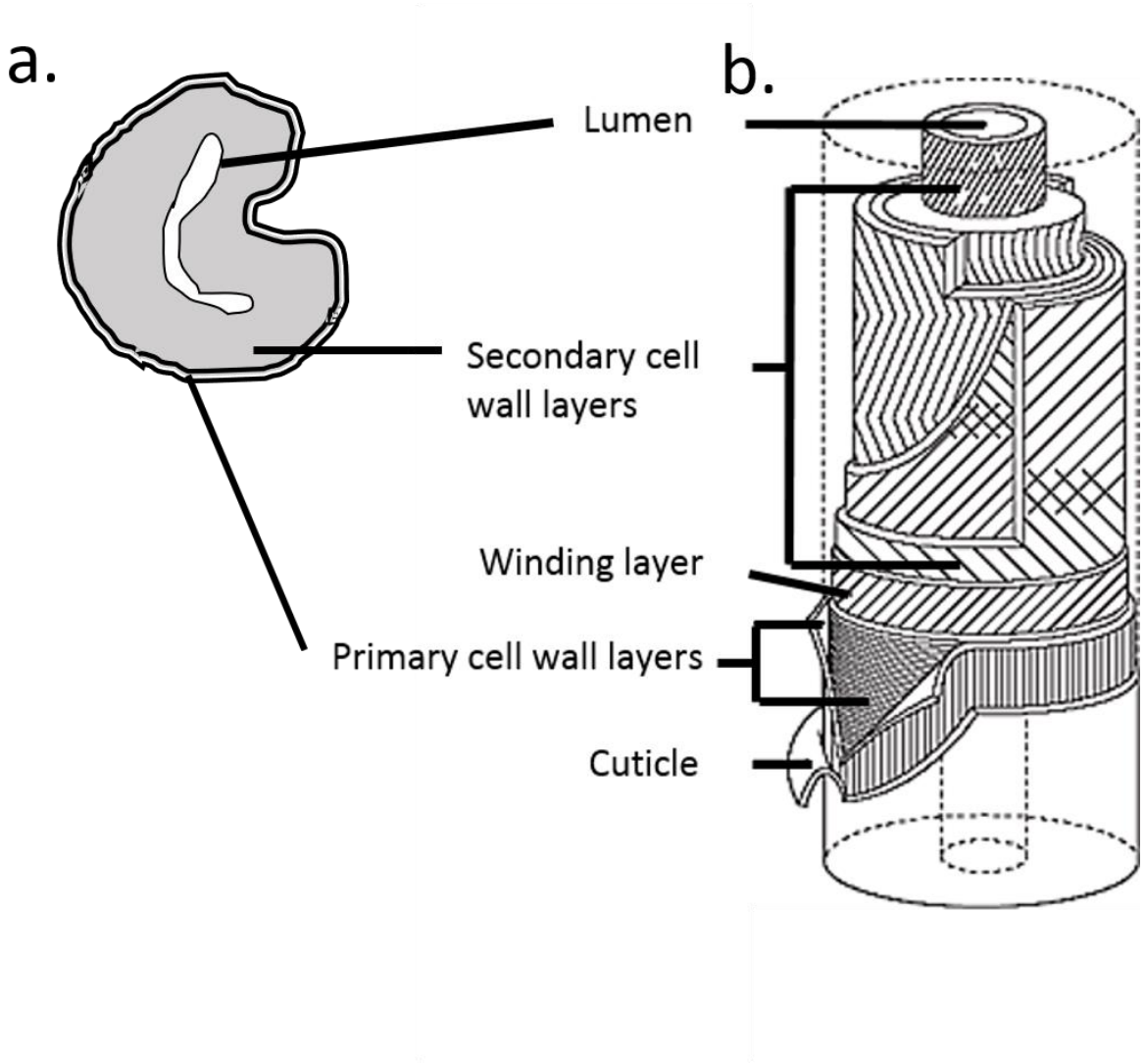


Figure 1.1. Structure of a cotton fibre. (a) Cross section of a mature cotton fibre as evident by the thickened secondary cell wall, and (b) an image of a whole cotton fibre showing primary and secondary cell walls which are made of several layers, surrounding a central lumen. Cotton fibre is covered by an outer cuticular layer. Figure adapted from http://textilebd.blogspot.com.au/2011/06/micro-structure-of-cotton-fiber.html

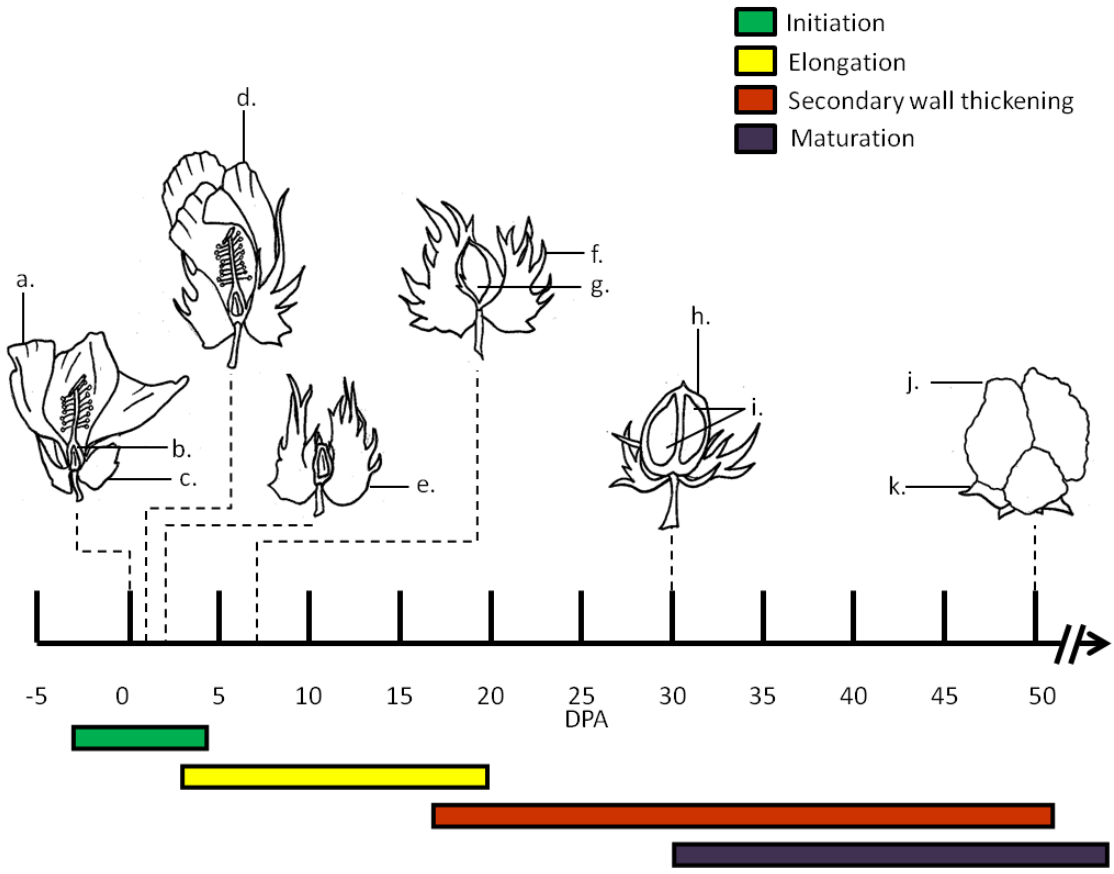


Figure 1.2. Cotton fibre development. Timeline of cotton fibre growth showing initiation -3 to 5DPA, elongation 3-20DPA, Secondary wall thickening 17-50DPA, and maturation 30-55DPA+. (a) Showing the petal of a fully open flower at 0DPA, (b) Ovule, (c) Bract, (d) Petals on flower beginning to close at 2DPA, (e) Remaining bract at 3DPA following the removal of the flower, (f) Bract remains at 7DPA, (g) Boll is forming as fibres elongate at 7DPA, (h) Boll is formed, (i) Fibres have elongated and maturation phase is beginning, (j) Mature fibres have erupted from boll, and (k) Boll has opened and desiccated to expose fibres.

The co-commencement of the fibre growth with anthesis means that we can describe the age of the fibre by days post-anthesis (DPA). This initial stage typically lasts several days. Stage 2 is the elongation of the fibre. Here the fibre tip begins to change from a bulbous to a sharp pointed tip and we see rapid primary cell wall synthesis leading to a large change in the length of the fibre which may be in excess of 2.2 inches (Haigler, 2010). This stage may last until between 14 and 20 DPA depending on species and the environmental growth conditions (Haigler, 2010). Stage 3 involves the thickening of the cell wall which occurs due to the deposition of a cellulose secondary cell wall. This stage overlaps the elongation stage and usually begins between around 12-20 DPA and is occurs during the period between 35 and 55 DPA. The 4th stage, the maturation of the fibre involves the death and desiccation of the fibre. The development of the secondary cell wall is sometimes also referred to as maturation of the fibre but for clarity with this naming system, this development is classed as a separate stage (stage 3) and maturity (stage 4) refers only to this death and desiccation stage. The entire fibre development typically takes between 0 and 55 DPA (Figure 2.) (Haigler, 2010).

Cotton fibres when mature are comprised of a multilayered secondary cell wall made up of cellulose and other polysaccharides surrounding a central lumen (Figure 1.1). This secondary cell wall is covered by a transitional layer called a winding layer comprised of a lacy network of cellulose microfibrils which is encapsulated by an outer primary cell wall also made of cellulose (Figure 1.1). The entire fibre is coated with a waxy cuticle (Figure 1.) (Haigler, 2010).

There are three major classes of plant polysaccharides that make up the cell wall; cellulose, hemicellulose and pectins (Caffall and Mohnen, 2009). Cellulose is an important structural polysaccharide that makes up 90% of the cotton fibre. The remaining fibre weight is made up of hemicellulose, pectins and other cuticular compounds.

Hemicelluloses are wall polymers comprising b-(1,4)-linked pyranosyl residues with the 0-4 in the equatorial position, which can be solubilised by alkaline solvents. Hemicelluloses are a class of polymers which include xylans, mannans and xyloglucans (Caffal and Mohnen, 2009). This includes compounds such as heteroxylan, xyloglucan, heteromannan, Type I and II arabinogalactan and arabinan.

Pectins are complex polysaccharides with a linear backbone comprised of (1-4) – linked α – D- galacturonan with regions of alternating (1-4) α – D- galacturonic acid and (1-2) - α – D-rhamnopyranosyl residues (Kačuráková et al., 2000). Pectins are located primarily within the primary cell wall. There are five main classes of pectic polysaccharides including homogalactonuran (HG), xylogalacturonan (XGA), apiogalacturonan (AGA), rhamnogalacturonan II (RG-II) and rhamnogalacturonan I (RG-I) (Caffal and Mohnen 2009).

1.1.2 Fibre quality, classing and valuation

Cotton fibre quality as related to its commercial value, is based on a number of physical attributes that indicate its ability to be processed and its end product performance. Due to the state-run classing of all U.S. produced bales of cotton, the United States Department of Agriculture (USDA) Agricultural Marketing Service (AMS) has determined a standard set of fibre attributes which has become a global benchmark for cotton valuation. These attributes are determined by High Volume Instrument (HVI) (Uster Technologies. Uster, Switzerland) and capture a mixture of cross sectional, length, tensile, colour, and impurity level, attributes. In response to these attributes, various industry stakeholders that deal in cotton along its processing chain, such as merchants and spinners, have created various economic premium and penalty systems to facilitate the trade of cotton. This system allows for a base line quality to be set by which all cotton is judged and then priced for sale (Haigler, 2010). For cross

sectional properties, micronaire is the most important. Micronaire is a measure that uses specific surface area per unit mass to reflect a combination of the cotton fibre perimeter or degree of coarseness (or fineness) and maturity, is measured using airflow to gauge the pressure difference obtained when air is passed through a bundle of fibres of known weight (Peirce and Lord, 1939, Gordon, 2004). Maturity, which is a measure of the degree of cell wall thickening, is a mathematical component of micronaire. So is fibre fineness, also termed linear density (weight per unit length of fibre) and is expressed as milligrams per kilometer (mtex) (Peirce and Lord, 1939). Lower perimeter (finer) fibres that have good maturity (secondary wall thickening) hold greater value than coarser less mature fibres (Gordon, 2004).

For length, there are three important attributes. Upper half mean length, which is determined by scanning the fibre photoelectrically, is the most important length attributes. According to USDA AMS classing there are 4 length standards; Short fibres which are less than 21mm, medium fibres which are 22-25mm in length, medium-long fibres which are 26-28mm long, long fibres which are 29-34mm in length, and finally extra-long fibres which are those in excess of 34mm in length (Haigler, 2010). Length uniformity is the second attribute, which is a measure of the ratio of the average length of all fibres vs. the average length of the longest 50% of the fibers in the sample. Short fibre index (SFI) is the third length attribute, which is an indirect measurement of the proportion by mass of fibres that are shorter than half an inch. The longer the fibres in a sample, with greater uniformity and with minimal short fibre content, the greater the value of the cotton. This is particularly so for how cotton contributes and performs via the ring spinning process, which is used primarily to spin fine count premium yarns (Haigler, 2010).

The tensile attributes of fibre are also measured and include: Fibre strength, which is determined by the amount of force required to break a bundle of fibres of known weight and is expressed as grams per tex (g/tex) (Gordon, 2004); and elongation, which is sometimes known as extension, being the percentage elongation of a bundle of fibres before it breaks (Gordon, 2004).The stronger the fibre the easier it is to process.

Finally trash and colour measurements are recorded including; Trash, which is a measure of the amount of seed coat, leaf and general debris remaining in the fibre samples after ginning. The presence of trash can lower the price obtained for the cotton fibre upon sale. Colour classing, which is the process by which the ginned cotton fibre is graded according to its level of whiteness and is determined by measurements of reflectance (Rd) and yellowness (Plus b). The whiter the cotton the higher the price for which it can be sold. There are numerous things that can influence the colour of the fibre such as varietal differences, weathering and pest damage and lengths are taken to reduce these influences in order to produce the purest white fibre possible (Haigler, 2010). Finally, the preparation measurement is a measure of the degree of matting (neps) or knotting present in the fibre samples after ginning which may make it more difficult to process (Haigler, 2010).

1.2 The Cotton fibre cuticle

The plant cuticle is a non-living hydrophobic extracellular layer that covers all aerial surfaces of the plant (Yeats and Rose, 2013, Bird and Gray, 2003, Riederer, 2007). On cotton fibres the cuticle is approximately 12nm thick and typically accounts for between 0.3 and 1% of the total fibre weight although it varies between genotypes (Agrawal et al., 2007).

1.2.1 Structure of the cuticle

The cuticle is a composite structure (Figure 1.3) which is made up of numerous components including cutin and various waxes (Yeats and Rose, 2013, Jetter et al., 2007). Cutin, a polyester of C16 and C18 hydroxy-fatty acids and glycerol makes up a matrix that forms the basis of the cuticle (Javelle et al., 2011). In *G. Hirsutum* these waxes may include true waxes such as gossypyl carnaubate, gossypyl gossypate, and montanyl montanate as well as other cuticular wax components including alcohols and higher fatty acids, hydrocarbons, aldehydes, glycerides, sterols, acyl components, resins and suberin (Hartzell-Lawson and Hsieh, 2000, Yeats and Rose, 2013). The cuticle is comprised of two primary layers; the outermost layer the Cuticle Proper (CP), and the innermost layer the Cuticular Layer (CL) (Figure 1.3). The CL is a cutin rich layer which contains embedded polysaccharides whilst the CP layer contains fewer polysaccharides, comprising mainly waxes which are embedded within the cutin matrix. Additionally the CP layer also includes a waxy film present on the outermost surface of the plant in which epicuticular wax crystals are embedded (Figure 1.3) (Yeats and Rose, 2013, Jeffree, 2007). The waxes present in the cuticle vary but are primarily derived from very-long-chain fatty acids (VLCFA's) which are those from C20-C34 (Figure 1.4). These individual components can vary greatly in concentration between different cotton genotypes.

1.2.2 Function of the cuticle

The cuticle has several crucial functions. Specifically, the cuticle plays an essential role in the control of movement of water, gases and vapours into and out of the plant as well as the control of loss and uptake of polar solutes and the transport of lipophilic substances into and out of the plant. It is also important for mechanical containment and as a separating

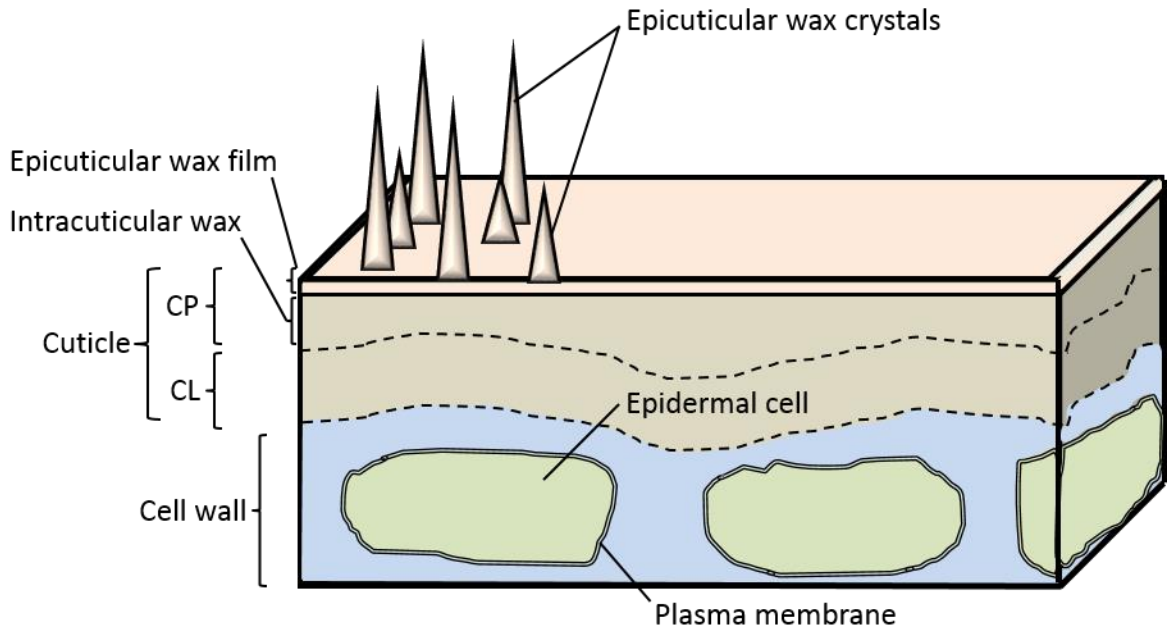


Figure 1.3. Cross section of the plant epidermis and cuticular layer. Cell wall is composed of polysaccharides. Directly above the cell wall lies the cuticle layer which is divided into three sections. The first, the cuticular layer (CL), is cutin rich with embedded polysaccharides. The second, the cuticle proper (CP), is a cutin rich layer with less polysaccharides and a greater proportion of intracuticular waxes. The remaining outermost layer is a thin epicuticular wax film embedded with epicuticular wax crystals. These layers are not entirely distinct and can vary between plant species. Figure adapted from Yeats and Rose 2013. Note, figure is not to scale and epicuticular wax crystals are a continuum of the epicuticular wax film and not distinct cells.

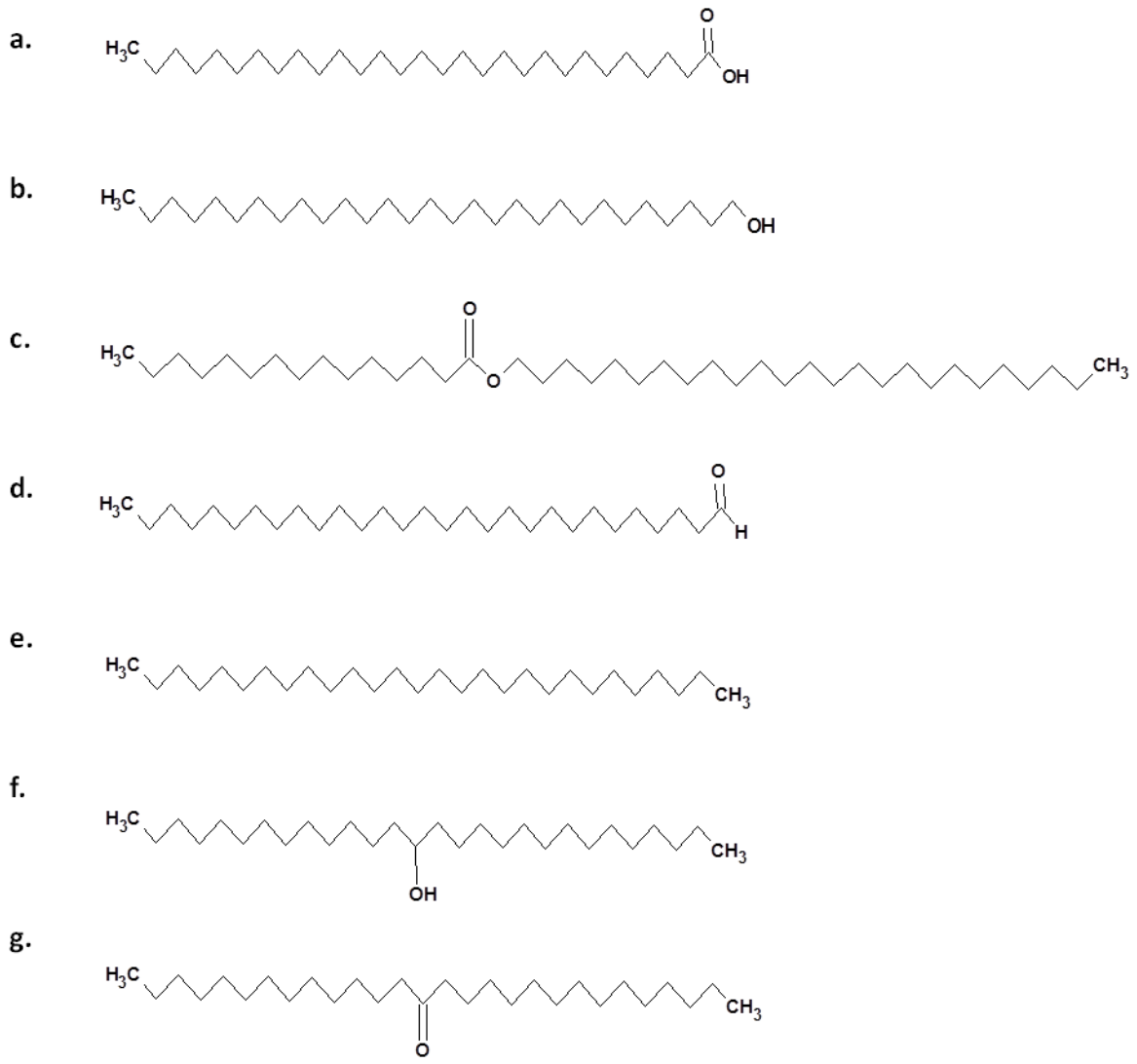


Figure 1.4. Lipid components of cuticular waxes. The average chain length of components found in the cuticle is shown. a. VLCFA, b. Primary alcohol, c. Wax ester, d. Aldehyde, e. Alkane, f. Secondary alcohol, g. Ketone.

agent in plant development. Additionally, it is the first line of defense for protecting the plant from outside stressors (Riederer, 2007).

Transpiration, the process by which water moves throughout a plant and is lost at aerial surfaces due to evaporation is controlled by stomata, small pores on the plant surface that can open and close in order to facilitate water and gas exchange and by the barrier provided by the cuticle (Riederer, 2007, Burghardt and Riederer, 2007). These stomata are closed for approximately 12h per day during which time all transport must be via the cuticle. The cuticle plays a vital role in minimizing the loss of water to the atmosphere due to its lipophilic and therefore hydrophobic nature. Because of this, water does not readily cross the cuticular membrane meaning most of the water loss is due to controlled transpiration via the stomata. This is also true of gas exchange whereby, gases are unable to move into or out of the plant via the cuticle and can only do so through controlled exchange via the stomata. In this way, the cuticle helps to stop unwanted gas exchange. Additionally, this hydrophobic nature hinders the uptake of polar solutes, i.e., solutes that will dissolve in other polar solvents such as water. Alternatively, due to its lipophilic nature, the cuticle allows the transport of lipophilic substances into the leaves, stems or fruits of the plant. These substances include secondary metabolites of the plant, as well as exogenously applied chemicals such as plant protection agents or other polluting chemicals. The hydrophobicity of the cuticle also allows the formation of water droplets which run off the plant surface collecting and removing foreign particles along the way such as dust, heavy metals and fungal spores which may otherwise collect on or infect the plant tissue. This self-cleaning mechanism is known as the lotus effect (Yeats and Rose, 2013, Riederer, 2007). The formation of these water droplets is facilitated by the presence of the epicuticular wax crystals (figure 1.3) which repel water and allow small

pockets of air to form underneath the droplets which aids in the movement of the water droplet off the plant surface (Yeats and Rose, 2013). The cuticle also provides a physical barrier to the underlying plant tissue whereby it limits the amount of penetrating ultraviolet radiation (UV) radiation (Riederer and Friedmann, 2007) which, whilst necessary for photosynthesis, in excess can cause damage to the plant cells as well as DNA damage. This physical barrier is also important for the protection of damage from bacterial and fungal pathogens and insects, and the deposition of water on plant surfaces which may increase the accumulation of polluting substances (Hartzell-Lawson and Hsieh, 2000).

Finally, the cuticle plays a role in defining boundaries during the development of the plant which helps to inhibit organ fusing during development (Riederer, 2007).

1.2.3 Biosynthesis of the cuticle

The current understanding of cuticle biosynthesis comes from studies involving model plants such as *Arabidopsis thaliana*. The section below describes the pathways as studied in *A. thaliana*. The synthesis of the wax portion of the cuticle occurs within the epidermal cells which lie directly beneath the cuticular layer (figure 1.3). The first step is the biosynthesis of C16 or C18 fatty acids (FA's) which are then converted to VLCFA's (Figure 1.5). Formation of C16 and C18 FA's occurs within the stroma of plastids beginning with two-carbon Acetyl-CoA units which undergo continuous elongation via a cycling reaction through the Fatty Acid Synthesis Complex (FAS) Each cycle involves a condensation of Malonyl-ACP to acetyl- CoA, a reduction of ß-ketoacyl-ACP, a dehydration of ß-hydroxyacyl-ACP, and a second reduction reaction of Trans Δ^2-anoyl ACP, each using an Acyl Carrier Protein (ACP) as a cofactor, which results in the addition of 2C -16C per cycle depending on the type of reducing enzyme

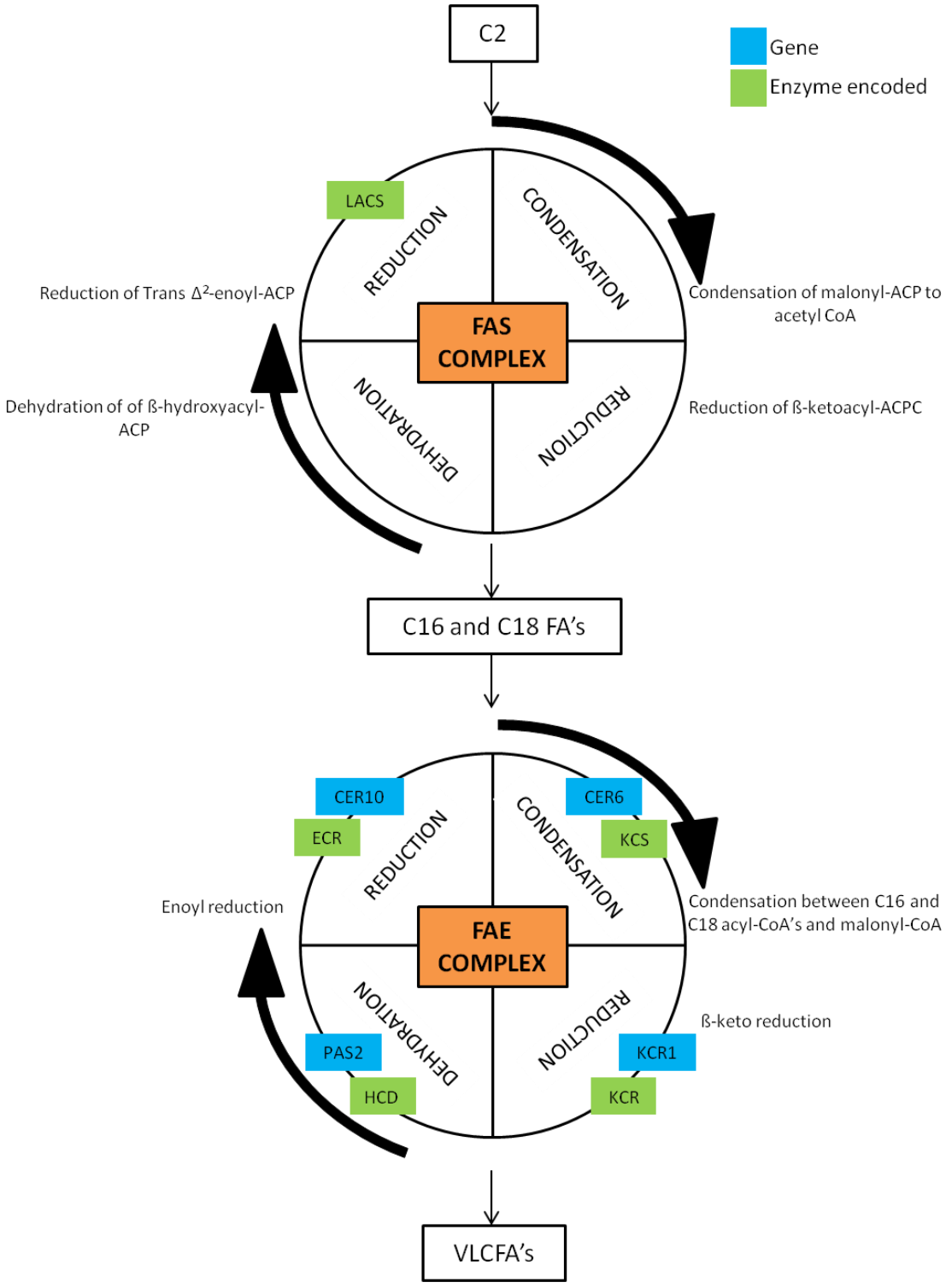


Figure 1.5. VLCFA biosynthesis. C16 and C18 FA's are synthesised via multiple cycles through the FAS complex. These C16 and C18 FA's are ultimately converted to VLCFA's via multiple cycles through the FAE complex with each cycle adding an addition 2C to the acyl chains. (Figure generated using information from Kunst and Samuels, 2003; Yeats and Rose, 2013; and Borisjuk et al., 2014)

involved in the reaction (Figure 1.5) (Borisjuk et al., 2014, Kunst and Samuels, 2003, Yeats and Rose, 2013). There are 3 types of FAS each with different reducing enzymes specific for different acyl chain length specificities. KAS III is specific for C2-C4, KAS I to C4-C16, and KASII to C16-C18 (Kunst and Samuels, 2003). Conversion of the C16 and C18 FA's to VLCFA's occurs in association with the Endoplasmic Reticulum (ER) and is done by converting the C16 and C18 FA's to CoA-thioesters utilising a long-chain acyl-coenzyme A synthase (LACS) isozyme before transporting them to the ER. Here the C16 and C18 acyl-CoA's then act as a substrate for the fatty acid elongase (FAE) complex through which VLCFA's are formed by the successive addition of two carbons to the fatty acid chains (Figure 1.5).

Like the reactions involved in the formation of the C16 and C18 FA's through the FAS complex, cycling through the FAE complex also involves a condensation, a reduction, a dehydration and a second reduction step (Figure 1.5). The first step occurring in the FAE complex is a condensation reaction. Here KCS, a ß-ketoacyl-CoA synthase encoded by ECERI-FERUM6 (CER6) gene catalyses a condensation reaction between C16 and C18 acyl-CoA's and malonyl-CoA. Next, a reduction reaction occurs catalysed by ß-ketoacyl-CoA reductase (KCR) which is encoded by ß-ketoacyl reductase 1 (KCR1) gene. Following this, ß-hydroxyacyl-CoA dehydratase (HCD) which is encoded by the gene PASTICCINO2 (PAS2) catalyses a dehydration reaction. Finally, enoyl-CoA reductase (ECR) which is encoded by ECERI-FERUM10 (CER10) gene catalyses an enoyl reduction reaction. These 4 reactions occur in a cyclical manner with each cycle adding an additional 2C resulting in the production of VLCFA's (Kunst and Samuels, 2003, Yeats and Rose, 2013, Borisjuk et al., 2014, Kunst and Samuels, 2009). These VLCFA's are the primary wax component of the cuticle and can undergo further modifications within

the FAE complex to produce the primary alcohols, esters, aldehydes, alkanes, secondary alcohols, and ketones also found within the cuticle (Yeats and Rose, 2013).

Following the production of VLCFA's the remaining cuticle components are formed via two divergent pathways within the ER before being transported to the cell membrane (Figure 1.6). The first, the Acyl Reduction Pathway is responsible for the production of primary alcohols and esters and has been well characterized in the model plant *Arabidopsis thaliana* where it has been shown that the production of primary alcohols from VLCFA's is a two-step process dependant on the CER4 gene which encodes a fatty acyl-CoA reductase which catalyses the process (Rowland et al., 2006, Kunst and Samuels, 2009).Following the production of primary alcohols, wax esters are produced from primary alcohols and acyl groups. In Arabidopsis this process is catalysed by a wax synthase/diacylglycerol acyl-transferase (WS/DGAT) family (Li et al., 2008, Kunst and Samuels, 2009) The second, the Decarbonylation Pathway is responsible for the production of aldehydes, alkanes, secondary alcohols, and ketones. (Yeats and Rose, 2013, Kunst and Samuels, 2003, Kunst and Samuels, 2009). This decarbonylation pathway is less understood although recent advances in the study of Arabidopsis genes involved in this pathway have identified one enzyme involved. Although the formation of alkanes from VLCFA's is not well understood, the conversion of alkanes to secondary alcohols and then the conversion of these secondary alcohols to ketones has been shown to be catalysed by a 2 step process involving a cytochrome-dependent midchain hydroxylase encoded by MAH1 gene (Kunst and Samuels, 2009, Greer et al., 2007).

The synthesis of the other major cuticle component cutin begins in the epidermal cells. Cutin is composed of interesterified C16 and C18 hydroxy fatty acids as well as glycerol,

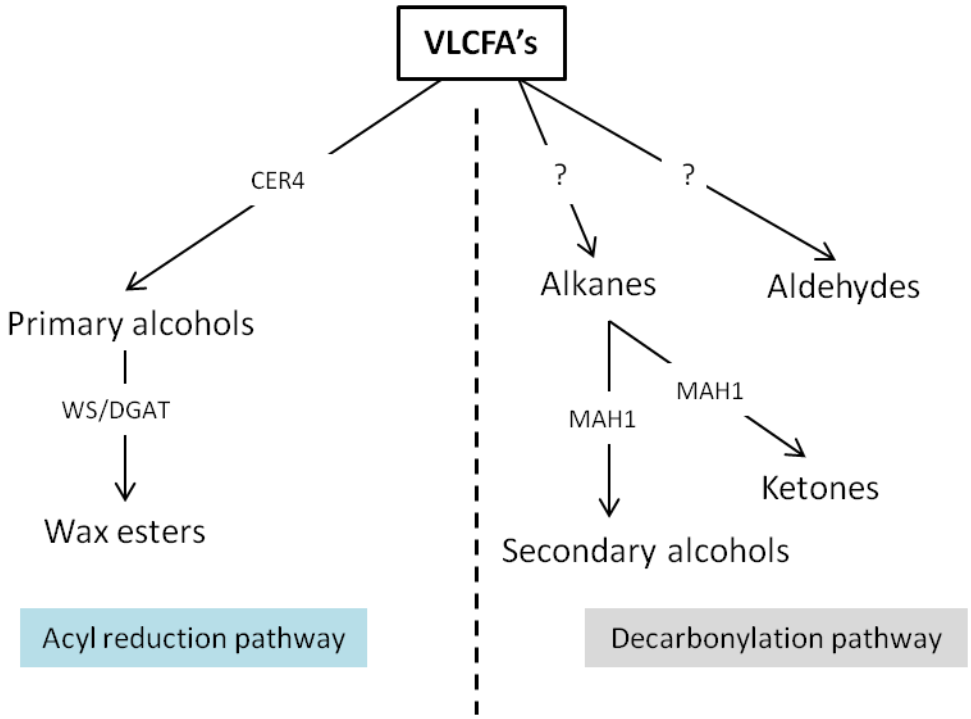


Figure 1.6. Cuticle wax biosynthesis; The Acyl reduction and Decarbonylation pathways. The acyl pathway is involved with the production of primary alcohols and wax esters through expression of CER4 and WS/DGAT genes, whilst the decarbonylation pathway is responsible for the development of alkanes, aldehydes, ketones and secondary alcohols through the expression of as yet mostly undetermined genes. Figure adapted from Greer et al., 2007.

phenylpropanoids and dicarboxylic acids. To begin with FA's are synthesized and are then transported to the ER where a dihydroxyhexadecanoic acid- CoA ester is formed via three steps; ω-hydroxylation, mid-chain hydroxylation and the synthesis of an acyl-CoA intermediate as shown in (Figure 1.5). The following process is not yet well understood however it is known that the final product of the intracellular phase of cutin biosynthesis is the production of 2-monoacylglyceryl esters of cutin monomers which are then transported out of the epidermal cell to the cuticular membrane where they are polymerized to form cutin biopolymers (Yeats and Rose, 2013).

1.3 Naturally coloured cottons

Although most commercially grown cotton has been bred to produce white fibres there are naturally occurring coloured cottons. These fibres are typically brown or green and have been shown to contain naturally higher percentages of wax within their cuticle. This has been recorded as high as 14% in comparison to the typical range of 0.3-1% found in white cottons (Conrad, 1941, Pan et al., 2010). Brown cotton typically contains twice the amount of cuticular wax compared to white varieties; approximately 2% of total fibre weight (Pan et al., 2010). White cottons have been exploited and augmented with breeding to improve their cross-sectional (micronaire), length, and tensile, properties, thus ensuring cotton that is more optimal for commercial use in yarn spinning, fabric manufacturing and dyeing. The same efforts have not been applied to naturally coloured cottons and as such they typically are shorter, with lower length uniformity, are weaker, and have less optimal cross-sectional properties (usually coarse and less mature), compared to their white counterparts (Pan et al., 2010). The higher wax levels on coloured cottons makes them useful in various studies

assessing the factors that influence cotton fibre wax, and research assessing the effect of wax on the dyeability of cotton fabrics.

1.4 The effect of abiotic stress on cotton fibre.

1.4.1 The effect of stress on standard and cross-sectional fibre attribute.

Australian upland cottons are adapted to high ambient temperatures within an optimal range of approximately 23-32°C (Burke et al., 1988, Farooq et al., 2015). Abiotic stress including heat stress and water deficit stress are known to affect plant species including upland cottons although the effect varies between genotypes. Numerous studies have investigated the effect of these stresses on standard fibre quality attributes that are measured by HVI, but limited work has been done investigating the effect on fibre cross-sectional properties such as maturity ratio, width, perimeter and wall area which can be measured using cottonscope (BSC Electronics. WA, Australia). Typically heat stress has been shown to increase fibre maturity, micronaire and fineness while decreasing fibre length (Reddy et al., 1999, Haigler et al., 2005, Singh et al., 2007). Water deficit stress has also been shown to influence fibre quality by increasing fibre length, elongation, strength, short fibre index and micronaire (Haigler et al., 2005, Singh et al., 2007, Nazar et al., 2012, Ahmad, 2013).

1.4.1 The effect of abiotic stress on cotton cuticular wax.

Whilst it has been well documented that stress is linked to increased production or thickening of the cuticle in a range of model and crop species such as *A. thaliana* and wheat (Shepherd and Wynne Griffiths, 2006) as well as in cotton leaf (Weete et al., 1978) (Oosterhuis et al., 1991, Bondada et al., 1996). There has been limited work done to assess the impact of stress on the amount and composition of waxes on cotton fibre.

One 1991 study that investigated water deficit effects on the cotton leaf cuticle and the effects of defoliation was able to demonstrate using Scanning Electron Microscopy (SEM) and GC-MS analysis that cotton grown in water-stressed environments produced a thicker cuticle layer which contained more epicuticular waxes of higher molecular weight (alkanes) (Oosterhuis et al., 1991) Here they applied continuous water stress (1 inch soil-water deficit) to field crops up until 14 days before defoliation when normal irrigation scheme was re applied to allow for potential stress-induced wax synthesis (Oosterhuis et al., 1991). Another study in 1996 examined the effects of water stress on the epicuticular wax composition of leaf, bract and boll found there was a significant increase in the amount of waxes present in the leaf and bract but no significant difference in the boll in response to water deficit induced stress and that there were increased percentages of alkane waxes in all three tissues (Bondada et al., 1996). Here glasshouse grown plants were watered as normal until flowering then water stress was applied by withholding water for 2-3 days until plants exhibited water-stress symptoms of stomatal closure. From this point water stressed plants received 1/3rd of the water of the control plants. The change of the composition of waxes to incorporate more long-chain alkanes is comparable to what has been reported in several other studies of different plant species. The lack of increase in the amount of wax present in the boll may be due to the lack of stomata and reduced photosynthetic activity occurring in the boll when compared to the leaves. We would expect less water loss through this fruiting tissue compared with vegetative tissues due to the reduced photosynthetic activity and therefore less of a response to the water stress. This study did not measure the effect on the fibre itself. An unpublished 2002 study by Gordon et al., aimed to understand the variation in wax content of fibre between Australian cotton varieties. They looked at the impact of both environmental factors (i.e. Growth region) and genetic factors (i.e. cotton variety) on the wax

content of Australian cotton varieties by comparing 5 cotton varieties, Sikala 40, Sicot 70, Siokra V16, Delta Sapphire and Delta Opal, grown across 13 regions across eastern Australia which varied from hot, central and cool locations. Average "growing degree days" (GDD) ranged from 25 to 35 degrees Celsius. They found that there were differences in the amounts of wax found on any one variety but when adjusted against micronaire this range was significantly reduced. When taking into consideration the influence of micronaire on wax amounts they concluded that the effect of variety on the amount of wax present was negligible and there appeared to be no interactions between hot, mild and cool growing regions and differences in the wax contents of the varieties grown in each region.

They also discovered that there were only subtle differences in the wax compositions between varieties, although this may have been influenced by the fact that the varieties did not differ greatly genetically, all coming from the same parentage. They did however note, that in some instances, low micronaire cotton samples were found to have a larger proportion of hydrocarbon waxes, a relationship they linked to plants that had suffered heat or water stress. High concentrations of hydrocarbon waxes were not found on all low-micronaire samples, so conclusions were only tentatively drawn and further analysis by controlled stress trials is required. Measurements of wax components were done using semi-quantitative methods including HPTLC and FTIR and a repeat of these measurements using a fully quantitative method such as GCMS may provide clearer answers. More recently, in 2017, Thompson et al., published a comprehensive study of water stressed field grown cotton in which 41 cuticular compounds were identified in the waxy cuticle using GC-MS. Of the 41 compounds characterised, 9 were shown to be significantly affected by the differing water treatments although the mechanism by which this change occurred was not investigated and requires further study. They showed that irrigation treatment did not influence the total

waxes present on the fibre although they do report a significant increase in C_{31} alkanes on fibres grown in water deficit conditions however no significant differences were reported for any of the other alkanes measured (C_{25}, C_{26}, C_{27}, C_{28}, C_{29} and C_{30}). They conclude that despite this increase in C_{31} alkanes, further study with field grown cottons, grown under well-watered and water stressed conditions are required to draw conclusions about the effect of stress on the deposition of cuticular waxes and that a more severe water stress may be required to elicit a response.

1.5 The effect of wax on the dyeability of cotton

The waxy cuticle is left on the cuticle during the ginning and spinning processes because it acts as a natural lubricant reducing the amount of fibre breakage occurring during processing. Following the processing of the cleaned fibre the fibre is spun into yarn which is further processed into textiles which can then be dyed before being used to manufacture a variety of goods including clothing and furniture. There are several different classes of dyes used on cotton fibre including direct dyes, vat dyes, sulfur dyes, azoic dyes and reactive dyes.

Direct dyes are water soluble anionic dyes widely used due to their ease of application, low cost and range of colours; however, their wash-fastness and vibrancy are only moderate which leads more towards the use of other dye types. Vat dyes which are water insoluble contain two carbonyl groups which are converted into water soluble leuco compounds under alkaline condition allowing the dye to be absorbed by the cellulose fibre. The most common application of vat dyes is in the use of indigo dyes to produce denim. Sulfur dyes are inexpensive and provide good wash fastness. They are used to dye fabrics deep muted shades. Azoic dyes are water insoluble compounds produced on the cotton fibres due to the reaction between a diazonium compound with a coupling component. Azoic dyes are used to produce

bright orange and red shades and some navy and black shades. Reactive dyes are dyes that comprise of a chromophore containing a substituent that reacts with a substrate. These dyes bond strongly and directly to the fibre resulting in high level colour fastness and are the most commonly used dye for the dyeing of cotton fibres (Lewis, 2011a).

Before a fabric can be dyed, the hydrophobic nature of the cuticle dictates the need for its removal. The waxy cuticle must be scoured so that dye can penetrate the cellulose portion of the fibre. Improper or incomplete removal of the cuticle is thought to be the reason behind many dyeing issues including irregular dyeing of theoretically identical fibre samples.

1.5.1 Scouring methods

The goal of scouring is to remove the hydrophobic non-cellulosic components of the cotton fibre to facilitate the dyeing process. There are a few methods by which cotton fibres can be scoured. Traditionally fibres are scoured using a caustic sodium hydroxide solution. This alkaline scouring method is effective, though costly due to the large quantities of energy and water required. It also poses serious environmental contamination risks due to the alkaline waste products particularly in developing countries where a large majority of commercial fabric dying takes place. Additionally, the high pH (pH 13-14) and high temperature (80-100°C) required for this process can be damaging to the cotton fibre (Mojsov, 2012, Hartzell and Hsieh, 1998). Despite this, it remains the most cost effective and efficient scouring method.

More recently enzymatic bioscouring processes which have specific actions, are less damaging to the cellulose and can be performed without the potential for harm to the environment have been investigated. There are a few types of enzymes that have industrial applications including pectinases which break down pectin, amylases which break down

starch molecules, cellulases which break down cellulose, proteases which break down proteins and lipases which break down lipids.

Removal of cuticular waxes by enzymes such as pectinase have been shown to be the most effective form of bioscouring and can remove enough of the cuticle to allow dye penetration without damaging the underlying cellulose (Mojsov, 2012, Tzanov et al., 2001). Pectinases remove the pectin found between cellulose fibrils which helps to increase dye penetration into the fibre and improves the ease with which waxes can be removed (Mojsov, 2012). Cellulases are perhaps the second most effective enzyme used for bioscouring. Cellulases work by catalyzing the hydrolysis of cellulose into smaller sugar molecules and in doing so cause damage to the cellulose on the outside surface of the fibre directly beneath the cuticle. This breakdown of the outer cellulose helps to loosen the hydrophobic cuticle layer allowing for increased dye penetration however damaging the cellulose layer in this way also reduces the quality and strength of the fibre (Hartzell and Hsieh, 1998).

The major limitation of this form of scouring is the high cost of the enzymes which mean that despite the reduced energy and water costs it is still an expensive process. The production of scour friendly cotton varieties that can be scoured faster and with less reagent will be useful in optimizing both the cost and efficiency of the scouring process especially where enzymatic bioscouring is concerned

1.6 Methods for the removal of cotton waxes for analysis.

Cotton wax was first removed for quantitative analysis in 1941 by a method that involved the cycling of hot ethanol over fibre for a number of cycles until no more wax was able to be removed. The number of cycles was found to be 90 using this method (Conrad, 1941). The ethanol extractable matter was then back extracted using chloroform to separate the waxes

from the other non-cellulosic ethanol extractable components removed then mixed with distilled water to facilitate purification of the wax by the removal of these other components which largely are comprised of sugars. This method produces accurate reproducible measurements but is labor intensive. More recently, techniques involving removal of cuticle from fibre by direct chloroform extraction followed by analysis using Gas Chromatograph Mass Spectroscopy to estimate total fibre wax have been employed (Thompson et al., 2017). Specific components can be identified through careful comparison of obtained spectra to known standards. Other simple methods include soaking plant tissue in chloroform to facilitate removal of the waxes, followed by evaporation to concentrate the waxy components. The latter method is more common with leaf tissue for plants such as *Arabidopsis thaliana* whose leaves are small. This method whilst simple and fast does not ensure complete removal of waxes and as such is not suitable for the quantification of total fibre waxes. The Conrad method is still the most commonly used and effective way to remove total wax from fibre samples when aiming to quantify the total cuticular wax.

1.7 Research aims and hypothesis

This project aims to further this area of study by developing a greater understanding of the effects that environmental abiotic stress applied during flowering and early to mid fibre development has on fibre quality attributes including cross-sectional, length and tensile attributes. Additionally, it aims to understand the effect this stress may have on the development of the fibre cuticle and its individual components and an understanding of the effect that the cuticle and its individual components have on the scouring and dying process. The following hypotheses will be tested;

1. Heat and/or water stress applied during flowering and early to mid fibre development will affect fibre cross-sectional, length and tensile attributes for both white and naturally coloured cottons.
2. Heat and/or water stress applied during flowering and early to mid fibre development will cause increased deposition of total wax on the cotton fibre cuticle.
3. Increased alkane wax concentration in fibre cuticle will negatively affect fabric dyeing and colour fastness.
4. Ethanol scouring will more efficiently remove cuticular waxes and low molecular weight sugars compared to traditional caustic scouring which will result in greater dye fastness in fabrics
5. Traditional caustic scouring will more efficiently remove polysaccharides compared with ethanol scouring
6. Total wax content will have a greater impact on fabric dyeability than polysaccharide content.
7. Alkane waxes are more difficult to remove by scouring and will therefore have the greatest effect on fabric dyeability and colour fastness.
8. Following fabric dyeing without any scouring, there will be a distinct change in fabric appearance following washing, and this change will be magnified for fabrics made of cotton with more cuticle wax and other components.

The following aims will address these hypotheses;

1. Assess the effect of heat and water deficit induced stress, in a commercial field production scenario, on standard fibre quality attributes of five cotton varieties. (Chapter 2)

2. Assess the effect of heat and water deficit induced stress, in a commercial field production scenario, on the amount of cuticular waxes on the fibre of five cotton varieties. (Chapter 3)
3. Develop a screening method for the quick determination of cotton fibres containing cuticles with high proportions of alkane wax using Scanning electron microscopy. (Chapter 3)
4. Determine the effect of water deficit induced stress, in a controlled glasshouse environment, on the standard fibre quality of naturally coloured cottons. (Chapter 4)
5. Determine the effect of water deficit induced stress, in a controlled glasshouse environment, on the amount of cuticular waxes on the fibre of naturally coloured cottons. (Chapter 4)
6. Assess a standard dye fastness test on fabric made from cottons varying markedly in the amount of cuticle components. (Chapter 5)
7. Assess the standard dye fastness test on fabric made from cottons varying markedly in the amount of cuticle components following the use of either traditional caustic scouring that disrupts a wide range of cuticle components, or ethanol scouring which is known to target more specifically waxes and some low molecular weight sugars. (Chapter 5)
8. Assess the effect of traditional caustic scouring and ethanol scouring on their ability to remove cuticle components including waxes, low molecular weight sugars and polysaccharides. (Chapter 6)

Chapter 2. The effect of heat and water deficit stress on the fibre quality of five field grown upland cotton genotypes.

Abstract

Heat stress and/or water deficit stress was applied to five upland cotton genotypes across two field experiments (Exp. 1 the 2013-2014 season, and Exp. 2 the 2014-2015 season). Sicot 71 was included as a control genotype. Siokra L23 and CS 50 were included for their good and poor water use efficiency respectively, and CIM-448 and Sicala V-2 were included for their good and poor heat tolerances respectively. Stress was applied during the flowering and early to mid fibre development phases. It was hypothesised that these abiotic stresses would impact cross-sectional, length and strength fibre properties, with the greatest impact thought to be on the genotypes with both poor heat stress and water stress tolerance. For all genotypes, water deficit increased measured cross-sectional properties affecting increased micronaire for both experiments, with the increases also seen following heat stress for fineness and maturity ratio in exp. 2. This was attributed to the field treatments causing initiating fibres to have larger perimeters. The stress treatments also promoted greater fibre secondary wall thickening and higher measured fibre maturity ratio with an associated increase measured in micronaire. For fibre length, either water deficit alone or a combination of water deficit and heat stress, reduced fibre length for all genotypes except for Siokra L23 which did not respond to water stress alone in exp. 1 or CS 50 which responded to neither stress for either exp. 1 or 2. Heat stress alone appeared to play the dominant role in reducing fibre length for Siokra L23 which reflected its good water use efficiency. The reduction in length was attributed to the abiotic stresses preventing adequate structural components being laid down and the prevention of normal fibre growth biochemistry during the

lengthening phase of fibre development. For fibre strength across all genotypes, either water deficit stress or a combination of both stress treatments, increased the strength of cotton fibres in exp. 1, while only a combination of both stress types in exp. 2 produced the same effect. One plausible reason for this, is that the abiotic stresses reduced the number of fruit or the size of fruit, such that when stress was removed for most of the final phase of secondary wall development, there was more photosynthetic and cellulose production capacity relative to control fruit and fibres. This also explains why stress treatments had higher fibre maturity ratio results.

2.1 Introduction

Commercial cottons have been selectively bred to produce plants that can generate fibres of high commercial quality. The value of cotton is based on a number of physical attributes that indicate its ability to be processed and its end product performance which are determined against a standard set of fibre attributes as designated by the United States Department of Agriculture (USDA) Agricultural Marketing Service (AMS). These attributes are determined by High Volume Instrument (HVI) (Uster Technologies, Uster) and capture a mixture of cross-sectional length, tensile, and other attributes. The most commercially important cross-sectional property is micronaire, which is a collective measure of the degree of fineness or coarseness of fibres and the amount of cellulose that is deposited in the secondary cell wall (Gordon and Hsieh, 2006). To assist in understanding what effects micronaire, several other fibre cross-sectional attributes, including maturity ratio, linear density (or fineness), width, perimeter and wall area, can be determined by other instruments such as the Cottonscope instrument (BSC Electronics, WA.).

Australian commercial cottons are suited to arid growing conditions when provided with adequate irrigation regimes. The same is true of varieties grown in other cotton producing nations such as U.S.A. Temperature conditions in cotton growing regions are typically above 30°C and can reach as high as 48-50°C (Ashraf et al., 1994).

Cotton generally has been shown to perform most efficiently at 23-32°C (Burke et al., 1988, Farooq et al., 2015) and heat stress is known to adversely affect cotton plants. This is particularly evident once temperatures reach around 35°C where decreased plant growth rates can be readily measured (Singh et al., 2007). Increased night time temperatures that lead to an increased cotton foliage temperature by as little as 4-5°C have been shown to significantly decrease vegetative dry matter production, fruit retention and lint yield (Singh et al., 2007) and there is a negative correlation between yields and temperatures above 32°C early in boll development (Singh et al., 2007). Heat stress also affects fibre quality. Greater Day Degree accumulation due to increased temperature can cause an increase in fibre maturity past what is considered commercially attractive due to the high optimal temperature requirement of cellulose synthesis, occurring primarily during secondary wall synthesis, which is around 28-37°C (Haigler et al., 2005). This in turn affects the fibre fineness and micronaire which have been shown to increase linearly with increasing temperature, while heat stress has also been shown to decrease mean fibre length and increase short fibre index (Reddy et al., 1999).

Water deficit stress has also been shown to influence fibre length, strength and elongation and therefore has an effect on the quality of yarn that can be manufactured from the fibre (Nazar et al., 2012). Water deficit has been shown to cause an increase in fibre length (Nazar et al., 2012, Ahmad, 2013) when deficit was applied throughout the cotton growth season

(Nazar at al., 2012) and when deficit was applied from specific stages of growth including from squaring and at first boll split (Ahmad, 2013). The application of excess water can lead to the development of more immature fibres (Haigler et al., 2005). Combined heat and water stress have also been shown to specifically affect additional fibre qualities such as length and micronaire with fibres becoming shorter with higher micronaire (Haigler et al., 2005, Singh et al., 2007).

In Australia there are many cotton genotypes grown that have different tolerances to variations in environmental conditions such as in temperature and water availability. Five genotypes were chosen for the research described in this chapter due to their varying tolerances to heat stress, and their varying tolerance to water deficit by way of their water use efficiency (Table 2.1).

Sicot 71

Sicot 71 is commercially grown Australian CSIRO genotype with dense foliage and a mean plant height of 76cm. It exhibits medium to late maturity (178 days to mature) and produces large bolls with strong (30.4 g/tex) medium length fibre (29.0 mm) with medium micronaire value (4.3) (Reid, 2003).

Siokra L23

Siokra L23 is an okra leaf genotype adapted to both dryland and irrigated cotton farming in N.S.W and Queensland which was included for its comparatively good water use efficiency and potential greater tolerance to water deficit stress. It has a mean plant height of 89.6cm and produces longer (30.22mm), weaker (28.0 g/tex) and lower micronaire (4.1) fibres compared with Sicot 71 (Reid, 1992).

CS 50

CS 50 is adapted to irrigated cotton growing areas of N.S.W and Queensland and was included for its relatively poor water use efficiency and therefore being less tolerant to water deficit stress (Reid, 1992). It produces medium length fibre (29.9mm) that is weaker (27.3 g/tex) and lower micronaire (4.1) fibre compared with Sicot 71 (Reid, 1992).

CIM-448

CIM-448 was developed by the Central Cotton Research Institute (CCRI) at Multan, Pakistan and released in 1996. It was developed under high testing temperatures and was included here for its good heat tolerance as measured by electron transport of mitochondrial enzymes (unpublished ACRI research).

Sicala V-2

Sicala V-2 was included for its relatively poor heat tolerance. Sicala V-2 plants are medium height with strong (29.30 g/tex) medium length fibre (29.30 mm) that has a lower micronaire (3.86) compared with Sicot 71 (Reid, 1995).

The aim of this work was to apply heat stress and water deficit stress to these genotypes, such that the majority of fruit were stressed from flowering through to early and mid fibre development. It was hypothesized that early stress at flowering might influence fibre initiation and fibre micronaire related components such as fibre perimeter. Stress during early fruit and fibre development would likely influence the fibre elongation phase and therefore fibre length properties. Stress during mid fruit and fibre development would also likely affect the final secondary cell wall thickening phase of fibre development, which in turn may well influence fibre maturity and tensile properties.

2.2 Materials and methods

2.2.1 Cultivation and treatment of Cotton plants.

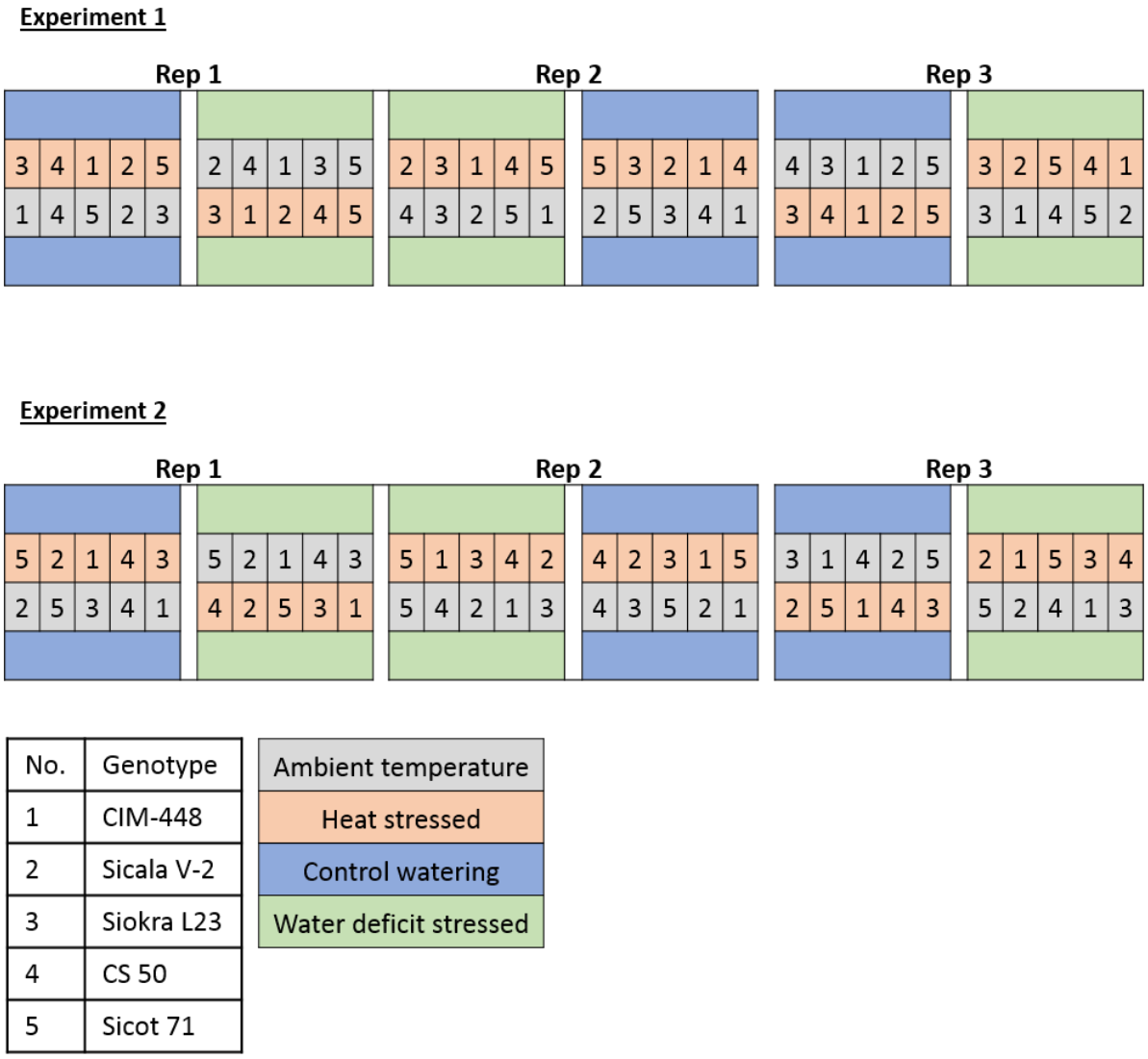
Two field experiments over two consecutive Australian growing seasons (Exp. 1 the 2013-2014 and Exp. 2 the 2014-2015 seasons) were conducted at the Australian cotton Research Institute (ACRI, 30° 12'S, 149°36'E), 22 km north-west of Narrabri NSW, Australia. This region is semi-arid with an annual rainfall of 646mm (Aust BOM, 2015). Each experiment was set up as a randomised complete block design with three repetitions (figure 2.1).

Experimental cottons

Five *Gossypium hirsutum* (upland cotton) genotypes were used for experiments. They were chosen for their range of heat tolerance and water use efficiency. The genotypes were Sicot 71, Siokra L-23, CS 50, CIM-448 and Sicala V-2 (Table 2.1). All were bred by the Commonwealth Scientific and Industrial Research Organisation (CSIRO), except for CIM-448 which was bred by the Central Cotton Research Institute (CCRI), Pakistan.

Cultivation of cotton plants

Experimental cottons were planted on Oct. 18th, 2013 and Oct. 22nd, 2014 (table 2.2). Each cotton genotype was planted on raised beds which were spaced 1m apart. The smallest experimental unit, or individual plots, consisted of a 13m long row of cotton, i.e. 13m^2. Plant density was established at approximately 10 plants m^{-2}. The soil at the site was uniform grey cracking clay soil (USDA soil taxonomy: Typick Haplustert; Australian soil taxonomy: Grey Vertosol). Nitrogen was applied as anhydrous ammonia approximately 12 weeks before planting at a rate of 200 kg N ha^{-1}. Planting occurred following an eleven-month fallow period which was preceded by a winter wheat crop.



No.	Genotype
1	CIM-448
2	Sicala V-2
3	Siokra L23
4	CS 50
5	Sicot 71

Ambient temperature
Heat stressed
Control watering
Water deficit stressed

Figure 2.1. Randomised block design for 2 seasons of field grown Australian cotton. Plots were allocated for each of the five genotypes and treatments using a random block design performed in triplicate. Four treatments were utilised; ambient temperature, heat stressed, control watering, and water deficit stressed as indicated. Experiment 1 is the 2013-2014 and Experiment 2 is the 2014-2015 season.

Application of heat stress

Plants were subjected to heat stress by placing Solarweave™ tents measuring seven metres in length, three metres in width and two metres in height over the plants for a period of five days to increase ambient temperature as per Cottee et al., 2010 (Table 2.2, Figure 2.2). Six tents were used in each experiment. Tents were constructed on site using Solarweave™ material. In addition to ACRI weather station data used to capture ambient temperature outside the tents, tiny tag data loggers (Hastings data loggers, N.S.W, Australia) were installed to capture the temperature inside the tents. Average temperatures for the two heat treatments were then determined using these measurements. Plants were considered heat stressed when the tents caused consistently raised temperatures as per Cottee et al., 2010 which has established that this constitutes a measurable heat stress.

Application of water stress

The irrigation schedule for cotton plants is outlined in table 2.3. Plots were furrow irrigated every 10-14d with approximately 1 ML ha^{-1} applied from December through to March apart from water stressed treatments as per Table 2.3. Water stress was applied by skipping two consecutive irrigations. Twenty-two days of continuous water stress were implemented. Specific water stress event dates as they relate to sowing date, flowering, heat stress event and harvesting date are presented in table 2.2. Plastic sheeting was placed over the soil on the water deficit plots to prevent the absorption of rain water so as not to affect the water deficit.

Measurement of field experimental weather conditions

Temperature, relative humidity, radiation and rain fall data for the time periods relevant to both experiments were obtained from a weather station located at the ACRI (Figures 2.3 – 2.6). Tiny tag data recorders (Hastings data loggers, NSW, Australia) were used to record

Table 2.1. Some details of the cotton genotypes used for both field experiments. All genotypes were produced by the CSIRO, except for CIM-448 which was produced by Central Cotton Research Institute in Pakistan.

Genotype	Reason for inclusion	Reference
Sicot 71	Control	Reid, 2003
Siokra L23	Good water use efficiency	Stiller et al., 2004; Stiller et al., 2005
CS 50	Poor water use efficiency	Reid, 2002; Stiller at al., 2005
CIM-448	Good heat tolerance	Pers. Comm. – Warren Conaty, ACRI
Sicala V-2	Poor heat tolerance	Pers. Comm. – Warren Conaty, ACRI

Table 2.2. Timeline including days after sowing (DAS) of some key agronomic events for both field experiments.

	Exp. 1	DAS	Exp. 2	DAS
Sowing date	18 Oct 13	0	22 Oct 14	0
Flowering	22 Dec 13 - 26 Jan 14	66 - 101	13 Dec 14 – 31 Jan 15	53 - 102
Heat stress event	10 Jan 14 – 15 Jan 14	93-98	16 Jan 15 – 22 Jan 15	87-93
Water stress event	06 Jan 14 – 28 Jan 14	89-111	13 Jan 15 – 03 Feb 15	84-105
Harvest date	17 Mar 14	159	23 Mar 15	153

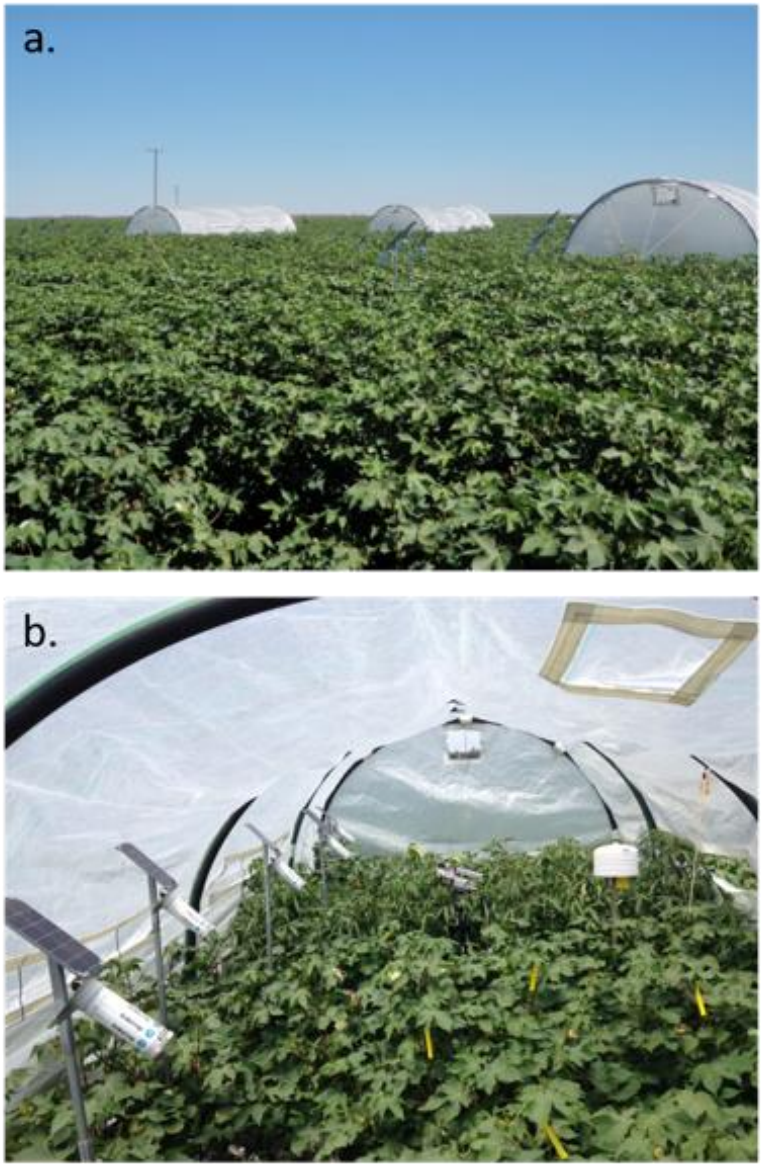


Figure 2.2. Application of heat stress in field grown cotton. Temporary Solarweave tents were placed over cotton plants for a period of six days to increase ambient temperature at the Narrabri field site. (a) Positioning of tents in field shown (b) Plants shown inside tents along with tiny tag recorders used to monitor ambient temperature.

Table 2.3. Field irrigation schedule for exp. 1 and exp. 2. The first irrigation was a pre-planting irrigation. All subsequent irrigations were in-crop irrigations. Irrigations were performed as indicated where Y= Yes, irrigated, and N = No, not irrigated.

Exp. 1			Exp. 2		
Irrigation date	Control	Water deficit	Irrigation date	Control	Water deficit
8 Oct 13	Y	Y	24 Oct 14	Y	Y
11 Dec 13	Y	Y	02 Dec 14	Y	Y
23 Dec 13	Y	Y	21 Dec 14	Y	Y
06 Jan 14	Y	N	13 Jan 15	Y	N
16 Jan 14	Y	N	23 Jan 15	Y	N
29 Jan 14	Y	Y	04 Feb 15	Y	Y
07 Feb 14	Y	Y	02 Feb 15	Y	Y
18 Feb 14	y	Y			

average daily (8am to 7pm) and maximum temperatures in the field and inside the tents during the stress periods (Table 2.6).

Measurement of leaf water potential to determine water stress

Leaf water potential (LWP) is the measurement of the negative hydrostatic pressure of a leaf and was measured using PMS instrument Co pressure chamber as per Conaty et al., 2014. Plants were categorised as either well-watered or water-stressed as per Cohen et al., 2005. Briefly a LWP > 1.4 MPa represents over-irrigated plants, LWP > 1.4 MPa to LWP > 1.7 MPa well-watered plants (WW), 1.7 MPa >LWP > _2.0 MPa low water stress (LWS), 2.0 MPa >LWP > 2.3 MPa medium water stress (MWS), 2.3 MPa >LWP severe water stress (SWS).

2.2.2 Harvesting of cotton fibre

Open cotton bolls were hand harvested from a 1 m^2 area of cotton plants within each relevant field plot. Hand harvesting of all open bolls occurred on multiple occasions (5 for exp. 1 and 4 for exp. 2) throughout crop maturity as bolls opened. Harvested bolls from each individual pick were stored separately. Day degrees, a measure of accumulated heat units which can be used to predict plant growth, were calculated using equation 2.1 (McMaster and Wilhelm, 1997) before being retrospectively applied to determine one harvest date per experiment for further analysis. It takes 750 day degrees for a flower to develop into a mature boll (Constable and Shaw, 1988), therefore the harvest date was selected by counting back 750 day degrees from each relevant harvest date to find the date of flowering from the most recent open boll in that maturity harvest. The harvest for each experiment where the majority of harvested bolls had been exposed to stress during flowering and early to mid fibre development was selected for further analysis. This time point was targeted due to the current understanding of when cuticular waxes are deposited during fibre development (Hartzell-Lawson and Hsieh,

2000) as this was the focus of thesis experimental work (Chapter 3). Seed cotton samples were ginned to separate the seeds from fibre using an experimental 20-saw Gin (Continental Eagle Corporation. Alabama, USA) located at the ACRI.

$$GDD = \frac{T_{max} + T_{min}}{2} - T_{base} \quad \text{(Equation 2.1)}$$

(Where T_{max} = daily maximum temperature, T_{min} = daily minimum temperature, T_{base} = the temperature below which cotton growth does not occur - approx. 20°C).

2.2.3 Assessment of fibre quality attributes

High volume instrument measurements

For each plot standard commercial classing testing and additional objective fibre testing was undertaken. Three replicate samples were taken from each plot sample for testing via an Uster Technologies Model 1000 High Volume Instrument (HVI) (Uster Technologies, Uster) located at ACRI. As part of this testing samples were conditioned under standard textile testing air conditions (20°C ±2, 65% RH ±3). Attributes measured were micronaire, upper half mean length, length uniformity index, short fibre index, fibre bundle strength and fibre bundle elongation.

Objective cross-sectional measurements

Three replicate 1 g samples were taken from each plot sample and subjected to standard textile testing conditions. Fibres were cut into 0.8mm snippets using a guillotine. 50mg (+/- 5mg) were accurately weighed then analysed via a Cottonscope instrument (BSC Electronics. WA, Australia) using Cottonscope V2.14 software. Attributes measured were Maturity Ratio (MR), Fineness (H) and Width (Wd). An estimate of the fibre cross section perimeter (P), and cross section wall area (A_w), were calculated via equations described previously (Montalvo,

2005, Montalvo and Von Hoven, 2005) (equations 2.2 and 2.3), using relevant measured micronaire, fineness and maturity ratio results.

$$P = 3.785 \times \sqrt{\frac{H}{MR}} \quad \text{(Equation 2.2)}$$

$$A_w = P \times \sqrt{\left\{\frac{(\text{Micronaire}+2.352)^2}{8.58} - 0.2525\right\}} \quad \text{(Equation 2.3)}$$

2.2.4 Statistical analysis

Statistical analysis was performed using a commercial package (Microsoft Excel) for basic descriptive statistics. GenStat Version 16 (Lawes Agricultural Trust, IACR. Rothamsted, UK) was employed for Analysis of Variance (ANOVA) of data. Raw fibre quality data for individual replicates for each genotype and treatment were added into this program. ANOVA was conducted as a two-way model with genotype and treatment being the two factors. Mean values were reported with preference given to significant two factor interaction, which was reported in bar chart format. Otherwise mean values for main effects with or without statistical significance were tabled where appropriate. The degree of the statistical significance of the ANOVA tests were indicated using standard star symbol convention, being when P values were either <0.05*, <0.01**, or <0.001***. Least significant difference (L.S.D) (5%) values were reported alongside significant ANOVA results to assist in mean value separation. The statistical analysis design for the ANOVA is shown in table 2.4a.

In separate statistical exercise, both season's experimental data (exp. 1 and exp. 2) was combined and a one-way ANOVA was undertaken where by season was the factor. This

allowed fibre quality attributes to be compared across seasons. The statistical analysis design for the ANOVA is shown in table 2.4b.

Table 2.4. Analysis of Variance Table. The degrees of freedom (d.f.) assigned to each component of the (a) two- factor model used to analyse the fibre quality data; and (b) the single factor model used to compare the data across two seasons (exp. 1 and exp. 2).

(a)

Source of variation	Degrees of freedom
Block	2
Genotype	4
Treatment	3
Genotype x Treatment	11
Residual	17
Total	37

(b)

Source of variation	Degrees of freedom
Season (exp.)	1
Residual	116
Total	117

2.3 Results

2.3.1 Measurements of applied stress and environmental conditions.

Assessment of water stress

According to the ranges determined by Cohen et al., 2005, the leaf water potential of the control plants indicated that the plants were in the well-watered category, whilst the stress plots were categorised as being in a water deficit (Table 2.7).

Seasonal weather conditions

Weather conditions recorded as seasonal averages showed that exp. 2 was hotter and more humid than exp. 1 which was evident in the faster accumulation of growing day degrees in exp 2 (Figure 2.4). Exp. 2 also experienced higher humidity compared to exp. 1 (Figure 2.5). There was no difference in average radiation (Table 2.5), however radiation during the stress application period (January) was higher for exp. 1 (Figure 2.6) Total rainfall (data not reported) was slightly higher in exp. 2 (Figure 2.7) with significantly higher rainfall occurring during the January stress application in experiment 2. Water deficit plots were protected from this extra water using plastic sheeting as described in the methods section.

The heat stress event for each experiment caused a significant increase in daily average ambient temperature and daily maximum temperature within the tents (Table 2.6).

2.3.2 Fibre cross-sectional attributes.

Experiment 1

A significant two-way interaction of genotype and stress was found for fibre fineness (Figure 2.8). There were no significant differences between fibre fineness for any of the genotypes

under control conditions except for CIM-448 which had significantly lower fineness than all other genotypes except for Sicala V-2. Heat stress alone had no effect on fibre fineness, while water stress alone caused significant increase in fibre fineness in all genotypes except CS 50 which was not significantly affected by water deficit. A combination of heat stress & water deficit caused a significant increase in fibre fineness in Sicot 71, CIM-448 and Sicala V-2 but did not have a significant effect on Siokra L23 or CS 50.

Significant main effects of genotype on cross sectional properties were found for all the measured cross-sectional properties (Table 2.8) ($P< 0.001$). Under control conditions CIM-448 had lower micronaire than all other genotypes and Siokra L23 was significantly higher than all other genotypes for which there were no significant differences (Table 2.8). A significant main effect of stress was measured for micronaire ($P<0.001$) where micronaire was significantly increased by both water deficit and a combination of heat stress & water deficit but not significantly changed by the other treatments (Table 2.9).

The highest maturity ratio under control conditions was measured for CS 50. Sicot 71 and Sicala V-2 had significantly lower maturity ratio compared with CS 50 but were not significantly different from each other. All remaining varieties had significantly different maturity ratios with the lowest maturity ratio measured in Siokra L23 (Table 2.8). Stress had a significant main effect on maturity ratio ($P= <0.05$). Heat stress alone caused a significant reduction in maturity ratio whilst water deficit alone and combined heat stress & water deficit stress caused significant increase in maturity ratio. No significant difference was measured between the water deficit alone and combined heat stress & water deficit treatments (Table 2.9).

Table 2.5. Seasonal weather conditions for Exp. 1 and Exp. 2. Weather data for both experiments from Oct – May obtained from the Myall Vale weather station.

	Exp. 1	Exp. 2
Avg. daily max temp (°C)	32.62	33.25
Avg. daily min temp (°C)	16.27	17.11
Total Rain (mm)	289.6	421.6
Average daily radiation (MJ/m²)	26.53	26.54
Avg. daily Max. Relative humidity (%)	70.72	73.51

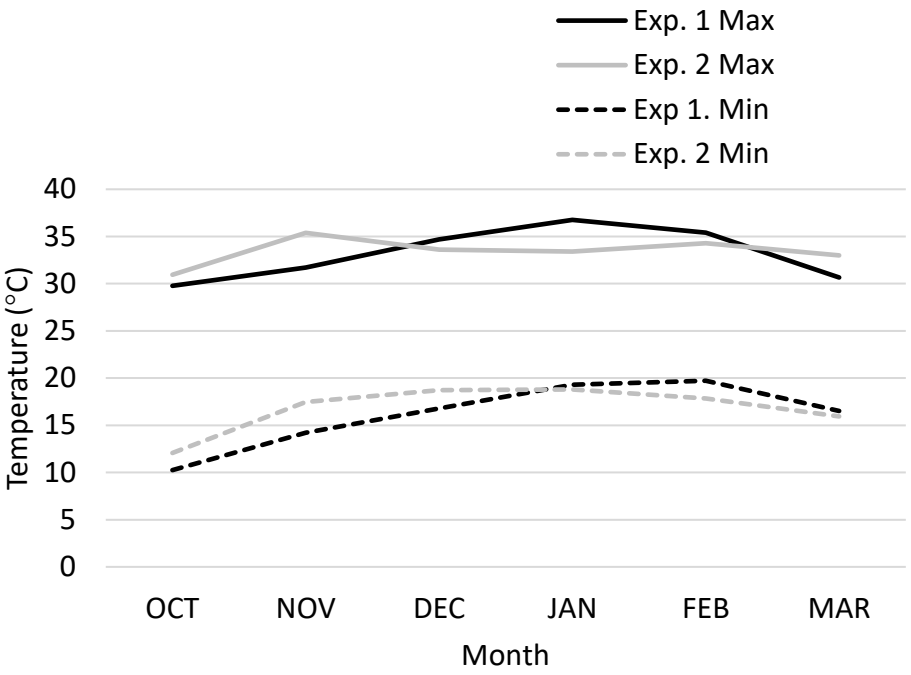


Figure 2.3. Average monthly temperatures for exp.1 and exp. 2. Graph illustrates the average minimum and maximum monthly temperatures for experiments 1 and 2. Average monthly temperature calculated from daily temperature data gathered at the Myall Vale weather station from October to March.

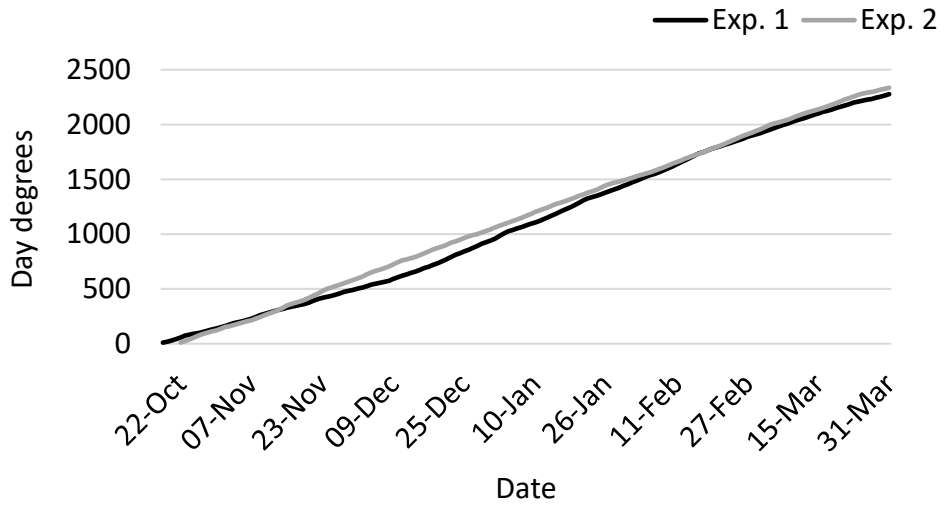


Figure 2.4. Calculated day degrees for exp. 1 and exp. 2. Graph illustrates the calculated day degrees **f**rom sowing date to harvest date for both experiments.

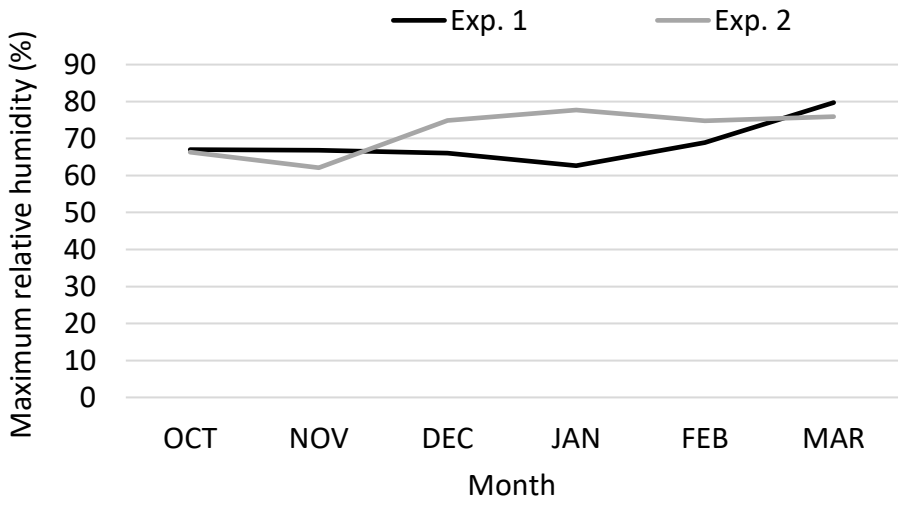


Figure 2.5. Average maximum relative humidity for exp. 1 and exp. 2. Graph illustrates the average monthly maximum relative humidity calculated using daily weather data gathered from the Myall Vale weather station.

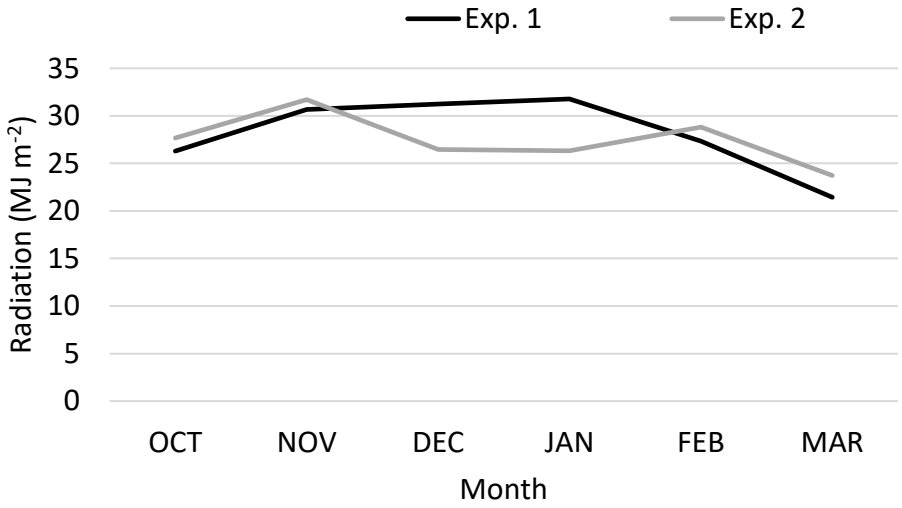


Figure 2.6. Average radiation for exp. 1 and exp. 2. Graph illustrates the average monthly radiation calculated using daily weather data gathered from the Myall Vale weather station.

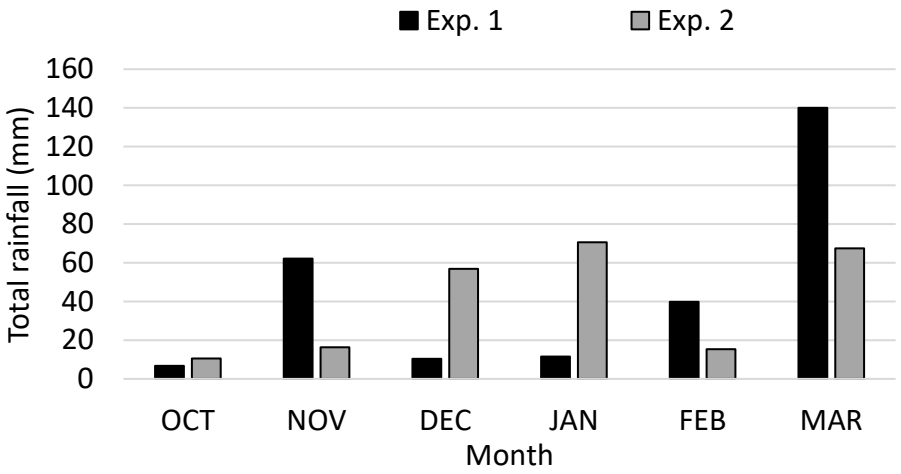


Figure 2.7. Total monthly rainfall for exp. 1 and exp. 2. Graph illustrates total monthly rainfall data obtained using daily weather data gathered at the Myall Vale weather station.

Table 2.6. Average and maximum daily temperature during the heat stress events. Daily average daytime temperatures (degrees Celsius) during the stress periods recorded for (a) Exp. 1 and (b) Exp. 2. Tents were applied to increase the ambient temperature. The average values presented are data from the Myall Vale weather station for the ambient plots, and tiny tag data logger data captured within heat stress tents.

(a)

	Average temperature		Maximum temperature	
Date	Ambient plot	Heat stress plot	Ambient plot	Heat stress plot
10 Jan 2014	27.48	28.75	31.92	34.67
11 Jan 2014	30.93	32.90	35.28	39.67
12 Jan 2014	34.81	36.29	38.79	42.50
13 Jan 2014	32.70	34.16	36.60	39.50
14 Jan 2014	32.92	34.54	36.80	39.67

(b)

	Average temperature		Maximum temperature	
Date	Ambient plot	Heat stress plot	Ambient plot	Heat stress plot
16 Jan 2015	29.91	35.37	32.37	38.94
17 Jan 2015	31.70	36.33	35.03	41.63
18 Jan 2015	31.71	35.56	34.74	40.12

19 Jan 2015	31.78	36.77	36.10	42.26
20 Jan 2015	29.31	32.59	34.00	39.50

Table 2.7. Leaf water potential of plants. Table shows average leaf water potential (Measured in MPa) of well-watered (WW) control plots and Water deficit stressed (WS) plots.

	LWP	P value
Exp. 1 - WW	1.436	P <0.001
Exp. 1 - WS	2.625	P <0.001
Exp. 2 - WW	1.491	P <0.001
Exp. 2 - WS	2.451	P <0.001

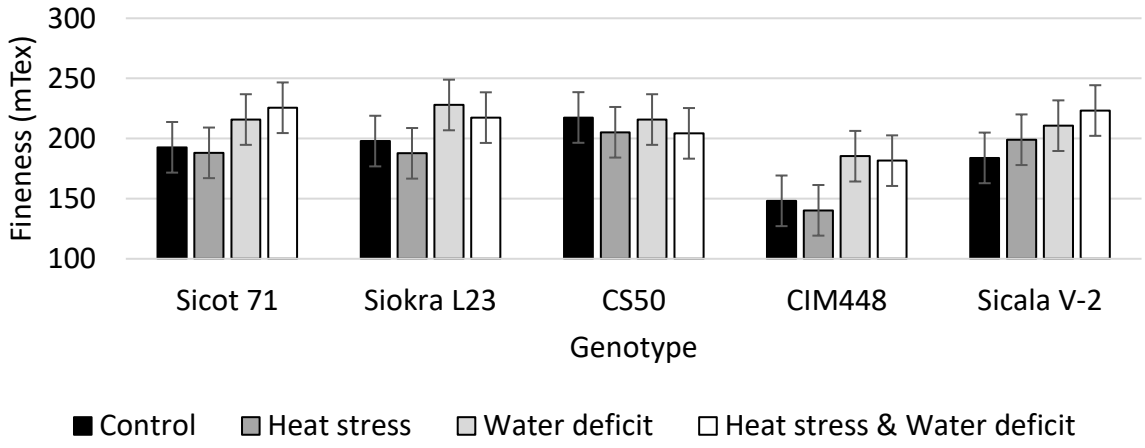


Figure 2.8. Average fibre fineness results for each genotype subjected to abiotic stresses for exp. 1. The graph illustrates the significant two-way interaction of stress and genotype. Mean values presented. Error bars are plus and minus L.S.D (5%) values to assist with mean separation.

Under control conditions all five genotypes had significantly different ribbon width with the smallest width seen in CIM-448 followed in ascending order by CS 50, Sicala V-2, Sicot 71, and Siokra L23 (Table 2.8). A significant main effect of stress was measured for width ($P<0.001$). No effect of heat stress alone was measured however increased width was measured for both water deficit stress alone and a combination of heat stress & water deficit treatments. No significant differences were found between water stress alone and the combined stress treatment (Table 2.9). The perimeter of Siokra L23 was significantly larger than all other genotypes under control conditions except for Sicot 71. No differences were seen between remaining genotypes except for CIM-448 which had a significantly smaller perimeter than all other genotypes (Table 2.8). The wall area of CIM-448 was significantly lower than Sicot 71, CS 50 and Siokra L23 for which there were no significant differences while the wall area of Sicala V-2 was significantly lower than all other genotypes. A significant effect of stress was measured on wall area with water deficit and combined heat stress & water deficit treatment causing an increase in total fibre wall area. Heat stress alone did not significantly affect wall area (Table 2.9).

Experiment 2

A significant main effect of genotype was measured for all fibre cross-sectional properties ($P<0.001$) (Table 2.10). Under control conditions CIM-448 had lower micronaire than all other genotypes for which there were no significant differences and Siokra L23 had a significantly larger micronaire than all other genotypes (Table 2.10). A significant main effect of stress was measured for micronaire ($P<0.001$) where micronaire was significantly reduced by heat stress alone and significantly increased both by water deficit stress alone and combined heat stress & water deficit treatments (Table 2.11).

Table 2.8. Effect of genotype on fibre cross sectional properties for exp. 1. The table illustrates main effects of genotype on fibre cross-sectional properties. Mean values presented. L.S.D (5%) values to assist with mean separation where differences are indicated with lettering. P values indicate significance where P ≤0.05 = *, P ≤0.01 = **, P ≤0.001 = Lettering system indicates numbers that are the same or different where matching letters are assigned to numbers for which no significant difference is measured.

	Sicot 71	Siokra L23	CS 50	CIM-448	Sicala V-2	L.S.D^{P}
Micronaire	5.19a	5.47b	5.17a	4.25c	5.17a	0.17***
Maturity ratio	0.90a	0.83b	0.97c	0.87d	0.87a	0.03***
Width (µm)	15.35a	15.88b	14.84c	14.53d	15.52e	0.14***
Perimeter (µm)	56.97ac	59.77b	55.79c	51.91a	57.86a	1.21***
Wall area (µm^2)	127.7a	119.1a	105.5a	89.9a	85.2b	40.5$^{N/S}$

Table 2.9. Effect of stress on fibre cross sectional properties for exp. 1. The table illustrates main effects of stress on fibre cross-sectional properties. Mean values presented. L.S.D (5%) values to assist with mean separation where differences are indicated with lettering. P values indicate significance where P ≤0.05 = *, P ≤0.01 = **, P ≤0.001 = ***. Lettering system indicates numbers that are the same or different where matching letters are assigned to numbers for which no significant difference is measured.

	Control	Heat stress	Water deficit	Heat stress & water deficit	$L.S.D^{P}$
Micronaire	4.73a	4.80a	5.28b	5.39b	0.15***
Maturity ratio	0.88a	0.87a	0.91b	0.90b	0.03*
Width (µm)	15.10a	15.10a	15.39b	15.30b	0.13***
Perimeter (µm)	55.29a	54.93a	57.76b	57.85b	1.09***
Wall area (μm^2)	96.6ab	89.1a	109.2ab	127.2ab	$36.3^{N/S}$

Table 2.10. Effect of genotype on fibre cross sectional properties for exp. 2. The table illustrates main effects of genotype on fibre cross-sectional properties. Mean values presented. L.S.D (5%) values to assist with mean separation where differences are indicated with lettering. P values indicate significance where P ≤0.05 = *, P ≤0.01 = **, P ≤0.001 = ***. Lettering system indicates numbers that are the same or different where matching letters are assigned to numbers for which no significant difference is measured.

	Sicot 71	Siokra L23	CS 50	CIM-448	Sicala V-2	L.S.D^{P}
Micronaire	4.31a	4.66b	4.34a	3.34c	4.11a	0.31***
Maturity ratio	0.91abd	0.90a	0.95b	0.85d	0.88d	0.04***
Fineness (mtex)	192.6a	197.0a	186.2a	150.7b	174.4c	11.5***
Width (µm)	15.14a	15.28a	14.82b	14.57c	15.00a	0.21***
Perimeter (µm)	54.97a	55.95a	53.00b	50.19c	53.23b	1.39***
Wall area (µm^2)	121.8a	124.0a	116.2a	88.8b	107.6c	8.0***

The highest maturity ratio under control conditions was seen CS 50 This was followed by Sicot 71, Siokra L23 which were not significantly different from each other and finally CIM-448 and Sicala V-2 which had the lowest maturity ratio (Table 2.10). A significant main effect of stress was seen on maturity ratio ($P<0.001$) with heat stress alone significantly reducing maturity ratio and both water deficit stress alone and combines heat stress & water deficit treatments causing an increase in maturity ratio (Table 2.11).

There were no significant differences in fineness for Sicot 71, Siokra L23 and CS 50 under control conditions however, CIM-448 and Sicala V-2 were both significantly finer than the other genotypes (Table 2.10). A significant main effect of stress was seen on fibre fineness ($P<0.001$) with heat stress alone significantly reducing fineness and both water deficit stress alone and combined heat stress & water deficit treatments causing an increase in fineness (Table 2.11).

CIM-448 had the smallest width under control conditions followed by CS 50. Sicot 71, Siokra L23 and Sicala V-2 were all significantly wider than either CS 50 or CIM-448 but not significantly different from each other (Table 2.10). Stress had no effect on fibre width. The biggest fibre perimeters were measured for Sicot 71 and Siokra L23, this was followed by CS 50 and Sicala V-2 who were significantly lower than Sicot 71 and Siokra L23 but not from each other. CIM-448 had the smallest perimeter under control conditions (Table 2.10). A significant main effect of stress on perimeter was measured ($P<0.001$) with both water deficit stress alone and combined heat stress & water deficit treatments causing an increase in fibre perimeter. Heat stress alone did not affect fibre perimeter (Table 2.11). CIM-448 had the smallest total wall area under control conditions. There were no significant differences in wall area for all other genotypes. A significant main effect of stress on wall area was seen with

Table 2.11. Effect of stress on fibre cross sectional properties for exp. 2. The table illustrates main effects of stress on fibre cross-sectional properties. Mean values presented. L.S.D (5%) values to assist with mean separation where differences are indicated with lettering. P values indicate significance where P ≤0.05 = *, P ≤0.01 = **, P ≤0.001 = ***. Lettering system indicates numbers that are the same or different where matching letters are assigned to numbers for which no significant difference is measured.

	Control	Heat stress	Water deficit	Heat stress & water deficit	$L.S.D^{P}$
Micronaire	3.69a	3.66a	4.51b	4.83c	0.27***
Maturity ratio	0.85a	0.81b	0.96c	0.96c	0.03***
Fineness (mtex)	166.2a	152.2b	204.4c	197.9c	10.3***
Width (µm)	14.99a	14.96a	14.99a	14.92a	$0.19^{N/S}$
Perimeter (µm)	52.79a	51.67a	55.15b	54.26b	1.24***
Wall area (μm^2)	102.1a	92.5b	127.8c	124.3c	7.1***

heat stress alone causing a significant reduction in total wall area, whilst both water deficit stress alone and combined heat stress & water deficit stress treatments resulted in a significant increase in total wall area (Table 2.11).

2.3.3 Fibre length attributes.

Experiment 1

A two-way interaction of genotype and stress on fibre length was measured (Figure 2.10). Here, under control conditions Sicala V-2 was the longest and significantly different than all other genotypes. There were no significant differences in length for Sicot 71, CS 50 and CIM-448 which were all shorter than Sicala V-2. Siokra L23 was significantly longer than Sicot 71 and CIM-448 but not longer than any other genotype. Heat stress alone caused a significant reduction in length compared to the control for Sicala V-2 in exp. 1 and Siokra L23 in exp. 2 but did not have an effect on any other variety. Both water deficit stress alone and combined heat stress & water deficit stress treatments caused a significant reduction in length in all genotypes except CS 50 which did not show a significant change following either treatment.

A two-way interaction was also seen for genotype and stress on length uniformity (Figure 2.10). In the control treatment there was no significant difference in length uniformity for any genotype except for CIM-44 which had significantly lower length uniformity compared to other genotypes. Both heat stress alone and water deficit stress alone caused significant increases in length uniformity in CS 50 and CIM-448 but did not have an effect on any other genotype. Combined heat stress & water deficit stress caused a significant increase in length uniformity for CIM-448 but not for any other genotype. A main effect of genotype was seen for short fibre index (Table 2.12). Under control conditions Sicala V-2 had the smallest short fibre index and CIM-448 had the largest. There were no significant differences seen for Sicot

71, Siokra L23 and CS 50. A main effect of heat on short fibre index was measured (Table 2.13). Heat stress caused a significant decrease in short fibre index whilst both water deficit stress alone and combined heat stress & water deficit treatments caused a significant increase in short fibre index.

Experiment 2

A two-way interaction of genotype and stress on fibre length was measured. Here, under control conditions there was no difference in length seen for any genotype (Figure 2.11). Heat stress alone caused a significant reduction in fibre length in Siokra L23 but did not have an effect in any other genotype. Water deficit stress alone significantly decreased fibre length in all genotypes except for Siokra L23 where it caused a significant increase. A significant reduction in length was found for all genotypes following application of combined heat stress & water deficit stress.

A main effect of genotype was found for length uniformity (Table 2.14). Sicot 71 had the greatest length uniformity. A significant main effect of genotype on Short fibre index was also measured (Table 2.14). There were no significant differences between Sicot 71, Siokra L23, CS50 and Sicala V-2 under control conditions. CIM-449 had a significantly larger short fibre index than Sicot 71 and Siokra L23. Stress did not influence length uniformity or short fibre index (Table 2.15).

2.3.4 Effect of heat stress and/or water deficit on tensile fibre attributes.

Experiment 1.

There was a significant main effect of genotype on strength and elongation of cotton fibres (Table 2.12). CIM-448 was significantly weaker with greater elongation compared with all

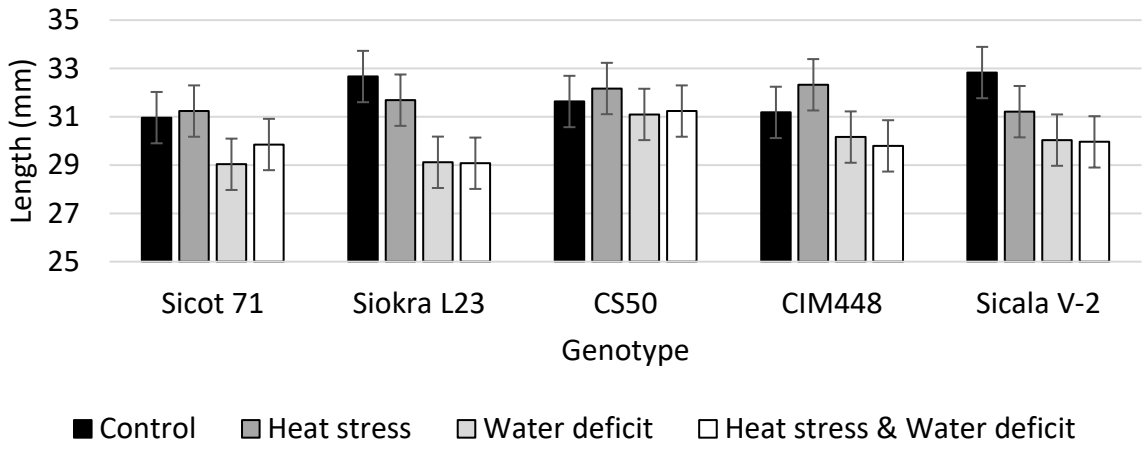


Figure 2.9. Average fibre length (mm) for each genotype subjected to abiotic stresses for exp. 1. The graph illustrates the significant two-way interaction of stress and genotype. Mean values presented. Error bars are plus and minus L.S.D (5%) values to assist with mean separation.

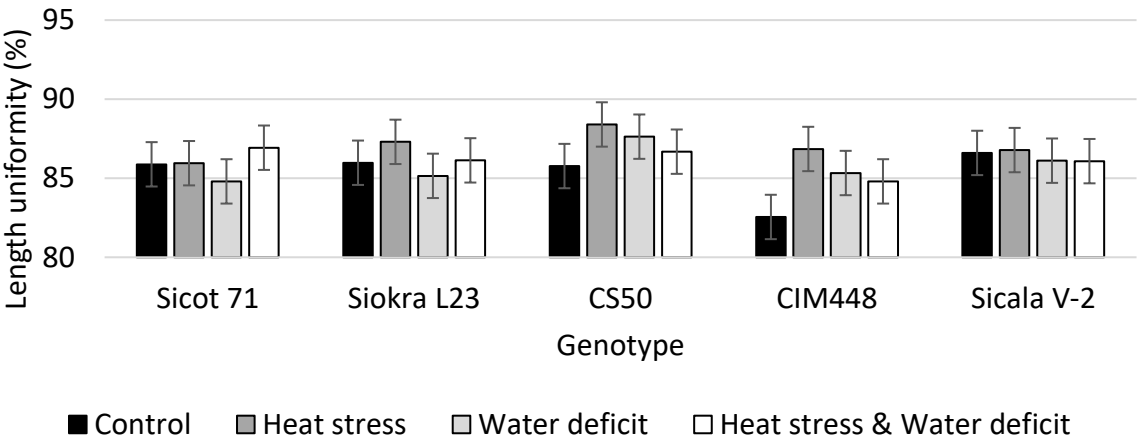


Figure 2.10. Average fibre length uniformity (%) for each genotype subjected to abiotic stress for exp. 1. The graph illustrates a significant two-way interaction of stress and genotype. Mean values presented. Error bars are plus and minus L.S.D (5%) values to assist with mean separation.

Table 2.12. Effect of genotype on HVI determined fibre attributes for exp. 1. The table illustrates main effects of genotype on fibre attributes. Mean values presented. L.S.D (5%) values to assist with mean separation where differences are indicated with lettering. P values indicate significance where P ≤0.05 = *, P ≤0.01 = **, P ≤0.001 = ***. Lettering system indicates numbers that are the same or different where matching letters are assigned to numbers for which no significant difference is measured.

	Sicot 71	Siokra L23	CS 50	CIM-448	Sicala V-2	L.S.D^{P}
Short fibre index (%)	6.17a	6.34a	6.21a	7.26b	5.85c	0.39***
Strength (g/tex)	36.48a	36.57a	37.14a	30.07b	37.74a	1.40***
Elongation (%)	6.0a	5.8a	4.7b	7.4c	5.3d	0.3***

Table 2.13. Effect of stress on HVI determined fibre attributes for exp. 1. The table illustrates main effects of stress on fibre attributes. Mean values presented. L.S.D (5%) values to assist with mean separation where differences are indicated with lettering. P values indicate significance where P ≤0.05 = *, P ≤0.01 = **, P ≤0.001 = ***. Lettering system indicates numbers that are the same or different where matching letters are assigned to numbers for which no significant difference is measured.

	Control	Heat stress	Water deficit	Heat stress & water deficit	L.S.D^{P}
Short fibre index (%)	6.28ab	6.10a	6.58b	6.49ab	0.35*
Strength (g/tex)	34.54a	34.13a	36.98b	36.75b	1.25***
Elongation (%)	5.7a	5.8ab	6.0ba	5.9ab	0.3$^{N/S}$

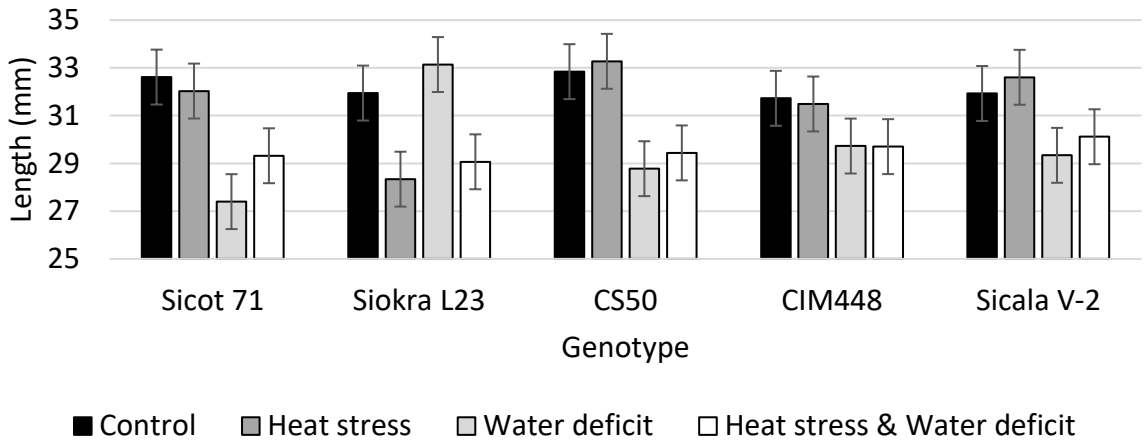


Figure 2.11. Average fibre length (mm) for each genotype subjected to abiotic stress for exp. 2. The graph illustrates a significant two-way interaction of stress and genotype. Mean values presented. Error bars are plus and minus L.S.D (5%) values to assist with mean separation.

other genotypes. There was no significant difference in strength for any other genotype. CS50 had the smallest elongation followed by Sicot 72 and Siokra L23 which were not significantly different from each other and Sicala V-2 which had significantly greater elongation compared to all genotype except CM-448. There were significant main effects of stress on strength and elongation with both water deficit stress and combined heat stress & water deficit causing a significant increase in fibre strength and elongation. Heat stress had no significant effect on strength or elongation (Table 2.15).

Experiment 2.

A significant main effect of genotype was seen on fibre strength ($P<0.001$) and elongation ($P<0.001$) (Table 2.14). Siokra L23 and Sicala V-2 fibres were strongest with the smallest elongation along with CS 5 for which there was no significant difference in elongation. Conversely, CIM-448 fibres were weakest with the greatest elongation. A significant main effect of stress was found for both strength and elongation ($P<0.001$ and $P<0.01$ respectively) where combined heat stress & water deficit caused a significant increase in strength and a significant decrease in elongation. There were no significant effects of any other stress treatment on strength or elongation (Table 2.15).

2.3.5 Across season comparison of maturity harvested fibres.

There were no significant differences between exp. 1 and exp. 2 however exp. 2 produced finer, narrower, smaller perimeter fibres (Table 2.16). There was no significant difference in length between the two experiments, however exp. 1 produced fibres that were more uniform with greater short fibre index. Fibres from exp. 1 were also significantly stronger with greater elongation compared with exp. 2 (Table 2.16).

Table 2.14. Effect of genotype on HVI determined fibre attributes for exp. 2. The table illustrates main effects of genotype on fibre attributes. Mean values presented. L.S.D (5%) values to assist with mean separation where differences are indicated with lettering. P values indicate significance where P ≤0.05 = *, P ≤0.01 = **, P ≤0.001 = ***. Lettering system indicates numbers that are the same or different where matching letters are assigned to numbers for which no significant difference is measured.

	Sicot 71	Siokra L23	CS 50	CIM-448	Sicala V-2	L.S.D^{P}
Length uniformity	86.3a	85.1bc	84.8bc	84.7b	85.5ac	0.8**
Short fibre index (%)	4.58a	5.63ac	5.69ac	6.98bc	5.41abc	1.75***
Strength (g/tex)	30.57ac	32.01b	31.06a	25.98d	32.22b	1.36***
Elongation (%)	5.4ac	5.0a	4.8a	6.3bc	4.6a	1.0**

Table 2.15. Effect of stress on HVI determined fibre attributes for exp. 2. The table illustrates main effects of genotype on fibre attributes. Mean values presented. L.S.D (5%) values to assist with mean separation where differences are indicated with lettering. P values indicate significance where P ≤0.05 = *, P ≤0.01 = **, P ≤0.001 = ***.

	Control	Heat stress	Water deficit	Heat stress & water deficit	L.S.D^{P}
Length uniformity	85.8a	85.1a	85.0a	85.2a	0.8$^{N/S}$
Short fibre index (%)	5.45a	5.73a	5.79a	5.67a	0.78$^{N/S}$
Strength (g/tex)	29.89a	29.07a	30.39a	32.13b	1.22***
Elongation (%)	5.3a	6.0a	5.3a	4.4b	0.9**

Table 2.16. Seasonal comparison of mean values for standard fibre cross sectional properties for exp. 1 and exp. 2. The table illustrates main effects of season on fibre attributes from exp. 1 and exp. 2 (Seasons 1 and 2 respectively). Mean values presented. L.S.D (5%) values to assist with mean separation where differences are indicated with lettering. P values indicate significance where P ≤0.05 = *, P ≤0.01 = **, P ≤0.001 = ***. Lettering system indicates numbers that are the same or different where matching letters are assigned to numbers for which no significant difference is measured.

	Exp. 1	Exp. 2	$L.S.D^{P}$
Micronaire	5.10a	4.17b	0.28***
Maturity Ratio	0.89a	0.90a	$0.03^{N/S}$
Fineness (mTex)	198.9a	180.2b	10.4***
Width (µm)	15.23a	14.96b	0.16**
Perimeter (µm)	56.50a	53.47b	1.14***
Aw (μm^2)	105.1a	111.7a	$14.6^{N/S}$

Table 2.17. Seasonal comparison of mean values for standard high volume instrument measurements for exp. 1 and exp. 2. The table illustrates main effects of season on fibre attributes from exp. 1 and exp. 2 (Seasons 1 and 2 respectively). Mean values presented. L.S.D (5%) values to assist with mean separation where differences are indicated with lettering. P values indicate significance where P ≤0.05 = *, P ≤0.01 = **, P ≤0.001 = ***. Lettering system indicates numbers that are the same or different where matching letters are assigned to numbers for which no significant difference is measured.

	Exp. 1	Exp. 2	$L.S.D^{P}$
Length (mm)	30.63a	30.74a	$0.60^{N/S}$
Length Uniformity (%)	85.9a	85.3b	0.4**
Short Fibre Index (%)	6.5a	5.7b	0.4***
Strength (g/tex)	35.59a	30.37b	1.08***
Elongation (%)	6.0a	5.2b	0.4***

2.4 Discussion

Following the application of abiotic stress from flowering through early to mid fibre development, the effect of these stresses was measured on the fibre of five cotton genotypes with varying tolerances to heat and water deficit stress. There have been several studies that have shown that abiotic stress can affect the fibre quality attributes that have been discussed below, however these studies have not included genotypes with known tolerances to abiotic stress. It was found that both heat stress and water deficit stress affected fibre cross-sectional, length and tensile attributes with the greatest effect seen following water deficit stress likely due to the longer period of stress application for water stress compared with heat stress. Significant main effects were measured for most fibre attributes however interactions between genotype and stress were seen for fibre fineness, length and length uniformity.

2.4.1 Heat stress affected fibre cross-sectional properties but may be reliant on the additional influence of higher average seasonal temperature.

Compared to other applied stress, heat stress had much less overall effect. This may be due to the timing of heat application which was early in fibre development. A recent paper by Bo et al., in 2017, determined that the greatest effects from heat stress on fibre quality occurred when stress was applied at between 13-19 DPA for a period of 2-7 days with 5 days been shown to be the critical time period. This study used a similar time frame, however plants were stressed earlier. The reduction of maturity ratio, fineness and wall area were only seen for exp. 2 but not for exp. 1. The general lack of response is most likely due to the timing of the heat stress which was applied very early in fibre development for a period of 5 days from 0DPA, whereas most of the fibre maturation and secondary wall thickening occurs during the later phases of fibre development from 15-50DPA. The response in the second experiment

that was absent in the first may be due to the average ambient temperature differences seen between the two experiments where exp. 2 on average was warmer than the first. This may have increased the effect of the applied heat stress to a point where it was able to have a significant effect on fibre. Additionally, there was a greater effect of the tents in the second experiment with the average temperature increase being significantly larger than that of the first, again indicating a greater level of heat stress.

The reduction in some cross-sectional properties in exp. 2 are in conflict with a previously reported study that showed that higher temperatures can cause increases in fibre maturity (Haigler et al., 2005). They posit that greater day degree accumulation due to increased temperatures caused increased fibre maturity because higher temperatures (around 27°C - 37°C) are more optimal for cellulose synthesis, the deposit of which is directly related to fibre maturation (Haigler et al., 2005). However, it has also been shown that although micronaire values do increase with increasing temperatures, at extreme temperatures there is a decrease in micronaire. One study that involved glasshouse grown cottons that were grown to squaring at 24/19°C day/night temperature before being transferred to a range of temperatures between 18/13°C and 36/31°C showed that micronaire increased with increasing temperatures up to 33/28°C and then decreased at higher temperatures (Hesketh and Low, 1968). This study did not explain why micronaire was reduced at these higher temperatures. It has been shown however that fibre maturity will be reduced by any factor that limits photosynthesis and therefore the generation of biomass (Haigler et al., 2005).

For exp. 1, the lack of response to heat stress has two likely explanations. The first being that the increases seen in fibre maturity seen in previous research (Haigler et al., 2005) was due to heat stress that occurred during the elongation stage of fibre development which occurs

later than where stress was applied in this experiment. The second reason may be due to the severity of the heat stress which may not have been extreme enough to affect fibre development in exp. 1.

2.4.2 Water deficit stress caused significant increases in measured fibre cross-sectional properties

Water deficit stress caused significant increases in all measured fibre-cross sectional properties and was repeatable across both experiments indicating a strong fibre response to water stress. This response to water stress was most likely due to the duration of water stress which occurred from 0DPA for a period of 22 days which means that the fibre was water stressed throughout the initiation, elongation and part of the secondary wall thickening phases of fibre development.

The increased micronaire, maturity ratio, width and fineness were found for both water stress alone and combined heat stress & water deficit stress treatments for both exp. 1 and exp. 2. Because no effect of heat stress alone was measured in exp. 1 for any fibre cross-sectional property and because the degree of increase was not greater for the combined stress compared with water stress alone for any fibre quality for either experiment it is most likely that the response observed in the combined stress treatment was due to the water stress component and not the heat stress. In the first experiment, there was a two-way interaction of genotype and fineness, where similarly to all other measured effects, water stress alone caused a significant increase in fineness for all genotypes except for CS 50 and combined heat stress & water deficit caused a significant increase in fineness in Sicot 71, CIM-448 and Sicala V-2 but not for Siokra L23 or CS 50. CS 50 is known to have poor water use efficiency, meaning it will produce less biomass per unit of water compared with the other varieties (Reid et al.,

2002) (Stiller et al., 2005) and for this reason is likely more susceptible to water stress. It is unsurprising that this variety did not have increased fibre fineness in response to either water stress alone or combined heat stress & water deficit stress treatments in exp. 1. This genotype would have reduced ability to produce new cellulose biomass under water stress than the other genotypes. In exp. 2 we did not see the same interaction of genotype and stress on fibre fineness for CS 50. It may be that the main effect of heat stress which caused decreased fibre fineness in exp. 2 is counteracting any effect of water stress. Siokra L23 was included for its good water use efficiency. It did not show an effect of combined heat stress & water deficit stress treatment on fibre fineness despite the increased fineness seen in all other genotypes in response to the combined stress. Conversely this may be due to the ability of this plant to function optimally with less water due to its lower water requirement meaning it may not have been suffering from water stress to the same degree the other genotypes were. Water stress has previously been shown to cause increased fibre micronaire when applied during late bloom stages (Nazar et al., 2012) and when applied either at first square or at first boll split (Ahmad, 2013) which supports the results of this study for which stress was also applied during late bloom/early fibre development stages. Application of stress during fibre initiation, as was done here, is likely to affect fibre perimeter. The stress treatments were also likely to promote greater fibre secondary wall thickening and higher measured fibre maturity ratio which affects higher micronaire. This was indeed shown here in response to both water stress and combined heat stress & water deficit stress where increased fibre perimeter and wall area due to greater secondary wall thickening were measured

2.4.3 Heat and/or water stress effect fibre length properties

Heat stress

There was a limited effect of heat stress on fibre length, length uniformity and short fibre index which may be due to the length of time that heat stress was applied. Heat stress was applied for five days that coincided with flowering and the early stages of fibre development (approximately 0-5DPA), whilst we know that fibre lengthening typically takes place during the elongation phase of fibre development which occurs from 3-20DPA. Therefore, the heat stress period only briefly crossed over into this elongation phase. The effect of heat stress on length was variety dependant with only Sicala V-2 fibre in the exp. 1 harvest and Siokra L23 in the exp. 2 harvest showing a significant response to heat stress with decreased fibre length. Sicala V-2 was included for its poor heat tolerance and of all varieties is the most likely to exhibit signs of heat stress so this response is unsurprising. It is likely the level of imposed heat stress was not severe enough to cause an effect in all genotypes, but this variety was more easily heat stressed due to its poor heat tolerance. Siokra L23 which produced the longest fibre of any included genotype under control conditions was included for its good water use efficiency. The effect of heat stress on length uniformity was also genotype dependant with responses seen only in CS 50 and CIM-448.

These results are consistent with previous work where heat stress has been shown to decrease mean fibre length (Reddy et al., 1999) and another more recent study that showed that length increased linearly with increasing temperatures from 18-22C and declined at higher temperatures (Lockhand and Reddy, 2014). They used growth chambers to grow plants with a range of conditions utilising five temperature regimes (1992 seasonal average and the seasonal average -2◦C, +2◦C, +5◦C, and +7◦C) in combination with varying CO_2 levels. They found that fibre fineness increased linearly with increasing temperatures up to 26◦**C** but decreased at 32◦C. They also found that short fibre content was increased at higher

temperatures which is consistent with this study where short fibre index was found to be reduced following application of heat stress in exp. 1.

Water stress

Previous studies have discussed how water stress can impact on fibre length when water stress is applied during fibre elongation but can also be affected generally at any time prior to boll opening (Nazar et al., 2012) which correlated with the timing of water stress in this study where water stress was applied during flowering and early to mid fibre development phases. In their study they looked at the continuous application of reduced irrigation throughout the cotton crop growth and found that fibre length decreased with the application of water deficit stress (Nazar et al., 2012).

In another study fibre length was shown to be decreased following a 30 day period of water stress applied at either first square or first boll split with both showing decreased fibre length as a result (Ahmad, 2013). This is consistent with the results of this study where water deficit stress alone and combined heat stress & water deficit stress treatments applied during flowering and early fibre development resulted in decreased fibre length for both experiments. This reduction in length can be attributed to the abiotic stresses preventing adequate structural components being laid down and the prevention of normal fibre growth biochemistry during the lengthening phase of fibre development.

Short fibre content has also been shown to increase when temperatures are increased above optimum leading to heat stress (Reddy et al., 1999). A possible explanation for this is that since high temperatures can speed up the alter and speed up the rate of pollen and ovule development leading to inefficient fertilization. Because of this inefficient fertilisation, which results in ovules being terminated shortly after fertilisation more short fibres are produced

(Reddy et al., 1999). Alternatively, increased short fibre content may be due to either a reduced rate of fibre elongation as above or a reduced duration of fibre elongation period which leads to a greater percentage of short fibres. Here although we only saw increased short fibre index in response to combined heat stress & water deficit treatment in exp. 1, because we applied heat stress during the early fibre development stage it is likely that any effects seen can be explained due to the effect of the high temperatures on the rate of pollen and ovule development leading to inefficient fertilisation.

2.4.4 Water stress has an effect on fibre tensile properties, but heat stress does not.

The effect of water deficit stress alone and combined heat stress & water deficit stress on fibre strength were of equally significant magnitude compared with the control. Because there was no effect of heat stress alone it can be concluded that it was the water stress in the combined treatment that resulted in increased length rather than a combination of heat and water stress. No effect of water stress was seen for fibre elongation. Water stress has previously been shown to cause increased fibre strength with decreased water application when water deficit is applied as a constant deficit throughout cotton growth (Nazar et al., 2012, Booker et al., 2006). Amongst these was an experiment which involved multiple cotton genotypes including CIM-448 which was also used in this study (Nazar et al., 2012). The amount of increase in strength also increased as water deficit level increased when comparing cottons receiving 100%, 75%, 50% and 25% total applied water irrigation (Booker et al., 2006). These studies differ in the method by which they applied stress in that they maintained water deficit throughout the entire cotton growing period while the current study applied a normal watering regime to all plots but skipped 2 consecutive irrigations on the water deficit stress plots to induce water stress. This skipped irrigation occurred during the flowering and early

to mid fibre development phases of the mature harvested fibre for each respective experiment. One possible explanation for the increased strength is that the abiotic stress may have reduced the number or size of the fibres such that when stress was removed for much of the final phase of secondary wall development, there was more photosynthetic and cellulose production capacity relative to control fruit and fibres. This also explains why stress treatments had higher fibre maturity ratio results.

2.4.5 Concluding remarks

There are certain fibre qualities that are considered more commercially valuable due to their importance when spinning the fibre into yarn and creating fabric that can be adequately dyed. These qualities include strength, length, whiteness and micronaire where stronger, longer, whiter cottons with ideally high micronaire are considered more valuable and those with lesser qualities attract a discounted sale price. Here heat and water stress generally increased fibre strength, reduced fibre length and increased fibre micronaire. Although the effect of heat stress on fibre strength may not be considered negative as stronger fibre is more valuable because it is less likely to break during the manufacturing of yarn, the reduced length and increased micronaire are not ideal.

Increased micronaire in response to heat stress may be a useful side effect in naturally lower micronaire varieties such as CIM-448 because low micronaire cotton fibre does not uptake dye as well as its higher micronaire counterparts (Haigler et al., 2005) however this needs to be taken into careful consideration against all other variables for plant growth and fibre quality that may be affected by these stresses. Care also needs to be taken because whilst increased micronaire can be a useful factor when it comes to fibre and fabric dyeability, a micronaire too high will attract a price discount due to the change this causes in fibre shape

which makes fibre knitting more difficult meaning growers should be especially careful when managing stress in higher micronaire varieties or varieties that have ideal micronaire values under control conditions. Ultimately, the micronaire ranges measured in this study were not outside the range of what may be considered ideal.

This study confirmed the importance of carefully managing both heat and water stress as both heat stress alone and water stress alone were shown to influence fibre cross-sectional, tensile and length properties.

Chapter 3. The effect of heat and water stress on the production of cuticular waxes on five field grown upland cottons.

Abstract

Heat and water stress were applied to five upland cotton genotypes with varying tolerances to heat and water stress for the 2013/14 and 2014/15 growing seasons to determine the effect of abiotic stress on fibre cuticular wax deposition. It was hypothesized that abiotic stress would increase fiber wax content. Sicot 71 was included as a control genotype. Siokra L23 and CS 50 were included for their good and poor water use efficiency respectively, and CIM-448 and Sicala V-2 included for their good and poor heat tolerances respectively. Each stress was applied as a single event that coincided with flowering and early to middle fibre development phases. Fibres from matured bolls that were in flowering and early fibre development stages during the stress events were harvested for analysis for the two seasons. Fiber wax was determined using a standard laboratory method formulated by Conrad in 1944. Across all genotypes and treatments, fiber wax content varied between 0.2 and 1.6%. There was a statistical interaction found between field treatment and genotype on the wax content of cotton fibre from both seasons. For Sicot 71, Siokra L23 and CIM-448 fiber wax content was either not affected or decreased following the application of abiotic stress. For CS 50 the abiotic stress treatments increased fiber wax content. For a Sicala V-2 either the heat stress or water stress treatments alone decreased fiber wax content, while the combination of both stress treatments markedly increased fiber wax content. Here a genotype dependant influence of stress on cotton fibre cuticular wax deposition was shown that has not previously been reported which further highlights the importance of carefully managing the heat and water stress that cotton crops may be exposed to.

3.1 Introduction

In the previous chapter the effects of abiotic stresses on some traditional cotton fibre quality attributes were captured with emphasis on the cross-sectional components that influence micronaire. While cotton cuticle wax is important to the fibre processing industry, little attention has been given to the question of how genotype and abiotic factors influence wax levels. Certainly, to date and to my knowledge, no wax assay or measure of wax levels have ever been used to value cotton. The cotton textile processing industry may well be aware of some differences in wax content of different genotypes such as naturally coloured cottons which have been shown to have 14-17% wax by total weight in comparison with white cottons which typically have between 0.4-0.7% wax content (Conrad, 1944). It appears however, that dye processors are content with the general acceptance that the wax that is present on commercial white cotton genotypes can be dealt with adequately using standard caustic scouring (Lewis, 2011a). This is despite the influence high wax cottons may ultimately have on the dye uptake and colour fastness of cotton fabrics.

The waxy cuticle plays a crucial role during plant grown where it acts as a barrier to water loss (Riederer, 2007) although water loss from fibre during cotton fiber development may not be significant and its presence may primarily be due to it being a type of epidermal cell. The fibre cuticle is made up of numerous components including cutin and various waxes and is made up of two primary layers; the outermost layer the Cuticle Proper (CP), and the innermost layer the Cuticular Layer (CL). The CP layer contains the majority of the waxes. It is comprised mainly of waxes which are embedded within a cutin matrix and also present as a waxy film present on the outermost surface of the fibre (Yeats and Rose, 2013, Jeffree, 2007). The waxes

present in the cuticle vary but are primarily derived from very-long-chain fatty acids (VLCFA's) which are those from C20-C34.

It has been shown that plants that have abnormalities in the waxy cuticle due to mutations in or the absence of necessary regulatory genes have numerous morphological changes and other physiological challenges as a result, including visible signs of stress from increased water loss (Barozai and Husnain, 2014). It has long been established that heat and water stress cause adaptations on plant aerial surfaces including increased deposition of cuticular waxes (Shepherd and Wynne Griffiths, 2006). This has been shown in the model plant *Arabidopsis thaliana* (Borisjuk et al., 2014) as well as a number of commercially grown plant species including wheat (Borisjuk et al., 2016), barley (Giese, 1975) and alfalfa (Ni et al., 2012).

There has been significant research undertaken in recent years by the Australian cotton industry assessing the range of tolerance to heat and water stress of cotton genotypes, and the effects that such stresses have on cotton production. These studies of upland cotton have demonstrated increased deposition of cuticular waxes on leaf tissue in response to drought conditions both under field and growth chamber conditions (Ashraf et al., 1994, Bondada et al., 1996). These studies have demonstrated increased cuticular thickness of cottons grown in drought conditions of as much as 33% on cotton leaf (Oosterhuis et al., 1991). More recently, research has shown an increased deposition of cuticular wax in response to water stress in leaf tissue as well as bract (Bondada et al., 1996). There has been one study of seven upland white cottons which examined the influence of well-watered and water-limited irrigation treatments on cuticular wax deposited on fibre (Thompson et al., 2017). Plants were grown under normal well-watered irrigation conditions until 50% of plots were at first flower and then water deficit treatment was applied and maintained throughout cotton growth

season. The study did not show an effect of stress on the amount of total cuticular wax on fibre although they did show an effect on some individual cuticular wax components. The authors posit that the water-limited treatment imposed in the study may not have been severe enough to cause a stress response in the plant that would lead to the deposition of more fibre cuticular wax (Thompson et al., 2017). If abiotic stress affects the deposition of wax on other cotton plant organs it is reasonable to assume it may also affect fruiting organs such as fibre and further experimentation utilising stress in upland cotton is warranted and is the focus of this chapter.

Ultimately there is a desire to be able to mitigate the negative impacts of changes in climatic conditions via management and potentially by the development of more tolerant genotypes. The aims of this study were to determine how heat stress and water deficit might affect the production of the wax portion of the fibre cuticle. The study applied intermittent heat stress, water deficit and a combination of both to two consecutive seasons of field grown upland cottons (exp. 1 and exp. 2) to study the effect on five cotton genotypes with varying heat and water stress tolerances. Fibres that developed during the stress event, which occurred from flowering through the early to mid fibre development phases, were selected for analysis for exp. 1 and exp. 2. It was hypothesised that heat stress and/or water deficit will cause increased deposition of cuticular waxes on cotton fibres.

3.2 Materials and methods

3.2.1 Cultivation and treatment of cotton plants.

The field experiments employed for this research are common to those reported in Chapter 2 (Sections 2.2.1 and 2.2.2). Following the cotton fibre quality analysis reported in chapter 2, the same experimental fibre was used for wax analysis.

3.2.2 Analysis of the ethanol extractable wax component of raw cotton fibre

Total ethanol extractable cuticle was removed from cotton fibre via a method first published by Conrad in 1944 where the wax portion is separated from the other non-cellulosic fibre components. The Conrad method has been utilised and modified for use with smaller batches of cotton and is considered the standard method with which to remove fibre cuticle. Following is a description of the method.

Preparation of cotton fibre samples

Cotton fibre samples were twice cleaned of leaf and vegetable debris using a Shirley Analyser then conditioned for greater than 48 hours under standard textile testing conditions (20^{o}C ±2, 65% RH ±3).

Preparation of glassware for cuticle extraction

Soxhlet apparatus glassware (Figure 3.1) was cleaned before use by running ethanol through the system for approximately 1h. Following this, hot ethanol was poured through the top of the condenser to ensure any remaining contaminants were removed. Round bottom flasks used for wax extractions were first cleaned by rinsing with ethanol, then acetone and once more with ethanol to ensure lipid contaminants were removed. This process was also used for storage vials and glassware used for storing wax samples.

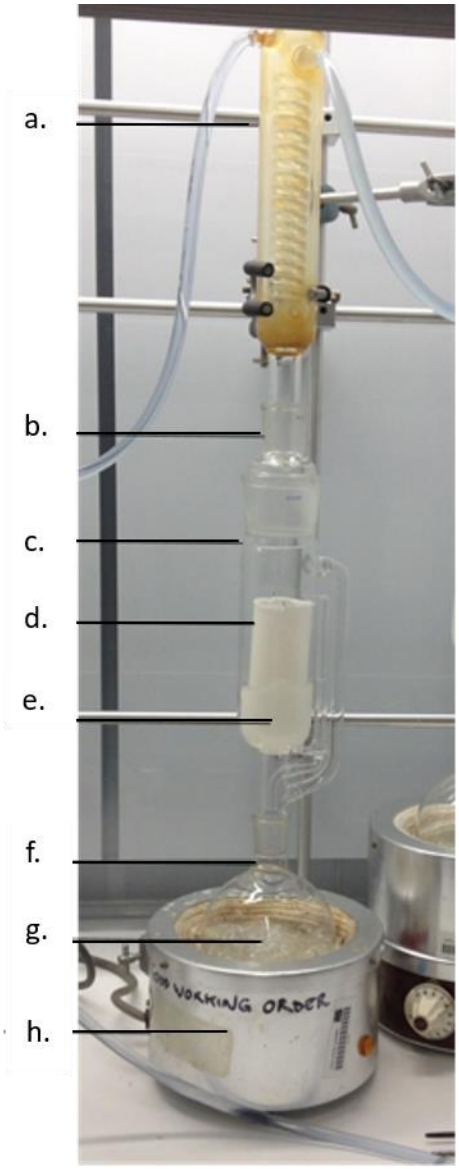


Figure 3.1. Soxhlet apparatus. a. Condenser, b. Adapter, c. Soxhlet, d. Cellulose thimble, e. Cotton fibres inside thimble, f. 250mL Round bottom flask, g. 100% Ethanol, h. Heating mantle. Ethanol is heated in a round bottom flask using a heating mantle and vapour is condensed in the condenser glassware which is kept cool using 18°C recirculating water. Condensed hot ethanol collects within the Soxhlet where the cellulose thimble which contains the cotton fibres is located and facilitates the removal of the ethanol soluble non-cellulosic cuticle components. Once full the ethanol containing the cuticle spills over and is collected in the round bottom flask where the ethanol cycle repeats whilst the extracted cuticle remains in the round bottom flask.

Hot ethanol Soxhlet extraction of cotton fibre cuticle

Wax and other non-cellulosic materials were extracted from cleaned and conditioned cotton fibre samples in triplicate using the Conrad method (Conrad, 1944). Pre-weighed cotton fibre samples weighing approximately 3-5g were placed inside 30x100mm cellulose thimbles. These thimbles were placed inside a Soxhlet apparatus (Figure 3.1) and hot ethanol extracted for 90 cycles to facilitate the removal of ~100% of the fibre cuticle.

Separation of wax from total extracted cuticle.

Following extraction, the wax portion of the total extracted cuticle was separated from the non-wax portion of the fibre cuticle by adding an equal measure of chloroform (~100ml) to the ethanol containing the extracted substances and finally back extracted using deionized (DI) water into a pre-weighed round bottom flask. The Chloroform solution was evaporated under a vacuum using a BUCHI Rotavapor R-210 rotary evaporator fitted with a BUCHI Heating Bath B-491 set at 40°C and a BUCHI Vacuum Controller V850 then incubated in a drying oven at 40^{o}C overnight before being placed in a desiccator to ensure removal of all remaining water. The remaining wax was conditioned at standard laboratory conditions and weighed and was compared with the weight of the original conditioned fibre samples.

Determination of total external surface area of fibre

Perimeter values determined using Equation 2.2 were used to find the radius of the fibre with Equation 3.1. Total height (length) of fibre was calculated using the known fibre fineness (g per 1000m) and total fibre weight using Equation 3.2. Fibre external surface area (SA) was then calculated using the traditional geometric equation for determining the area of a

cylinder (Equation 3.3). Wax content on fibre was calculated as grams of wax per square meter of fibre external surface area (Equation 3.4).

$$r = \frac{Perimeter\ in\ \mu m}{2\pi} \quad \text{(Equation 3.1)}$$

$$h\ in\ metres = \left(\frac{Sample\ weight\ in\ grams}{Fibre\ fineness\ in\ g/1000m}\right) \times 1000 \quad \text{(Equation 3.2)}$$

$$Surface\ area\ of\ fibre = 2\pi rh + 2\pi r^2 \quad \text{(Equation 3.3)}$$

$$Wax\ in\ milligrams\ m^{-2} = \frac{Total\ wax\ (mg)}{Total\ surface\ area\ of fibre\ (m^{-2})} \quad \text{(Equation 3.4)}$$

3.2.3 Analysis of surface wax morphology using Scanning Electron Microscopy

A sub sample of combined CS 50 whole fibre replicate samples for each treatment were placed on an aluminium sample holder using double sided, conductive carbon tape. It was then coated in a Cressington 208HRD magnetron coater with approximately 3 nm of a Pt/Pd (80/20) alloy to provide electrical conductivity and imaged in a FEI Phenom Desktop Scanning Electron Microscope (Thermofisher Scientific, MA, U.S.A) which has a fixed voltage of 5 kV.

3.2.4 Statistical analysis

Statistical analysis was performed using Microsoft Excel for basic descriptive statistics. GenStat Version 16 (Lawes Agricultural Trust, IACR. Rothamsted, UK) was employed for Analysis of Variance (ANOVA) of data. Raw fibre wax data for individual replicates for each genotype and treatment were added into this program. ANOVA was conducted as a two-way model with genotype and stress treatment being the two factors, with the field replicate designated as a blocking term (Table 2.4).

Mean values were reported with preference given to significant two factor interaction, which was reported in bar chart format. The degree of the statistical significance of the ANOVA tests were indicated using standard star symbol convention, being when P values were either <0.05*, <0.01**, or <0.001***. Least significant difference (LSD) (5%) values were reported alongside significant ANOVA results to assist in mean value separation. The statistical analysis design for the ANOVA is shown in table 3.1.

Table 3.1. Analysis of Variance Table. This table details the degrees of freedom (d.f.) assigned to each component of the two-factor model used to analyse the wax content data.

Source of variation	Degrees of freedom
Block	2
Genotype	4
Stress	3
Genotype x Stress	12
Residual	36
Total	57

3.3 Results

3.3.1 Quantification of cotton fiber wax content

Cotton fiber wax content was influenced by both genotype and abiotic stress treatments in both experiments, and statistically significant interaction was captured between these treatment factors in both experiments for both wax % and wax mg m^{-2} measurements (ANOVA P for both experiments. <0.001) (Fig. 3.2 and 3.3). Across treatments, wax content varied between 0.2 and 1.3% in exp. 1 and varied between 0.4 to 1.6% in exp. 2.

Calculation of total wax as a percentage of total fibre weight (%)

Considering the effect of genotype alone, in exp. 1 control treated Sicot 71 had the same amount of fiber wax as CIM-448 and Sicala V-2, which was higher than Siokra L-23 and CS 50. Siokra L-23 had significantly more fiber wax than CS 50 (Fig. 3.2a). For control treated genotypes in exp. 2, all had similar amounts of fiber wax except for CS 50 which was significantly lower than the others (Fig. 3.3a).

Considering the effects of abiotic stress, in both experiments for Sicot 71, the industry standard genotype, either water deficit or a combination of heat stress and water deficit reduced the amount of fiber wax equally. Heat stress alone did not affect wax levels (Fig. 3.2 and 3.3).

For Siokra L-23, the genotype with good water use efficiency, the heat stress treatment in exp. 1 increased fiber wax content compared to the other treatments which had the same wax levels (Fig. 3.2a). This was different to the response in exp. 2, with wax levels for the control and heat stress treatments being the same and higher than the other two treatments (Fig. 3.3a).

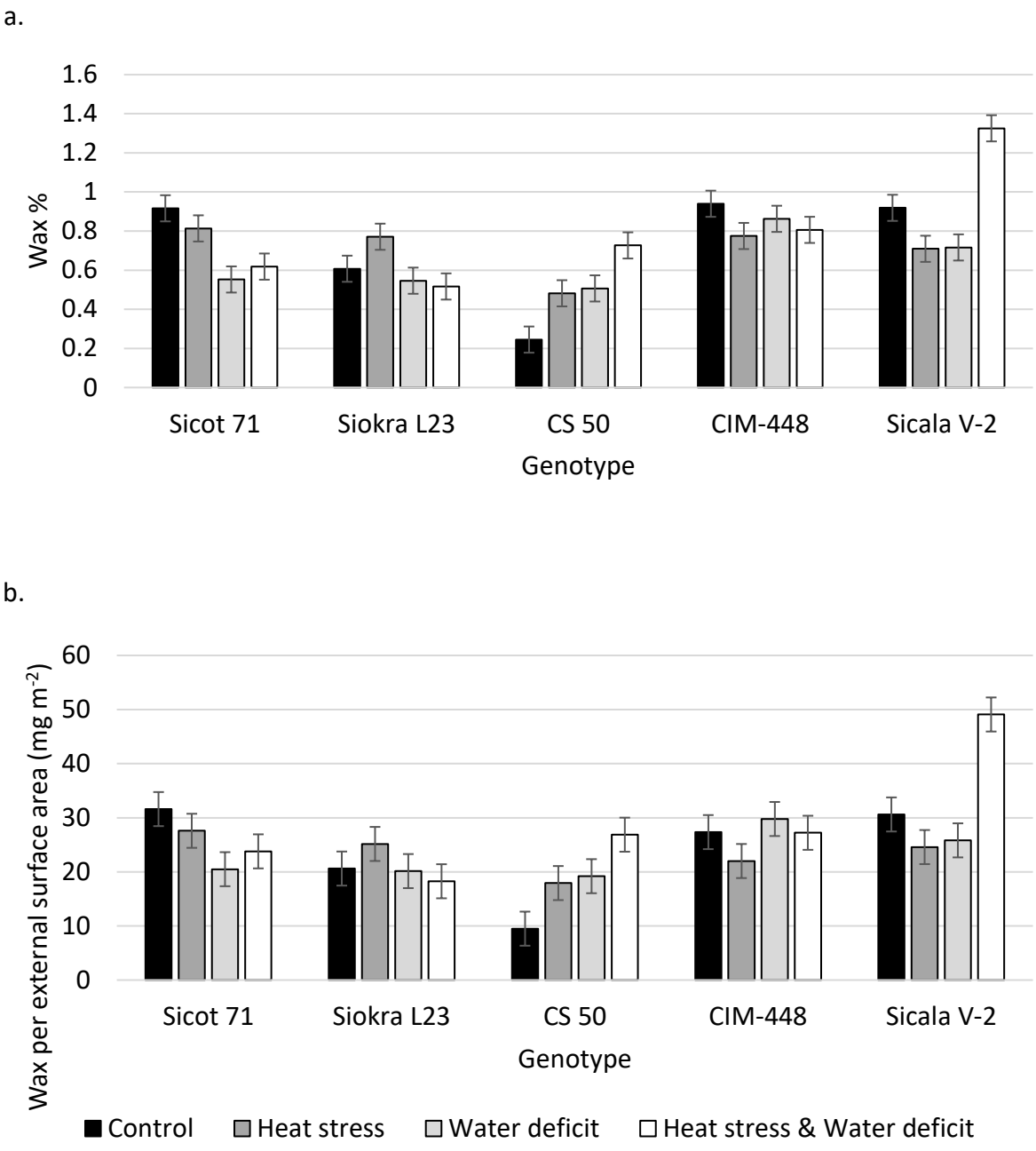


Figure 3.2. Average wax content results for each genotype subjected to abiotic stresses for exp. 1. The graph illustrates the significant two-way interaction of stress and genotype. Mean values presented for for (a) the amount of wax as a percentage of total fibre weight and (b) total wax amount per external surface area (mg m^{-2}). Error bars are plus and minus L.S.D (5%) values to assist with mean separation.

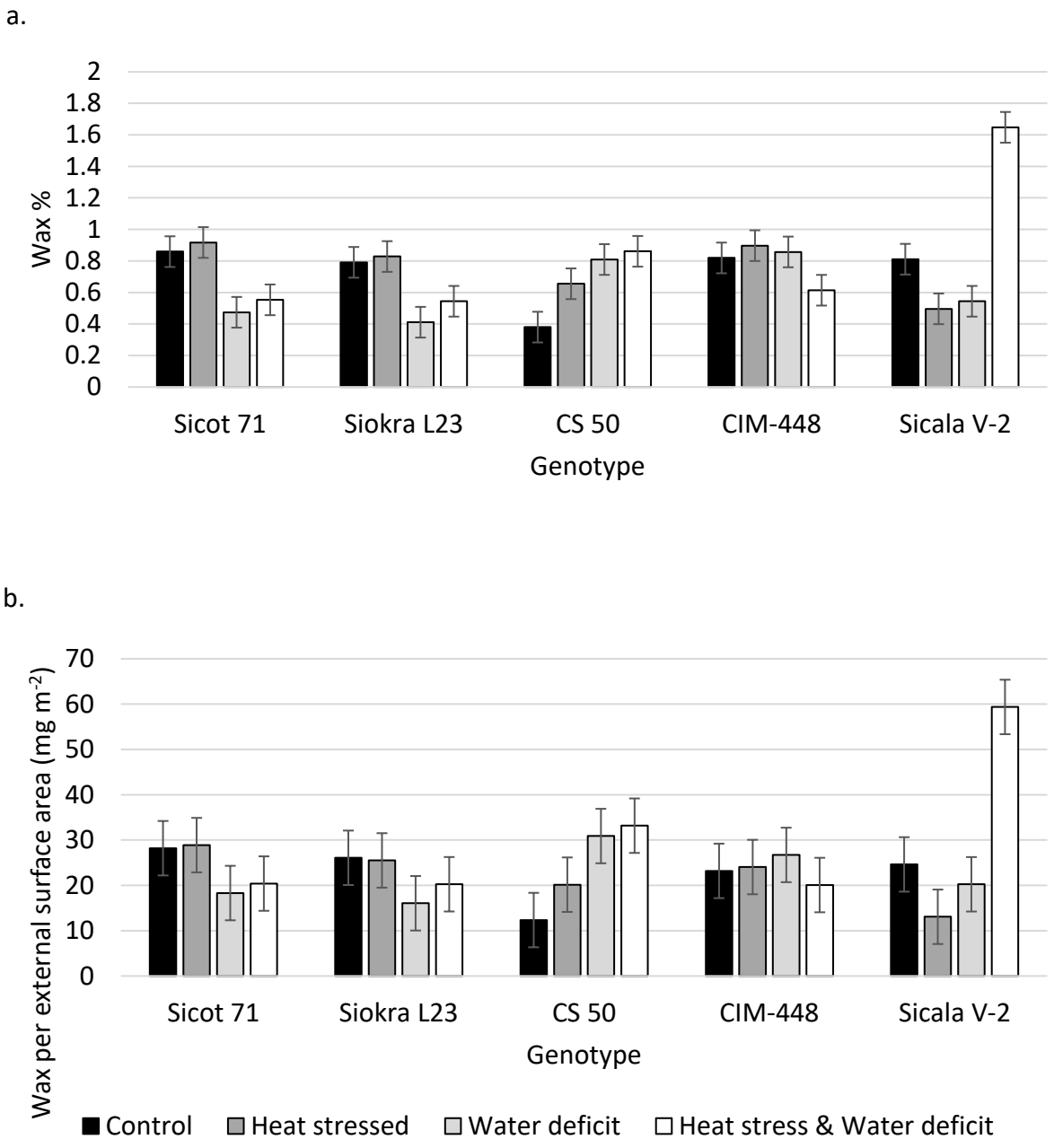


Figure 3.3. Average wax content results for each genotype subjected to abiotic stresses for exp. 2. The graph illustrates the significant two-way interaction of stress and genotype. Mean values presented for for (a) the amount of wax as a percentage of total fibre weight and (b) total wax amount per external surface area (mg m^{-2}). Error bars are plus and minus L.S.D (5%) values to assist with mean separation.

For CS 50, the poor water use efficient genotype, in exp. 1 either the heat stress treatment or the water deficit treatment increased fiber wax content equally, while a combination of both stress treatments increased wax content more than either treatment alone (Fig. 3.2a). In exp. 2 a similar trend was also noted, except that the fiber wax content for the treatment that combined heat stress and water deficit was the same as the water deficit treatment (Fig. 3.2a).

For CIM-448, the genotype with good heat tolerance, in exp. 1 either the heat stress treatment or the combined stress treatment reduced fiber wax content equally, while water deficit stress did not affect fiber wax content (Fig. 3.2a). In exp. 2, a similar trend was also noted for this genotype except that the fiber wax content for the heat stress treatment was the same as both the control and the water deficit treatment (Fig. 3.3a).

For Sicala V-2, the genotype included for its poor heat tolerance, in both experiments either the heat stress or water deficit stress treatments reduced fiber wax content to the same degree whilst the combined stress treatment significantly increased fiber wax content (Fig. 3.2a). In exp 2, the same trend was observed (Fig. 3.3a).

Calculation of total wax per external surface area (mg m^{-2}).

Similar trends and results were evident when wax was measured as total wax per external surface area (mg m^{-2}) (Figures 3.2b and 3.3b). However, although the majority of the results were consistent with the reported wax % values, there were a few inconsistencies and loss of clarity in results due to larger error including the following; For exp. 1, the significant decrease in wax percentage following application of combined heat stress & water deficit treatment

on CIM-448 was no longer significant. In exp. 2 the significant decrease in wax percentage following application of combined heat stress & water deficit treatment on both CIM-448 and Siokra L23 were no longer significant. Additionally, water deficit stress alone no longer significantly decreased wax deposition on Sicala V-2.

3.3.4 Visual assessment of cotton fibre

Assessment of surface wax morphology by Scanning Electron Microscopy (SEM)

Visual differences in the outer cuticle layer of the fibre were apparent in the CS50 genotype that showed significantly increased wax deposition in response to both heat and water stress (Figure 3.4) Ridges that run along the length of the fibre were present in all three stressed fibres but not present in the control. In the combined heat stress and water deficit stress treatment there were also apparent differences in surface morphology. Here large ridges are noted that have the appearance of cracks on the surface of the fibre which again was not present in any of the control samples.

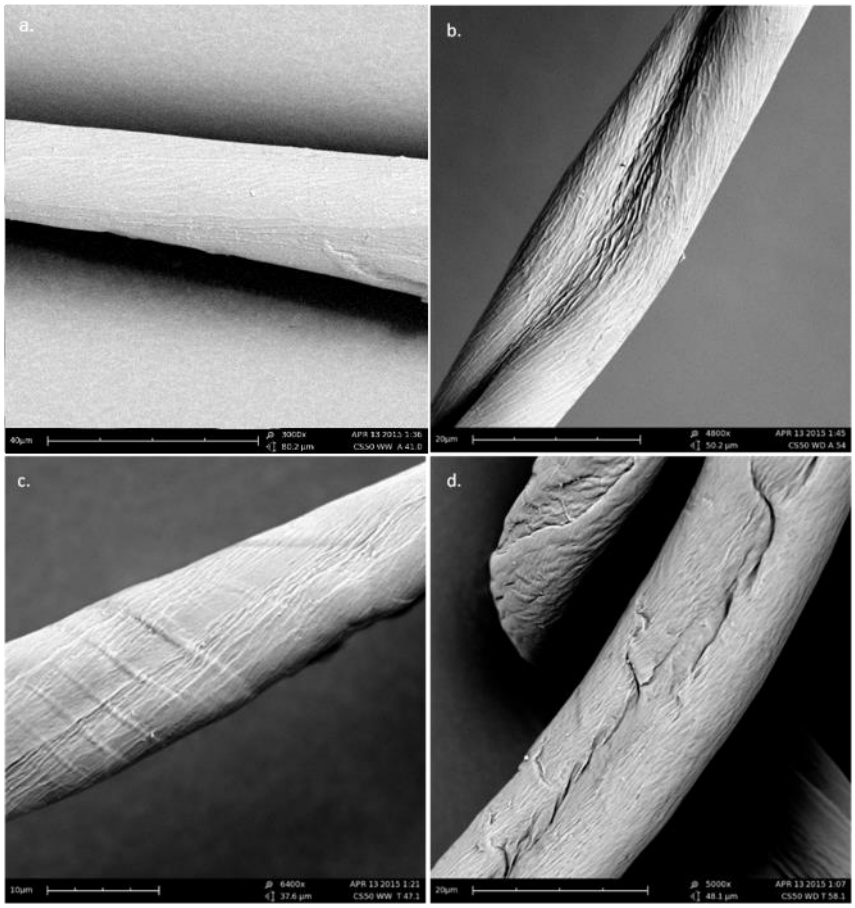


Figure 3.4. Morphological changes in the cuticular wax layer of *Gossypium hirsutum* CS 50 cotton fibres in response to heat and water stress. SEM images of whole, unscoured cotton fibres from CS50 poor water use efficiency genotype that were subjected to varying stress treatments. (a) CS50 Well-watered, non-heat stressed (control) fibres, (b) CS50 Water-deficit, non-heat stressed fibres, (c) CS50 Well-watered, heat stressed fibres, and (d) CS50 Water-deficit, heat stressed fibres.

3.4 Discussion

To determine the effect of abiotic stress on fibre cuticular wax deposition, heat and water deficit stress was applied to five upland cotton genotypes with varying tolerances to heat and water stress in two separate experiments across the 2013/14 and 2014/15 growing seasons. Stress was shown to significantly influence fibre cuticular wax deposition in both experiments, but the effect was genotype dependant. Whilst effects were measured in all genotypes the greatest effect was measured in Sicala V-2 and CS 50 the two varieties included for their poor heat and water tolerances. Although several studies have investigated the effect of abiotic stress on fibre quality, to my knowledge there has only been one study where the effect of abiotic stress was evaluated. This recent study showed no increase of total cuticular wax amount on fibre following abiotic stress application although there was an influence on individual wax components (Thompson et al., 2017) In the research discussed in this chapter, increased fibre cuticular wax deposition was measured in response to abiotic stress which, to my knowledge, has not previously been shown.

3.4.1 Heat and water stress alter wax deposition on cotton fibre

An effect of heat stress and/or water stress was observed for both experiments despite some differences between treatments across the two years indicating a strong response in the fibre to stress that has not been shown previously. Typically, the wax content of cotton fibre is measured and reported as a total percentage of fibre weight (Conrad, 1944), however for other plant organs, cuticular wax content is typically reported as total wax per unit of surface area. Here an attempt was made to include a novel calculation of wax on fibre as an amount of wax per external surface area in mg m^{-2}. This was done by utilising the measured wax amount in a sample of fibre of known weight in conjunction with a number of measured cross-

sectional fibre quality attributes as described in section 3.2.2. Using this novel approach to measure wax per external surface area in mg m^{-2} generally gave the same result and overall trends, although it did affect the significance of some of the treatments when a small, yet significant wax percentage result was seen. This largely affected the control genotype Sicot 71 where small reductions in fibre wax percentages were no longer significant following assessment of total wax per external surface area of the fibre. This loss of resolution is likely attributed to the fact an assumption is made when calculating fibre perimeter that all fibre has the same perimeter and does not take into account the differences due to the presence of immature fibre within the sample which may be affecting the results.

It was shown that in the cotton fibre from each of the five included genotypes, there is a response to increasing stress that leads to altered wax deposition although the degree and type of change was dependant on genotype with some genotypes responding by decreasing wax deposition and others by increasing. Greater responses were seen in some genotypes compared with others which was expected considering they were chosen for inclusion in the study based on their range of different tolerances to heat stress and for their water use efficiency.

Heat stress.

Heat stress is known to influence plants in numerous ways including generation of plant biomass, specific fruiting yield, seed germination efficiency and pollination rates (Reddy et al., 1999). More specifically it is known to have an effect on cotton plants despite it being a crop that is adapted for growth in warm climate (Stewart et al., 2009). It has been shown in several cotton genotypes that at high temperatures an increase in the ambient temperature results in increased epicuticular wax deposition on cotton leaves. One such study demonstrated that

an increase in ambient temperature from 30°C to 48°C caused increased epicuticular wax content on leaf tissue of five genotypes of cotton that ranged from a 66.3 % increase to as high as 223.3 % increase in wax (Ashraf et al., 1994).

Water deficit stress

Adequate irrigation is a crucial factor in determining fibre yield and several other fibre qualities such as length (Stewart et al., 2009) although no effect on the amount of fibre cuticle has been shown. It has, however, been shown that water deficit stress has an effect on cotton in leaf, bract and boll tissues (Bondada et al., 1996). The study by Bondada et al., (1996) involved glasshouse grown cotton that received adequate watering until flowering followed by 3 days of skipped daily irrigations. They showed significant increases in wax concentration in leaf tissue and bract tissue following the application of water deficit stress, but no significant change in boll tissue. This study did not assess the effect on fibre however the increased wax on bract provide further evidence of another cotton plant organ responding to stress with increased wax deposition. Recently there has been one study (Thompson et al., 2017) of seven upland white cottons which examined the influence of well-watered and water-limited irrigation treatments on the amount of cuticular wax deposited on the fibre of field grown cotton. The water-limited treatment was started when 50% of the plots were at first flower. The study did not show an effect of stress on the amount of total cuticular wax on fibre although they did show an effect on some individual cuticular wax components. Of the 41 individual wax components measured, 9 were shown to be significantly affected by water deficit stress, however only 1 component, C31 alkanes, was significantly increased. The authors posit that the water-limited treatment imposed in the study may not have been

severe enough to cause a stress response in the plant that would lead to the deposition of more fibre cuticular wax and that further study is warranted.

The genotypes included in this study were specifically selected for their tolerances to stress so a greater response was measured in those with poorer tolerances to abiotic stress.

Sicot 71

Sicot 71 is a commercial genotype that will have undergone breeding selection for good tolerance to abiotic stress. The decrease in cotton fiber wax for that genotype across both seasons appears to be primarily associated with the water stress treatment. The response by Sicot 71 indicates that generally commercial cottons in the Australian production systems subjected to abiotic stress will produce fiber that will not harbor excessive or problematic levels of cuticle waxes. If anything, stress will cause wax levels to decrease.

Siokra L-23

The response of Siokra L-23 being similar to that of Sicot 71 in exp. 2 can be attributed to the fact that Siokra L-23 is known to have good water use efficiency. This genotype has been shown to be drought tolerant due to its high stomatal resistance and high water potential (Voloudakis et al., 2002).

It also has an okra leaf shape which offers less total leaf area compared to normal leaf shape which is associated with better water use-efficiency (Stiller et al., 2004). The increased wax deposition seen following heat stress alone in exp. 1 can be potentially attributed to higher maximum temperatures during the month that the stress event was implemented and higher radiation for that experimental season (Fig. 2.3 and 2.6). Alternatively, the ability of this plant to increase stomatal resistance in response to water stress may indicate it is also

able to do so in response to heat stress where reduced transpiration rate would protect the plant from heat stress-related water loss. This ability to reduce stress could explain why a consistent increase in wax deposition on the fibre was not found though it is a known response to heat stress in other cotton plant organs. Further work with this particular genotype would be required to support this statement.

CS 50

For CS50, a potential advantage was that this genotype naturally had low fiber wax compared to the other genotypes. This can be attributed to its known poor water use efficiency, and while not measured in this study, it's likely that generally it will have had low cuticle wax content on other plant organs. Following stress, although this genotype had the greatest increase in wax deposition following application of water stress alone relative to control conditions the increased wax deposition was still within the range observed in the other genotypes under control conditions. This genotype has previously been shown to have significantly reduced photosynthetic rate in response to drought stress (Nepomuceno et al., 1998) indicating that it is not a drought tolerant genotype. It is therefore unsurprising that the greatest response to water stress occurred in this genotype. Other than the increase seen only in exp. 1 for Siokra L23 which was attributed to the higher temperature in that season during the stress period, CS50 was the only genotype to show increased wax deposition in response to heat stress alone. Given the effect that heat stress can have on the water balance of plants through increased water loss via transpiration it is also not unexpected that an effect of heat stress was seen in this genotype. Although the increased wax deposition here on fiber is novel, it is in line with previously published work showing

that drought stress causes increased wax deposition in cotton leaf and bract (Bondada et al., 1996).

CIM-448

The good heat tolerant genotype CIM-448 had no notable response to abiotic stress, only demonstrating a reduction in wax in exp. 2 in response to combined stress. This was likely due to the higher seasonal temperatures contributing to greater stress. This response of this genotype was similar to that of Sicot 71 and Siokra L23 in that there was a reduction in wax in response to stress indicating it may have been similarly bred for good tolerance to abiotic stress.

Sicala V-2

The markedly different response to the abiotic stress by the poor heat tolerant Sicala V-2 genotype may be due to numerous physiological factors. One theory is that the accumulative stress from the two individual stress treatments led to an effect that was greater than either stress alone. This is supported by a review of combined heat and water stress in plants that emphasized that the response to a combination of abiotic stress such as heat stress and water deficit stress is unique and can't be directly extrapolated from the response of the plant to the individual stress treatments when applied individually (Mittler, 2006).

The increased wax deposition measured in this study, although novel when compared to previous work (Oosterhuis et al., 1991; Ashraf et al., 1994; Bondada et al., 1996; Thompson et al., 2017) may be due to the specificity of methodology. For this study, stress application occurred during flowering and the early to mid fiber development phases during which

cuticular waxes have been shown to be deposited (Hartzell-Lawson and Hsieh, 2000). The study also imposed a higher level of water stress compared to previous studies and included genotypes with known intolerance to heat and/or water stress. In comparison to the more recent Thompson et al., study which also applied water deficit at flowering, a complete lack of irrigation was implemented here opposed to the 50% irrigation rate used by Thompson. This indicates that the severity of the water stress was greater and in combination with the inclusion of poor abiotic stress tolerant genotypes was likely responsible for the increased wax deposition measured.

3.4.4 Stress causes morphological changes in the cotton fibre cuticle in response to stress

In the study by Bondada et al., (1996) the morphology of the wax on the surface of leaf, bract and boll were also imaged using SEM. This study found that the epicuticular wax morphology in the form of wax ridges appeared similar in all three tissues before and after stress. Here, they observed wax ridges that ran both parallel and at random directions in all tissues. Those results differ from those found in the current research where increased stress appeared to result in a greater proportion of ridges running parallel and along the length of the fibre. Also, for fibre samples where both heat and water deficit stress had been applied rough deep cracks on the surface were apparent. This research examined only fibre from the four treatments for CS50 which had shown a significant increase in wax for all three treatments with the largest significant difference observed in the combined heat stress & water deficit stress treatment group. The increased wax percentages found on these fibres support the likelihood of visual differences between these treatments. The use of a plant highly

susceptible to stress and the application of a greater degree of stress is likely responsible for the differences observed.

3.4.5 Concluding remarks

A response in cotton fibre to abiotic stress resulting in increased fibre cuticular wax deposition was shown here, to the best of my knowledge, for the first time. This may be due both to the timing of the stress application which was during flowering and the early to mid fibre development phases during which cuticular waxes a have been shown to be deposited (Hartzell-Lawson and Hsieh, 2000) and the severity of the stress which was implemented which for water deficit was potentially more severe than previously reported studies (Oosterhuis et al., 1991, Ashraf et al., 1994, Bondada et al., 1996, Thompson et al., 2017). While the fiber wax content level was shown to vary following the application of abiotic stress (0.2 – 1.6%), the total wax content of fiber remained within processable limits for current commercial caustic scouring methods. However, with more research being undertaken investigating the use of alternative and possibly more environmentally friendly scouring methods such as enzymatic scouring, it is possible that increased wax deposition could become problematic.

Chapter 4. The effect of water deficit stress in glasshouse grown naturally coloured cotton.

Abstract

Water stress has been shown to impact cotton fibre qualities in white cotton varieties. These qualities are important for the determination of the commercial value of the cotton. The specific response of coloured cottons to water stress and resulting changes to fibre quality have not yet been documented so comparisons can only be made to white cottons. To determine the effect of water deficit stress on the production of wax on coloured cotton fibres and to better understand the production of wax, three cotton genotypes, including a white and a green Siokra L22 genotype as well as another white genotype, CS 50, which had previously been shown to have increased fibre wax deposition in response to stress (Chapter 3) were subjected to water deficit stress. The stress was applied throughout the experiment from first flower onwards to study the effect of water deficit stress during the entire fibre development period. In addition to a number of standard and cross-sectional fibre quality attributes, the amount of wax present on the fibre was measured. It was hypothesised that water stress applied throughout entire cotton fibre development will impact on fibre quality attributes, and more specifically that it will cause increase cuticular wax deposition. Further to this, it was hypothesised that the effect will be greatest for CS 50 due to its poor water use efficiency. Here it was shown that water stress did not have a significant effect on fibre cuticular wax content or any fibre cross-sectional properties for any genotype other than fibre fineness which was significantly increased by water stress. Additionally, a significant main effect of water deficit stress on fibre length and strength were recorded where water deficit decreased fibre length and increased fibre strength.

4.1 Introduction

In addition to commercial white cotton genotypes, there are also naturally coloured cottons in a variety of shades of brown and green. Coloured cottons produce fibres that more closely resemble older cotton genotypes, producing fibre with qualities that match those of fibres produced on cottons grown before the introduction of farming and breeding processes. These processes have helped optimise the development of fibres that occur today with qualities that make them more commercially attractive due to their suitability towards the manufacturing of yarn, and the knitting and dyeing of fabrics (Tang et al., 2013). Several studies have attempted to quantify the fibre qualities of naturally coloured green cottons. There appears to be consensus that in comparison with white cottons, green cottons have lower maturity and lower micronaire (Richards et al., 1999), are finer and weaker (Richards et al., 1999, Murthy, 2001, Pan et al., 2010) but with greater extension (Richards et al., 1999; Pan et al., 2010). They also produce shorter fibre compared to white genotypes (Richards et al., 1998, Murthy 2001, Pan et al., 2010). Additionally, naturally coloured cottons have higher fibre wax content. Typically, white cottons have around 0.4 – 0.7% wax as a percentage of total fibre weight (Conrad, 1944), brown has 1-2% (Pan et al., 2010), and green cottons have as high as 14-17% (Conrad, 1944). Naturally coloured fibre is typically of low commercial value due to many of the measured fibre quality attributes.

Water stress has been shown to impact cotton fibre qualities in white cotton varieties as described in chapters 2 and 3. Some of these qualities are important for the determination of the commercial value of the cotton. The specific response of coloured cottons to water stress and the resulting changes to fibre quality have not yet been documented so comparisons can only be made to studies involving white cottons. To determine the effect of water deficit

stress on the production of wax on coloured cotton fibres and to better understand the production of this wax, three cotton varieties including a white genotype, CS 50, which has previously been shown to have increased wax deposition in fibre in response to stress (Chapter 3) as well as two Siokra L22 genotypes, a white and a naturally coloured green variety, were grown and subjected to water deficit stress. The stress was applied from first flower until the conclusion of the experiment to study the effect of water deficit stress during the entire fibre development period. In addition to a number of standard and cross-sectional fibre quality attributes, the amount of wax present on the fibre was also measured. This study may further the understanding of the effect of water deficit stress on fibre wax content in an attempt to mitigate the effect this excess wax may have on the manufacturing process.

4.2 Materials and methods

An experiment designed to study the effects of water stress on the fibre quality of white and naturally coloured cottons was conducted in a glasshouse at the Australian Cotton Research Institute (ACRI, 30° 12'S, 149°36'E), 22 km north-west of Narrabri NSW, Australia.

4.2.1 Experimental design

The experiment was set out as a randomised complete block design with three replications. Cotton plants were grown in individual pots, with one pot or plant being a single experimental unit (Figure 4.1).

4.2.2 Cultivation and treatment of cotton plants

Experimental cottons

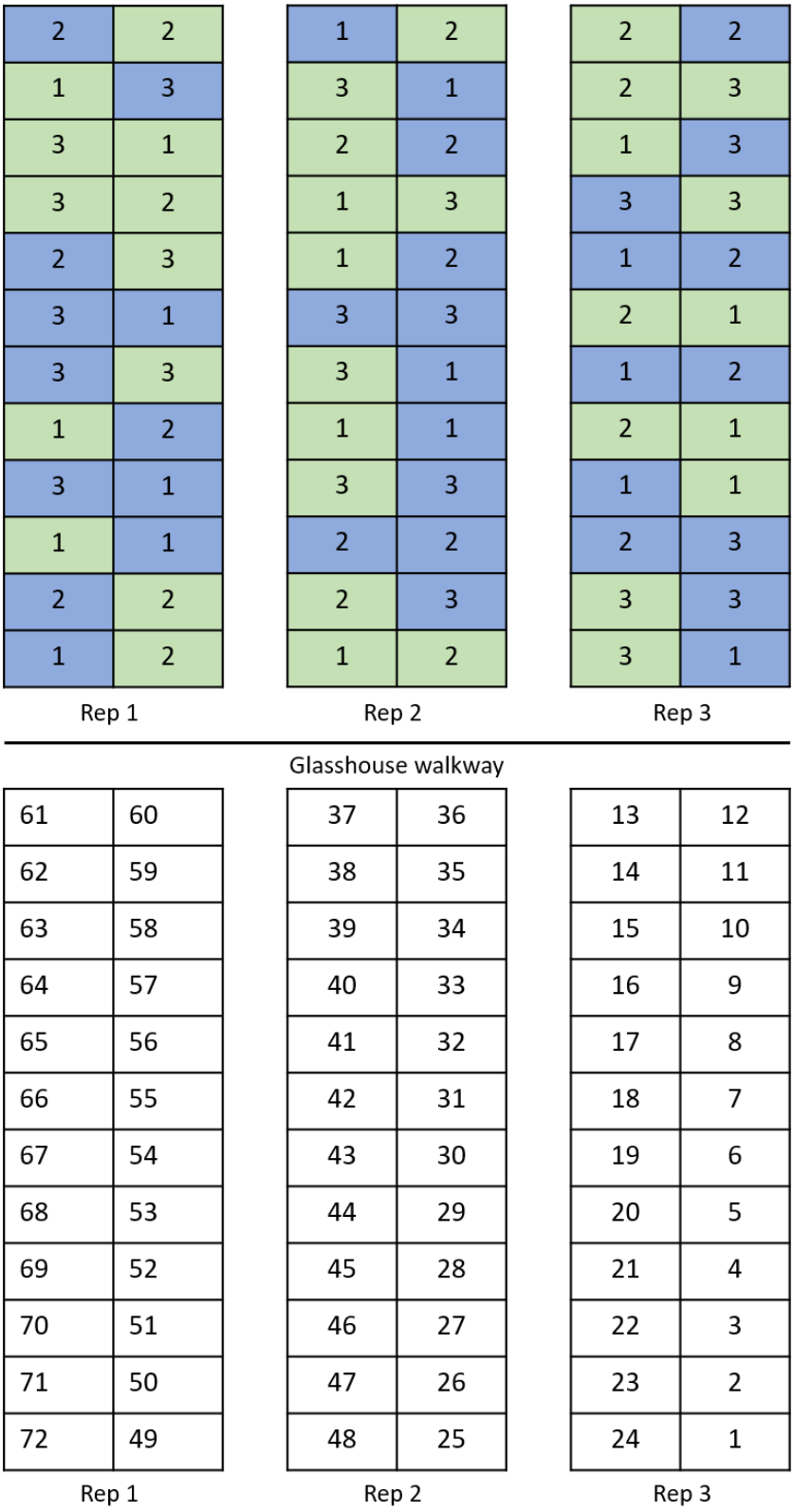


Figure 4.1. Experimental design. Randomised block design on 3 benches in a glasshouse where each bench is one block and individual plots representing a single plant were identified with labels 1-72. Blue shading indicates control watered plots and green shading indicates water stress plots. Genotypes for each plot are numbered 1-3. CS 50 = 1, Siokra L22 = 2, Siokra L22 Green =3.

Three CSIRO *Gossypium hirsutum* cotton genotypes were included in this study. CS 50 white was included for its previously shown poor water use efficiency (Reid, 1992), Siokra L22 was included as a white control and Siokra L22 Green was included as the naturally coloured genotype.

Cultivation of cotton in the glasshouse

Cotton plants were grown in 9-inch pots using potting mix topped with a sand layer using Osmocote fertilizer at a density of 4 plants per pot before being thinned to 1 plant per pot at the appearance of the first true leaf. Glasshouse temperatures were maintained at 31°C during the day and 17°C overnight.

Application and assessment of water stress

Cotton plants were watered using a standard drip irrigation system until anthesis, from which time control plants continued to receive daily watering using standard drip irrigation whilst irrigation nozzles were removed from water stress treatment pots. Water stress treatments were then hand watered daily providing the minimum amount of water required to maintain growth whilst keeping plants in a state of stress (Approximately 400mL). Stress was monitored both qualitatively by assessing leaf wilt and quantitatively by monitoring stomatal conductance. Stomatal conductance was measured and recorded in glasshouse grown cotton plants using a SC-1 Porometer (ICT International. NSW, Australia) as per the manufacturer's instructions. Briefly, the porometer was first calibrated by placing paper disks soaked in de-ionized water inside the porometer apparatus before being used to measure stomatal conductance by clamping the apparatus onto the 3rd from the top apex leaf of cotton plants. Measurements were made at the same time of day (11am) to ensure consistency. Due to the

interstate travel requirements of this experiment, data was recorded at 0DPA, 3DPA, 7DPA, and 9DPA then reduced to weekly measurements to ensure plants remained in a stressed state. Note that visual assessment of stress was performed daily for the duration of the experiment. Data was recorded on the porometer device and then analysed statistically.

Harvesting of cotton

Cotton bolls were hand-picked as they matured and stored in paper bags for future analysis until all bolls were collected. Seed cotton from each plant was ginned using an 8-saw experimental sample gin to separate fibre from seeds.

4.2.3 Assessment of standard fibre quality attributes

Fibre quality was assessed using High Volume Instrument (HVI) and the Cottonscope instrument as described in Chapter 2 (2.2.3).

4.2.4 Analysis of the ethanol extractable cuticle component of raw cotton fibre.

Preparation of fibre samples, extraction of the ethanol extractable cuticle components and subsequent quantitative wax analysis were performed as described in chapter 3 (3.2.2)

4.2.5 Statistical analysis.

Statistical analysis was performed using Microsoft Excel for basic descriptive statistics. GenStat Version 16 (Lawes Agricultural Trust, IACR. Rothamsted, UK) was employed for Analysis of Variance (ANOVA) of data. Raw fibre quality, wax, and stomatal conductance data for individual replicates for each genotype and treatment were added into this program. ANOVA was conducted as a three-way model with genotype, stomatal conductance and date being the three factors. Mean values were reported with preference given to significant two factor interaction, which was reported in line chart format. Otherwise mean values for main

effects with or without statistical significance were tabled where appropriate. The degree of the statistical significance of the ANOVA tests were indicated using standard star symbol convention, being when P values were either <0.05*, <0.01**, or <0.001***. Least significant difference (LSD) (5%) values were reported alongside significant ANOVA results to assist in mean value separation. The statistical analysis design for the ANOVA can be seen in table 4.1a. In a separate statistical exercise, a two-way ANOVA was undertaken where genotype and stress were the two factors. This allowed the effects of stress on fibre quality attributed to be determined. Mean values for main effects were represented in table format. The degree of statistical significance of the ANOVA tests were indicated as above. The statistical analysis design for this ANOVA is shown in Table 4.1b.

Table 4.1. The analysis of variance table. Details of the degrees of freedom assigned to each component of the (a) three-factor model used to analyse stomatal conductance data, and (b) two-factor model used to analyse fibre quality attribute data.

(a)

Source of variation	Degrees of freedom
Genotype	2
Genotype x Date	14
Genotype x Water	2
Genotype x Date x Water	14
Residual	495
Total	574

(b)

Source of variation	Degrees of freedom
Block	2
Genotype	2
Water stress	1
Genotype x water stress	2
Residual	8
Total	15

4.3 Results.

4.3 Induction and measurement of water stress in cotton plants

There was no significant main effect of genotype and water stress on stomatal conductance (Table 4.2). There was a significant two-way interaction of date and water stress ($P \leq 0.001$) which showed a significant decrease of stomatal conductance in the water stressed plants compared with the control (Figure 4.2).

Visual assessment of leaf wilt indicated wilt present in all three genotypes following application of water stress that was not observed in the control plots for any of the genotypes (Figure 4.3).

4.3.3 Effect of water stress on the fibre properties of naturally coloured cottons

Cross-sectional properties

No statistical interaction was captured between abiotic stress and genotype. There were no effects of the water stress treatment on any of the cross-sectional properties except fineness, with water stressed fibre being higher by approx. 20mtex (Table 4.3). For genotype, there were significant differences captured for all cross-sectional properties. Siokra L22 had the highest micronaire followed by CS50 and then Siokra L22 Green which had the lowest micronaire (Table 4.4). CS 50 had the highest maturity ratio followed by Siokra L22 and finally Siokra L22 Green which had the smallest maturity ratio. No significant difference in fibre fineness was measured between CS 50 and Siokra L22, however Siokra L22 Green was significantly finer than the two white genotypes.

There were significant differences in fibre width (µm) between all three genotypes with Siokra L22 Green being the widest followed by Siokra L22 and then CS 50. There were also

Table 4.2. Main effect of genotype stomatal conductance (mm m^{-2}. s). LSD of 5%. P represents significance where P≤0.05 = *, P≤0.01 = **, P≤0.001 = ***, N/S = Not significant.

CS 50	Siokra L22	Siokra L22 Green	$L.S.D^{P}$
245.9a	249.3a	253.3a	$26.5^{N/S}$

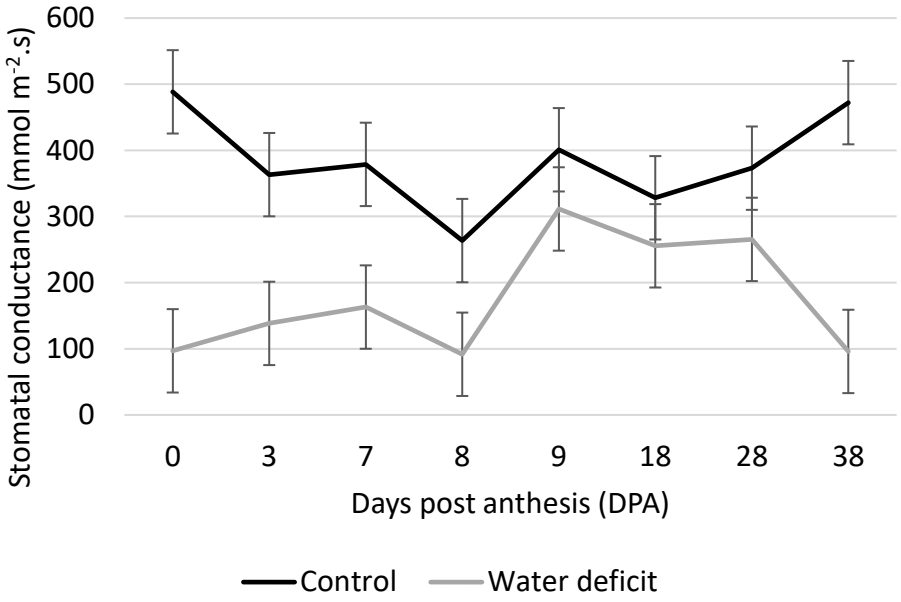


Figure 4.2 Average stomatal conductance (mm m^{-2}. s) of cotton plant apex leaves. Graph illustrates a two-way interaction captured between date (DPA) and water treatment. Mean values presented. Error bars representing L.S.D of 5% included to assist with mean separation. Note, x axis is not linear. From day 9, measurements were taken weekly.

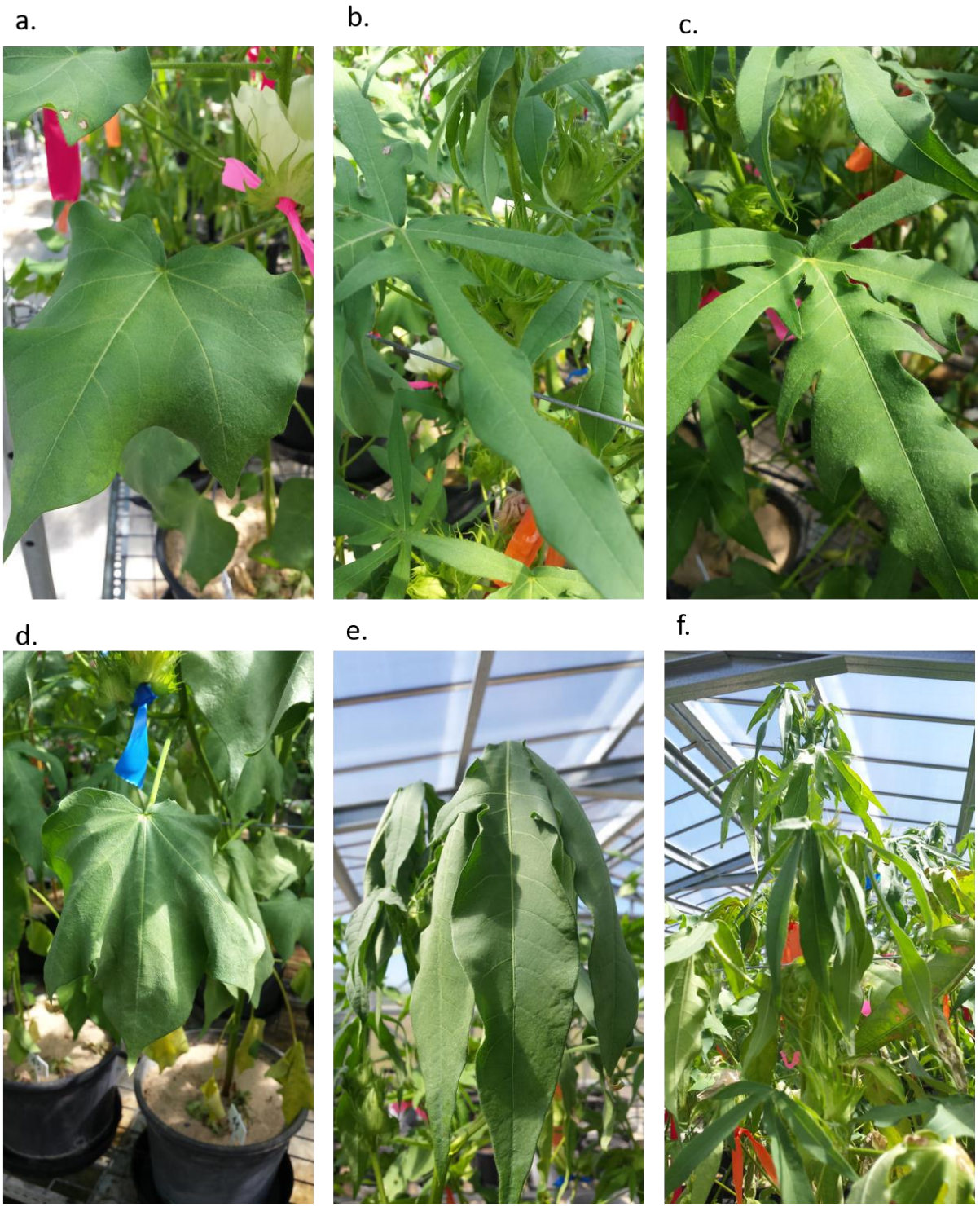


Figure 4.3. Qualitative visual assessment of stress in cotton leaves. Comparison of glasshouse grown cotton plants under control and water stress conditions. (a) CS 50 control leaf, (b) Siokra L22 control leaf, (c) Siokra L22 Green control leaf, (d) CS 50 water stressed leaf, (e) Siokra L22 water stressed leaf, (f) Siokra L22 Green water stressed leaf.

Table 4.3. Main effect of water stress treatment on measured fibre cross-sectional properties. Mean values presented with L.S.D of 5% where differences are indicated by standard lettering convention. P values indicate significance where P ≤0.05 = *, P ≤0.01 = **, P ≤0.001 = ***, Not significant = N/S. Lettering system indicates numbers that are the same or different where matching letters are assigned to numbers for which no significant difference is measured.

	Control	Water stressed	$L.S.D^{P}$
Micronaire	3.87a	4.05a	$0.18^{N/S}$
Maturity ratio	0.81a	0.81a	$0.03^{N/S}$
Fineness (mtex)	208.8a	225.4b	15.1*
Width (µm)	15.81a	16.01a	$0.28^{N/S}$
Perimeter (µm)	60.45a	62.78a	$2.42^{N/S}$
Wall area (μm^2)	125.2a	134.5a	$11.2^{N/S}$

Table 4.4. Main effect of genotype on fibre cross-sectional properties. Mean values presented with L.S.D of 5% where differences are indicated by standard lettering convention. P values indicate significance where P ≤0.05 = *, P ≤0.01 = **, P ≤0.001 = ***, Not significant = N/S. Lettering system indicates numbers that are the same or different where matching letters are assigned to numbers for which no significant difference is measured.

	CS 50	Siokra L22	Siokra L22 Green	$L.S.D^{P}$
Micronaire	4.67a	5.19b	2.00c	0.23^{***}
Maturity ratio	0.96a	0.88b	0.59c	0.04^{***}
Fineness (mtex)	250.7a	258.7a	141.8b	18.6^{***}
Width (µm)	15.09a	16.22b	16.41b	0.35^{***}
Perimeter (µm)	61.27a	64.95b	58.64c	2.96^{**}
Wall area (μm^2)	143.6a	164.0b	82.0c	7.9^{***}

significant differences between all three genotypes for both perimeter and wall area with Siokra L22 Green had the smallest perimeter and total wall area followed by CS 50 and then Siokra L22 which had the largest perimeter and wall area (Table 4.4).

Length properties

There were no effects of the water stress treatment on any of the fibre length attributes except fibre length which was significantly reduced following water deficit stress ($P<0.001$) (Table 4.5). For genotype, there were significant differences captured for all fibre length attributes. CS 50 produced the shortest fibre followed by Siokra L22 Green then Siokra L23 which produced the longest fibre (Table 4.6). There were significant differences in length uniformity between all three genotypes with Siokra L22 Green being the least uniform followed by CS 50 then Siokra L22 which was the most uniform (Table 4.6). Siokra L22 Green had the highest % of short fibre followed by CS 50 and then Siokra L22 which had the smallest short fibre index (%) (Table 4.6). The Immature Fibre Count (IFC%) of the three genotypes showed significant differences between each with the greatest percentage measured in Siokra L22 Green followed by Siokra L22 then CS 50 (Table 4.6).

Tensile properties

A significant main effect of stress on strength was captured ($P<0.05$) where the application of water deficit stress led to the production of stronger fibre (Table 4.5) but no significant effect of stress was found for elongation. For genotype, there were significant differences captured for both strength and elongation. Siokra L22 produced the strongest fibre followed by CS 50 then Siokra L22 Green which produced the weakest fibre (Table 4.6). Siokra L22

Table 4.5. Effect of water deficit stress on HVI determined fibre attributes. Mean values presented with L.S.D of 5% where differences are indicated by standard lettering convention. P values indicate significance where P ≤0.05 = *, P ≤0.01 = **, P ≤0.001 = ***, Not significant = N/S. Lettering system indicates numbers that are the same or different where matching letters are assigned to numbers for which no significant difference is measured.

	Control	Water stressed	$L.S.D^{P}$
Length (mm)	31.39a	30.32b	0.60**
Length uniformity	84.99a	85.38a	$0.94^{N/S}$
Short fibre index (%)	5.1a	4.7a	$0.8^{N/S}$
Immature fibre count (%)	3.3a	3.3a	$0.8^{N/S}$
Strength (g/tex)	32.96a	35.13a	1.59^{*}
Elongation (%)	4.8a	4.9a	$0.3^{N/S}$

Table 4.6. Effect of genotype on HVI determined fibre attributes. Mean values presented with L.S.D of 5% where differences are indicated by standard lettering convention. P values indicate significance where P ≤0.05 = *, P ≤0.01 = **, P ≤0.001 = ***, Not significant = N/S. Lettering system indicates numbers that are the same or different where matching letters are assigned to numbers for which no significant difference is measured.

	CS 50	Siokra L22	Siokra L22 Green	$L.S.D^{P}$
Length (mm)	29.94a	31.78b	30.84c	0.74^{***}
Length uniformity	85.0a	86.6b	84.0c	1.2^{***}
Short fibre index (%)	5.2a	3.4b	6.0a	1.0^{***}
Immature fibre count (%)	1.0a	1.5a	7.4b	1.0^{***}
Strength (g/tex)	35.43a	37.89b	28.81c	1.95^{***}
Elongation (%)	4.4a	4.5a	5.7b	0.3^{***}

Green had significantly greater elongation than either CS 50 or Siokra L22 which were not significantly different from each other.

4.3.2 Effect of water stress on the fibre cuticular waxes of naturally coloured cottons

There were no effects of the water stress treatment on fibre cuticular wax for any genotype (Table 4.7). For genotype, there were significant differences captured for wax content. Siokra L22 Green control had significantly more wax than either CS 50 or Siokra L22 which were not significantly different from each other (Table 4.8). This was true when measured both as a percentage of total fibre weight and as wax per external surface area (mg m^{-2}) (Table 4.8).

Table 4.7. Effect of water deficit stress on the wax content of three naturally coloured cottons. Values illustrate no significant main effect of stress on the wax component of cotton fibre. Mean values presented with L.S.D of 5% where differences are indicated by standard lettering convention. P values indicate significance where P ≤0.05 = *, P ≤0.01 = **, P ≤0.001 = ***, N/S = Not significant. Lettering system indicates numbers that are the same or different where matching letters are assigned to numbers for which no significant difference is measured.

	Control	Water stressed	$L.S.D^{P}$
Wax %	1.75a	2.00a	$1.11^{N/S}$
Wax per surface area mg m^{-2}	48.2a	54.6a	$19.1^{N/S}$

Table 4.8. Average wax content of three naturally coloured cottons. Values illustrate a significant main effect of genotype on wax content. Mean values presented with L.S.D of 5% where differences are indicated by standard lettering convention. P values indicate significance where P ≤0.05 = *, P ≤0.01 = **, P ≤0.001 = ***. Lettering system indicates numbers that are the same or different where matching letters are assigned to numbers for which no significant difference is measured.

	CS 50	Siokra L22	Siokra L22 Green	$L.S.D^{P}$
Wax %	0.44a	0.60a	4.57b	0.79^{***}
Wax per surface area mg m^{-2}	22.4a	21.8a	11.0b	23.4^{***}

4.4 Discussion

Three cotton genotypes, including a white and a green Siokra L22 genotype as well as another white genotype, CS 50, which was shown in chapter 3 to have increased fibre wax deposition in response to stress (chapter 3) were subjected to water deficit stress from flowering through early to mid fibre development. The effect of this abiotic stress on fibre quality attributes including cuticular wax deposition was assessed. Water stress did not have a significant effect on fibre cuticular wax content for any genotype. There was no effect of water deficit on any fibre cross-sectional properties for any genotype other than fibre fineness which was significantly increased by water stress. Additionally, a significant main effect of water deficit stress on fibre length and strength were recorded where water deficit decreased fibre length and increased fibre strength.

4.4.1 Water deficit stress has a significant impact on measured fibre quality attributes

Following the application of water deficit stress significant effects were measured on a number of fibre quality attributes however, the specific response of coloured cottons to water stress and the resulting changes to fibre quality have not yet been documented so comparisons can only be made to studies involving white cottons. Under control conditions, the cross sectional, length and tensile properties of the naturally coloured cotton were in line with previously reported fibre attributes for coloured cottons (Richards et al., 1999, Murthy, 2001, Pan et al., 2010).

Cross-sectional properties

The current literature varies in its reports of the effect of water deficit stress on cotton micronaire, with one study showing it caused a decrease in fibre micronaire water deficit stress (Padmalatha et al., 2012). In that study water deficit stress was applied from 70 days

after sowing, whereby water was withheld, and soil moisture content was measured until soil moisture content reached 50% of the control plot measurement. At this point fibre samples were taken (Padmalatha et al., 2012). Another study discussed increased micronaire following water stress applied in late bloom stages of cotton growth (Nazar et al., 2012). Yet another study indicated no significant effect of water stress on micronaire (Nazar et al., 2012) where a pivot irrigation system was used to deliver a base rate of irrigation as well as base rate -20% for the water deficit treatment. The timing of these irrigations was not stated. The timing of application of water stress in this study most closely resemble the Padmalatha study of 2012, as does the approximate irrigation rate. The continuous application of water stress ensured the entire fibre development occurred during water deficit stress. Here no effect of water stress on micronaire or maturity ratio was observed for any of the three included genotypes. This may be explained by the different growing conditions used between this glasshouse study and the field grown cottons of Padmalatha's study or may be due to the different genotypes used in the studies. In chapter 2 a significant main effect of water deficit stress on micronaire was measured where water deficit caused increased micronaire on field grown cotton genotypes when water stress coincided with flowering and early to mid fibre development however other than CS 50, the genotypes used in chapter 2 differed from those used in this study. Because cotton genotypes vary in their tolerances to water stress it is likely that an effect of stress on micronaire is genotype dependent. Because of these variations, it remains unclear as to whether micronaire is influenced by water stress and further study utilising a larger number of cotton genotypes may be required to discern the true effect of water deficit stress on micronaire.

Although there was no significant effect of water deficit stress on micronaire or maturity ratio, there was a significant main effect of water stress on fineness with the application of water deficit stress leading to an increase in fibre fineness. Fibre fineness has not previously been shown to be affected by water deficit stress.

Length properties

Similar decreases in fibre length have been previously reported for white cottons (Nazar et al., 2012, Ahmad, 2013, Padmalatha et al., 2012). One such study that utilised water deficit stress for a period of 30 days at squaring and first boll split showed a significant decrease in fibre length in the water stress treatment (Ahmad, 2013). Another which utilised water stress to implement and maintain a 50% reduction in soil moisture content from 70 days after sowing until the conclusion of the experiment showed that fibre length was significantly reduced following water deficit stress (Padmalatha et al., 2012). The results from these studies demonstrates a similar response to water stress on length despite the differences in water stress application timeline. Here, the water stress application most closely resembles that of Padmalatha (2012) and a similar reduction in fibre length was measured. Other fibre length attributes including length uniformity, short fibre index and immature fibre count were not significantly affected by water stress. This indicates that the effect of length was occurring due to water deficit during the elongation phase, rather than the initiation phase which if effected would have likely increased the number of immature fibres present. Again, this is supported by Padmalatha where studies of the expression of genes important during fibre elongation were shown to be altered resulting in reduced fibre length (Padmalatha et al., 2012).

Here Siokra L22 Green produced fibres that were by far the lowest quality when considering commercially attractive fibre quality attributes as describes in chapters 1 and 2. The only measured exception to the overall lower fibre quality was fibre length where Siokra L22 green was significantly longer than the CS 50 white genotype although it did measure significantly shorter than the Siokra L22 white variety which is consistent with the literature. The differences in fibre length between the two Siokra varieties and CS 50 can be related to the okra leaf shape. Cottons with okra leaf shapes have been shown to produce fibres which are longer than fibres produced by normal leaf shape varieties (Stiller et al., 2004) such as CS 50.

Tensile properties

The increased strength that resulted from the water deficit stress treatment is in agreement with previous reports where water deficit was shown to cause an increase in fibre strength (Booker et al., 2006, Nazar et al., 2012, Ahmad, 2013). Strength has been shown to increase linearly with increasing water stress when applied as a constant deficit throughout cotton growth (Booker et al., 2006; Nazar et al. 2012) as was the case in this study where constant water deficit stress led to increased fibre strength. It is likely that the decreased fibre length found in response to water stress is responsible for the increased fibre strength. Additionally, elongation has previously been shown to be significantly reduced following water deficit stress (Padmalatha et al., 2012) however it was not significantly affected by water deficit stress in this study.

4.4.2 Water deficit stress did not increase deposition of fibre cuticular waxes.

Although the wax % in the water deficit stressed fibre appeared to be higher than the control fibres (1.75% wax vs. 2.00% wax) there was no significant main effect of water deficit stress on the wax content of fibre. This result is in accordance with a recent study of the response

of total waxes and the individual wax components to water deficit stress. The study showed that despite a significant increase in the proportion of C31 alkanes present in water deficit stressed fibres, there was no significant increase in total wax content (Thompson et al., 2017). However, results from research presented in this thesis and described in chapter 3 demonstrated that in multiple genotypes a response to water deficit stress in fibre wax was found, including for the CS 50 genotype that showed an increased deposition of wax on fibre in response to water stress and a combination of heat and water stress that was repeated across 2 growing seasons (Chapter 3).

One possible explanation for the increased wax response of CS 50 in chapter 3 is that unlike this experiment, the CS 50 fibres that showed a response to water deficit stress were grown in the field which is a less controlled environment where additional stress may have influenced the results. Additionally, the level of water stress was not quantified in the field experiment. Rather than modifying the irrigation amounts daily to maintain a constant state of stress that still allowed the plants to grow and maintain bolls, the field experiments skipped 2 consecutive irrigations out of a total of the 8 irrigations that were implemented (numbers 2 and 3, “in-crop’ irrigations). This meant the plants were intermittently stressed but had larger periods of no watering compared the glasshouse grown plants in this experiment. This period without any watering may have resulted in a greater level of stress in the field grown plants which was high enough to stimulate a stress response that led to increased wax deposition.

The fact that cotton fibre is not a photosynthesizing cell may also influence results when it is considered that increased wax deposition has been shown in leaf and other aerial parts of the plant in response to water stress (Oosterhuis et al., 1991; Bondada et al., 1996). It may be

that the plants are first prioritizing the deposition of additional wax onto the leaf surface and other aerial parts that carry out photosynthesis and where transpiration and the resulting water loss would occur before the deposition of additional waxes on fibre cells.

4.4.3 Concluding remarks

Stress is not a one-dimensional factor and, rather, one variable amongst many conditions to which plants are exposed (Rizhsky et al., 2004). A glasshouse study is typically able to more closely limit these variables in comparison to a field experiment where the plants are exposed to a greater range of environmental factors such as heat and water stress but also, where other weather factors like wind, radiation, and biotic stress factors are present. Conversely, glasshouse environments may provide additional stresses such as increased humidity. Additionally, the timing of stress application has been shown to vary the response of the fibre depending on the period of fibre development at which the cotton plant was stressed. The plant response to stress is complex and many of these stresses overlap (Rizhsky et al., 2004). It is possible that in the glasshouse study described here, despite maintaining a constant level of water stress the overall stress level of the plants was lower than the stress level of the plants in the field experiment described in chapter 3. The lower level of stress may explain why an increase in wax deposition in response to water deficit stress was not seen in this study on the CS 50 fibres despite the increase measured in this genotype in chapter 3. Further analysis using a range of water deficit stress treatments and an analysis of the known plant defence gene pathways may be useful in helping to gain a clearer understanding of the plant response to water deficit stress in white and naturally coloured cottons

Chapter 5. The dyeability and colour fastness of three naturally coloured upland cottons.

Abstract

An investigation into the dyeability of fabrics made from three different naturally coloured upland cottons with naturally varying fibre wax content was undertaken. One common white cotton, and two un-common coloured cottons, one brown and one green, were used for experiments. These cottons were grown under standard growing conditions and were not subjected to abiotic stress. As part of the production of smaller sample amounts of yarn and fabric to facilitate the study, standard fibre quality attributes were measured and discussed, as well as brief discussions around standard yarn performance results for each cotton type.

The wax content of the white cotton was found to be 0.51%, the brown cotton was 0.78% and the green cotton was 7.88% wax. It was hypothesised that following dyeing, fabrics that were not scoured would have inferior colour fastness following a standard fabric wash test and the effect was expected to be more prominent for fabric made from the higher wax content coloured cottons. Fabrics made from these cottons were subjected to either traditional NaOH caustic scouring or hot ETOH scouring before being dyed and washed. The hot ethanol Conrad method of scouring was compared to the more practical approach of using sodium hydroxide scouring via a shorter industrial method. While traditional scouring techniques disrupt waxes and a number of other non-cellulosic fibre components including polysaccharides, ethanol scouring provides a more complete scouring of the entire wax component of the fibre cuticle which aided in the identification of wax compounds that may be influencing fibre dyeability and colour fastness. For the standard white commercial cotton, scouring was not required to ensure the colour fastness of fabric. This counterintuitive result is likely due to the colour fast test used and the dye type; a more rigorous wash test and or the use of a lighter or different

dye type would likely give a different outcome. For the coloured cotton fabrics, no scouring prior to dyeing negatively influenced fabric colour fastness, which was attributed to the elevated wax levels in these genotypes. Regardless of wax content in the different genotypes, for all fabrics, NaOH adequately disrupted the hydrophobic fibre cuticle allowing for colourfast dyeing. Analysis of wax components using FTIR demonstrated that increased alkane waxes correlated with decreased dyeability. ETOH scouring was shown to more efficiently remove cuticular waxes including alkanes compared with traditional caustic scouring on all three fabrics which facilitated greater dyeability in the naturally high wax coloured green fabric whilst NaOH scouring facilitated greater dyeability in the naturally coloured brown fabrics. Discussions of these results included the potential consequences of the scouring and dyeing of future cottons that might have high wax content.

5.1 Introduction

To facilitate the dyeing of raw cotton fabrics the cuticular layer of fibre must first be disrupted or removed by scouring using various methods to reduce the hydrophobicity of the fibre surface to allow dye access to the underlying cellulose to which the dye is uptaken. Traditionally this scouring involves the use of caustic sodium hydroxide to disrupt a wide range of hydrophobic cuticular components (Lewis, 2011a) as well as removal of other non-cellulosic polysaccharides which are bonded to the cellulose. In contrast, synthetic fibre (polyester) based fabrics can be dyed via a simpler process, which is partly due to the absence of cuticle material meaning it does not need to undergo scouring prior to dyeing. Therefore, this physical attribute is one of a number that allow synthetics to effectively compete with cotton.

The amount of cuticular wax present on cotton fibre varies between genotypes. Traditionally, a standard white cotton will contain approximately 0.4 - 0.7% wax by total weight of the fibre (Conrad, 1944) whilst naturally coloured cottons contain a much higher concentration. For example, brown cottons generally have around 1.0 - 2.0% wax by total weight of the fibre (Pan et al., 2010), whilst green cottons have the highest fibre cuticular wax component which has been recorded as high as 14% (Conrad, 1944).

Naturally coloured cottons that are grown today are not directly related to older "wild" pre farming era coloured cottons, rather they are typically mutant versions of white genotypes that originated in Texas (Elesini et al., 2002). Coloured cottons are not widely used for manufacturing although they do supply a niche market where they are sold as organic alternatives that do not require dyeing due to their natural colour and therefore do not require scouring (Tang et al., 2013, Matusiak and Frydrych, 2014). In the context of this work, they were included not for their colour, but for their naturally high wax content compared with commercial white cottons.

The aim of the research presented in this chapter was to assess a standard dye fastness test on fabric made from cottons varying markedly in the amount of cuticle components. Further the aim was to test these cottons following the use of either traditional caustic scouring that disrupts a wide range of cuticle components, as well as assessing ethanol scouring which is known to tend to target more specifically waxes and some low molecular weight sugars. It was hypothesised that following fabric dyeing without any scouring, there would be a distinct change in fabric appearance following washing, and that this change would be magnified for fabrics made of cotton with more cuticle wax and other components. To undertake this work, three industrially produced and processed upland bales of cotton were used for comparisons;

one was a standard white cotton, one a naturally coloured brown cotton, and one a naturally coloured green cotton. Experimental fibre in bales was also characterised via various fibre quality testing to enable the commercial production of yarn and fabric to facilitate the dye fastness experiments.

5.2 Materials and methods

5.2.1 Experimental cotton bales

Three bales of *Gossypium hirsutum* upland cotton were sourced from the CSIRO cotton processing facility and used for experiments. One bale was a standard high yielding common commercial white cultivar produced by Australian commercial growers. The other two were less common naturally coloured upland cultivars, one brown and one green *G. hirsutum* genotype obtained from Bidstrupp farming. From each experimental bale, approx. 1 kg was sub-sampled to provide a stock sample for all experimental testing.

5.2.2 Assessment of standard fibre quality attributes

Standard high volume instrument measurements

Three replicate samples were taken from each stock sample for testing via an Uster Technologies Model 1000 High Volume Instrument (HVI) located at a commercial cotton classing facility (Auscott Classing Facility. Sydney, Australia). As part of this testing samples were conditioned under standard textile testing air conditions (20^{o}C ± 2, 65% RH ± 3). Attributes measured were micronaire, reflectance (Rd), yellowness (plus b), Trash grade (TrID), trash count, trash content area (TrAr), upper half mean length, length uniformity index, short fibre index, fibre bundle strength and fibre bundle elongation.

Objective cross-sectional measurements

Fibre cross-sectional properties including micronaire, maturity ratio, fineness, width, fibre perimeter and total fibre wall area were determined as per chapter 2.

5.2.3 Determination of wax on fibre samples

Fibre samples were cleaned and wax content determined via the Conrad method as detailed in Chapter 3.

5.2.4 Generation of fabric from experimental fibre using a miniature spinning line

Yarn spinning and testing

Experimental cotton samples (6 x 20g lots for each experimental cotton) were spun into 20 tex yarns with a typical knit twist using a miniature spinning line (Tianjin Jiacheng Mechatronic Equipment Co., Ltd, China). Yarns were conditioned at standard textile air conditions. Yarn bobbins were tested using 100m yarn sample, to determine yarn linear density/count (tex g/1000m) using a yarn wrap wheel, and for the determination of twist (turns per meter) using a Zweigle yarn twist machine (Uster Technologies. Uster, Switzerland) at 10 replicate tests per bobbin. An Uster Technologies Tester 4-SX was used to measure the mass unevenness [% coefficient of variation (CV %) or 'evenness'] which gauges the variation in the mass of multiple 0.01m portions of yarn. Yarn strength (tenacity) (cN/tex) which is the force to break divided by the Tex of the yarn, was determined using a Zellweger Uster Tensorapid 3 (Uster technologies, Uster, Switzerland).

Fabric manufacturing

Experimental yarns were knitted into single jersey fabrics using a Lawson Hemphill 25.4cm "10 inch" F.A.K. knitting machine (Lawson Hemphill. MA, USA). Each of the three experimental fabrics was then cut and overlocked to make smaller fabric samples of

approximately 3.5g weight as shown in figure 5.1. For each experimental fabric, there were four treatments for which each had three replicate overlocked pieces of fabric. A total of 12 pieces of fabric were utilised per experimental cotton genotype with a total of 36 pieces of experimental fabric being made. Experimental treatments included control, fabrics to be dyed without scouring (raw) and fabrics to be dyed following either NaOH or ETOH scouring.

5.2.5 Fabric experiments

Traditional caustic scouring of fabrics

Fabric samples were placed together inside a wire basket within a Thiess Eco Bloc Dye machine (Thiess, Coesfeld Germany) and immersed in a scour solution comprising 110g NaOH, 11g of Albaflow wetting agent, 88g of Croscolour Surfactant and 44g of Irgasol Dispersing agent dissolved in 22L of water. The solution was heated and held at 100°C for 30 mins before being rinsed with water to remove residual scour solution.

Ethanol scouring of fabrics

Fabrics were put inside 30mm x 100mm cellulose thimbles and hot ethanol extracted for 90 cycles. This was identical to the first stage of the Conrad fibre wax determination method described in chapter 2. Each thimble contained three identical replicate pieces of fabric to ensure consistent scouring for each treatment replicate.

Fabric dyeing

Fabric samples were dyed together using a Thiess D-4420 Eco Bloc Dye machine. Total fabric weight dyed was 105.9g. Fabric was dyed using a solution comprising 1.06g Novacron Navy blue S-G dye (1% strength) (Hunstsman, Gateway West, Singapore), 11g Albaflow FFA (0.1g/L) (Hunstsman, Gateway West, Singapore), 22g Irgasol CoNEW (1.0g/L) (Novartis, Switzerland),

Figure 5.1. Experimental fabrics. Overlocked experimental fabric samples generated from white (left), green (centre) and brown (right) experimental cottons. Scale bar equals 1cm

1100g Salt (sodium carbonate) (50g/L) (Chem supply, SA, Australia) and 264g/L Soda Ash (12g/L) (Chem supply, SA, Australia) dissolved in 22L water. Solution was run for 1hr 40 mins total at 60°C. Soda ash was added in increments after the first hour of dyeing. 26.4g was added after 1hr, an additional 52.8g was added 10 mins later and the remaining 184.8g added after a following 10 mins. All other components were added at the commencement of the dye cycle. The dye solution was then drained, and fabric was rinsed with water several times until the waste water ran clear.

Commercial washing of treated fabrics

To determine the colour fastness behavior of fabric specimens, fabric was washed for 30 mins in a solution of Standard Soap without optical Brightening agent using an Atlas Electric Devices Co. Launder-Ometer washing machine as per International Standard ISO 105-C06; 1994 (Textiles – Test for colour fastness Part C06: Colour fastness to domestic and commercial laundering). Fabric samples were grouped based on individual scour-dye treatment replicates, such that each of the 3 replicate fabrics were placed together in individual stainless-steel wash capsules. The nine capsules were placed inside the Launder-Ometer where they were spun to facilitate washing. Following washing, the solution was drained, and fabrics were rinsed using 40°C tap water until all visible traces of solution were removed. Fabrics were then laid to dry at room temperature.

Fabric sample weight determination

Conditioned fabrics were weighed prior to any treatment to establish an initial fabric weight. Following scouring treatment fabrics were allowed to dry before being reconditioned and weighed to determine the weight lost by scouring. Fabrics were conditioned under standard

textile testing conditions and weighed and recorded to 4 decimal places. Fabric weight measurements were repeated until three consecutive identical measurements had been taken to ensure accuracy. The change in weight was then calculated as a percentage and reported for each treatment.

Colour spectroscopic fabric testing

Colour spectroscopic analysis of fabric was performed using the GretagMacbeth™ COLOR-EYE® 7000A spectrophotometer (X-rite, MI, USA) according to manufacturer's instructions using the associated Color iQC Professional with SLITaper® Version 7.5.10 software. Four spectroscopic repeats were captured per piece of fabric following re-positioning in the instrument. The 15 mm dimeter aperture was used to acquire colour spectra. Colour differences between fabrics were determined by the instrument software using Delta E calculations as specified by the Colour Measurement Committee (CMC) giving a DEcmc value (Equation 5.1) (Chrisment, 1998). Delta E is a single number that is calibrated to human perceptions of colour and shade and represents the "distance" between two colours with a DEcmc value of 1 representing the smallest colour difference between two objects discernible by the human eye. The instrument software calculated an average of the four replicate readings which was used to produce an average set of L a b coordinates, where L represents lightness from black (0) to white (100), a is the colour spectrum from red to green, and b is the colour spectrum from blue to yellow (Chrisment, 1998), which were combined along with each single other relevant set of coordinates to produce a single DEcmc result used to compare any two samples of interest.

Fabrics were analysed before and after each of the scouring, dyeing and washing treatments. For each batch of three replicate treated fabrics, a single average DEcmc value and associated

standard deviation value, was calculated. CIELab spectra plots were generated using the instrument software which allowed fabric brightness and colour hue to be simultaneously presented. These plots were produced for each cotton type for each of the three scour treatments, without and with, the dye and wash treatment.

Colour photographs of fabric samples were taken with a digital camera under ambiant lab lighting. To ensure accurate comparison and colour balance images were taken at the same time of day (11am) at a measured distance of 30cm using the same camera without the use of a flash.

$$DE_{CMC} = \left[\left(\frac{\Delta L*}{1S_L}\right)^2 + \left(\frac{\Delta C*}{cS_C}\right)^2 + \left(\frac{\Delta H*}{S_H}\right)^2\right]^{1/2} \quad \text{Equation 5.1}$$

Where L = lightness, C = Chroma (Saturation), H = Hue, S = corrective terms (weighting coefficients specific to CMC) (Chrisment, 1998).

Summary of experimental steps for the analysis of dyeability of naturally coloured cottons with varying wax concentrations.

An outline of when relevant weight and colour spectroscopic measurements were undertaken before and after scour and dye treatments of fabrics, is summarized in Figure 5.2.

5.2.6 Compositional analysis of fabrics using FTIR

Following dye fastness wash testing, recovered fabrics were cut into small pieces and ~100mg ground in 20mL Stainless Steel jars with a 20mm Stainless Steel ball at 30Hz, 60 sec using a Qiagen Tissue-lyser (Qiagen, Hilden, Germany). FITR analysis was performed using a Bruker Vertex 70 FTIR with an ATR (Pike) accessory (Bruker, Massachusetts, USA). Spectra were obtained in transmission mode with a resolution of 4cm-1 with 16 scans on three replicates

per sample. Background corrections were made between samples and spectra were baseline corrected and normalised using Bruker OPUS software.

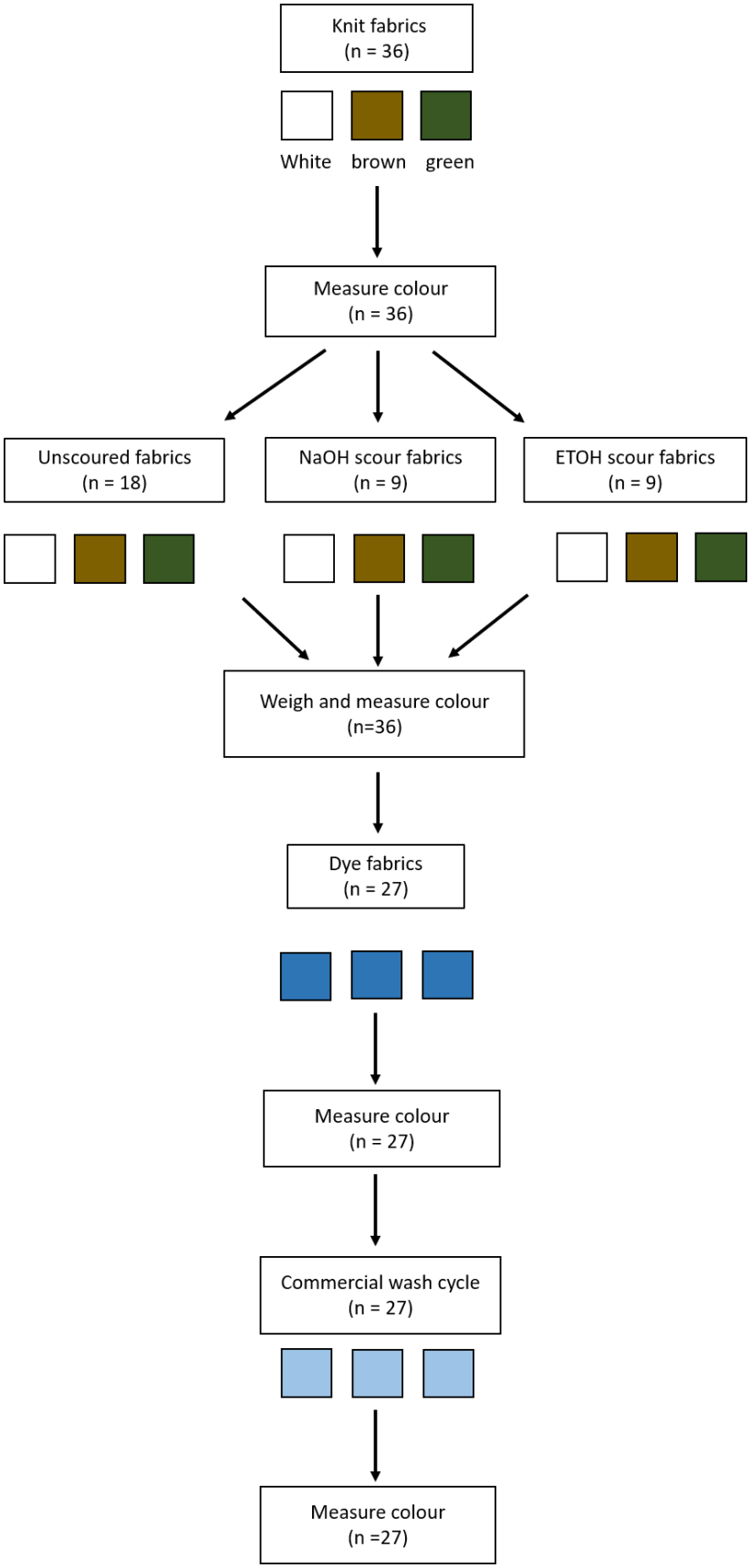


Figure 5.2. Experimental design. Flow chart illustrating experimental steps for the analysis of dyeability of naturally coloured cottons with varying wax concentrations.

5.2.7 Statistical analysis

Statistical analysis was performed using Microsoft Excel for basic descriptive statistics. GenStat Version 16 (Lawes Agricultural Trust, IACR. Rothamsted, UK) was employed for Analysis of Variance (ANOVA) of standard and cross-sectional fibre quality attributes, fibre wax content, and yarn performance data. Average result values were reported, and significant results were indicated using standard star symbol convention, being when P values were either <0.05*, <0.01**, or <0.001***. Least significant difference (LSD) (5%) values were reported alongside significant ANOVA results to assist in mean value separation. The statistical analysis design for the ANOVA can be seen in table 5.1. Average DEcmc measurements were recorded alongside standard deviation to assist with mean separation.

Table 5.1. Analysis of Variance Table. This table details the degrees of freedom (d.f.) assigned to each component of the single factor model used to analyse both the fibre and the yarn quality data sets.

Source of variation	Degrees of freedom
Genotype	2
Residual	6
Total	8

5.3 Results

5.3.1. Standard and cross section fibre attributes

Cross-sectional properties

Significant differences in micronaire values were measured between all three cotton varieties (Table 5.2). The highest micronaire was measured for the white fibre followed by brown and then green which had the lowest micronaire. A similar pattern of significant differences was captured for maturity ratio and fineness with the white fibre shown to be the most mature and fine followed in order by the brown and green fibre. Significant differences in width and perimeter and wall area were also captured for all three genotypes. Green fibre had the greatest width, largest perimeter and smallest wall area of all three cottons. This was followed by the brown which had significantly greater width, smaller perimeter and larger wall area than the green fibre. The white fibre had the smallest width, smallest perimeter and the largest wall area of all three genotypes (Table 5.2).

Length properties

The white cotton fibre was significantly longer and more uniform than either of the coloured cottons with a significantly lower short fibre content. The green fibre was significantly shorter than the brown however no significant differences in uniformity and short fibre content were measured between the brown and green fibre (Table 5.3).

Tensile properties

Significant differences in strength were measured between all three cottons. The white cotton fibre was strongest followed by brown then green which was the weakest. There was

Table 5.2. Effect of genotype on fibre cross-sectional properties. The table illustrates main effects of genotype on fibre cross-sectional properties. Mean values presented. L.S.D (5%) values to assist with mean separation where differences are indicated with lettering. P values indicate significance where P ≤0.05 = *, P ≤0.01 = **, P ≤0.001 = ***. N/S = Not significant. Lettering system indicates numbers that are the same or different where matching letters are assigned to numbers for which no significant difference is measured.

	White	Brown	Green	L.S.D
Micronaire	4.55a	3.25b	2.46c	0.14***
Maturity ratio	1.00a	0.75b	0.50c	0.01***
Fineness (mtex)	193.50a	158.30b	125.80c	6.75***
Width (µm)	14.75a	15.87b	16.66c	0.13***
Perimeter (µm)	52.56a	55.11b	60.24c	0.81***
Wall area (μm^2)	121.0a	101.6b	94.3c	2.9***

Table 5.3. Effect of genotype on HVI determined fibre attributes. The table illustrates main effects of genotype on fibre HVI determined fibre attributes. Mean values presented. L.S.D (5%) values to assist with mean separation where differences are indicated with lettering. P values indicate significance where P ≤0.05 = *, P ≤0.01 = **, P ≤0.001 = ***. N/S = Not significant. Lettering system indicates numbers that are the same or different where matching letters are assigned to numbers for which no significant difference is measured.

	White	Brown	Green	$L.S.D^{P}$
Length (mm)	29.28a	26.00b	24.87c	0.77^{***}
Length uniformity (%)	82.2a	77.1b	77.7b	1.0^{***}
Short fibre index (%)	8.8a	16.9b	17.8b	1.4^{***}
Strength (g/tex)	30.07a	21.45b	19.29c	0.74^{***}
Elongation (%)	5.2a	5.3a	6.1b	0.2^{***}

no significant difference in elongation between the white and brown cottons however the elongation of green cotton was significantly higher than both the white and brown (Table 5.3).

Colour and trash properties

Significant differences in reflectance (Rd) were measured between all three genotypes (Table 5.4). The white cotton had the greatest reflectance, followed by green and then brown which had the lowest reflectance. There were also significant differences in the degree of yellowness between all three genotypes with white cotton being the least yellow followed by the green and finally the brown which had the greatest degree of yellowness (Table 5.4).

There were no significant differences in TrID or TrAr for any of the three genotypes however the green fibre had a significantly lower trash count than either the white or brown fibre which were not significantly different from each other (Table 5.4).

5.3.2 Quantification of the wax content of experimental cotton fibre.

The wax component of the cuticle showed no significant difference in total wax percentage between the industry standard white variety and the naturally coloured brown cotton which had 0.51% and 0.78% total wax respectively, however the naturally coloured green cotton fibre which contained 7.88% wax was significantly different from both the white and brown fibres (Table 5.5).

5.3.3 Analysis of yarn quality

Yarn quality was different between the three genotypes. The white and brown yarn had significantly higher tex than the green yarn although there was no significant difference in

Table 5.4. Effect of genotype on HVI determined colour and trash measurements. The table illustrates main effects of genotype on fibre attributes. Mean values presented for Reflectance (Rd), Yellowness (Plus b), trash grade (TrID), trash count and trash content area (TrAr) for the three experimental cottons. L.S.D (5%) values to assist with mean separation where differences are indicated with lettering. P values indicate significance where P ≤0.05 = *, P ≤0.01 = **, P ≤0.001 = ***. N/S = Not significant. Lettering system indicates numbers that are the same or different where matching letters are assigned to numbers for which no significant difference is measured.

	White	Brown	Green	L.S.D^{P}
Rd	77.67a	37.93b	46.81c	0.58***
Plus b	8.62a	21.40b	20.74c	0.42***
TrID	3.00a	2.67a	2.00a	N/S
Trash count	30.00a	21.70a	9.70b	10.49**
TrAr (%)	0.34a	0.46a	0.22a	N/S

Table 5.5. Effect of genotype on the amount of cuticle component present on fibre. The table illustrates main effects of genotype on the amount of cuticle component present. Mean values presented for total extractable cuticle and total cuticular wax per weight of fibre for the three experimental cottons. L.S.D (5%) values to assist with mean separation where differences are indicated with lettering. P values indicate significance where P ≤0.05 = *, P ≤0.01 = **, P ≤0.001 = ***. N/S = Not significant. Lettering system indicates numbers that are the same or different where matching letters are assigned to numbers for which no significant difference is measured.

	White	Brown	Green	L.S.D^{P}
Total extracted cuticle %	0.88a	1.87b	8.33c	0.91***
Wax %	0.51a	0.78a	7.88b	0.68***

twist between yarns. The greatest tenacity was measured in the white yarn showing that it was much stronger than either the brown or green fibre whilst the brown yarn was more uneven than either the white or green yarns (Table 5.6).

5.3.4 Analysis of scouring efficiency on cotton fabrics.

There were no significant differences in scouring efficiency between the traditional NaOH scouring and the hot ETOH scouring for either the white or brown fabrics. Significantly more cuticle was removed on the green fibres using hot ETOH scouring when compared to traditional NaOH scouring. Using the traditional NaOH caustic scouring a total weight reduction in the fabrics of 0.53%, 0.75% and 3.63% was recorded for white, brown and green fabrics respectively. Using the hot ETOH scouring method a total weight reduction in the fabrics of 0.53%, 0.65% and 7.08% was measured for the white, brown and green fibres respectively (Figure 5.3).

5.3.5 FTIR analysis of experimental fabrics.

In all three cotton fabrics, major peaks were evident as marked in figures 5.4 at approximately 3300 cm^{-1} (Alkynes) (Figure 5.4a, b, c - a), 2850 cm^{-1} (Alkanes) (Figure 5.4a, b, c - b), 1640-1680 cm^{-1} (Alkenes) (Figure 5.4a, b, c - c), 1350-1470 cm^{-1} (alkanes) (Figure 5.4a, b, c - d) and 1000 cm^{-1} (Alkenes) (Figure 5.4a, b, c - e). Identification was made using comparisons to published work (Lambert et al., 1987, Pavia et al., 1979, Church and Woodhead, 2006).

In the white fabric, there was very little difference measured in the alkynes between treatments nor were there great differences between the alkanes at 1350 cm^{-1} (Figure 5.4a, d) or the alkenes at 1000 cm^{-1} (Figure 5.4a, e). Observable differences were measured between treatments for the alkanes at 2850 cm^{-1} (Figure 5.4a, b) with both the raw control and the

Table 5.6. Effect of genotype on yarn quality attributes. The table illustrates main effects of genotype on yarn quality attributes. Mean values presented for count (tex = g/1000m), twist (tpm = turns per meter), mass evenness (CV %), and tenacity (cN/tex) for the three experimental cottons. L.S.D (5%) values to assist with mean separation where differences are indicated with lettering. P values indicate significance where P ≤0.05 = *, P ≤0.01 = **, P ≤0.001 = ***. N/S = Not significant. Lettering system indicates numbers that are the same or different where matching letters are assigned to numbers for which no significant difference is measured.

	White	Brown	Green	$L.S.D^{P}$
Count (tex)	21.49a	20.61a	18.79b	1.33^{**}
Twist (tpm)	778.67a	798.00a	795.67a	$27.93^{N/S}$
Mass evenness %	17.66a	23.44b	18.95a	1.54^{***}
Tenacity (cN/tex)	15.50a	11.56b	13.10c	0.70^{**}

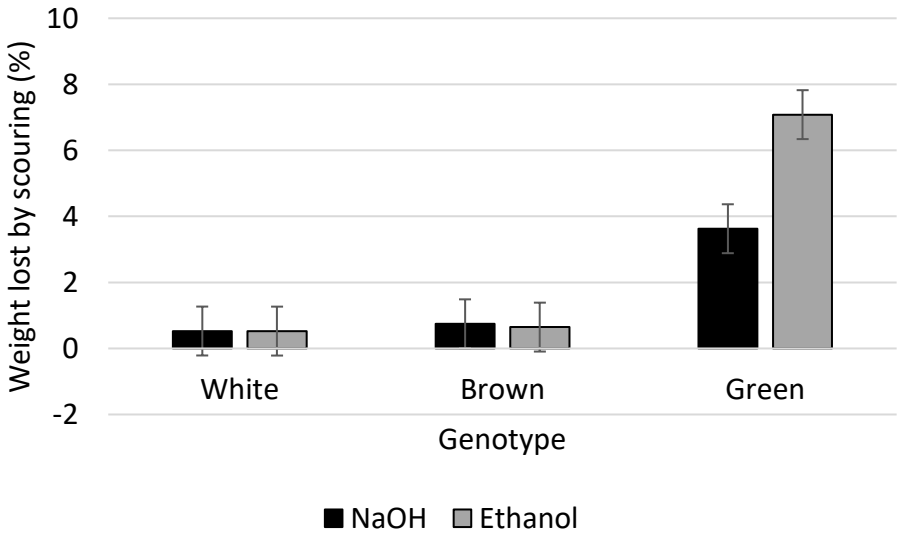
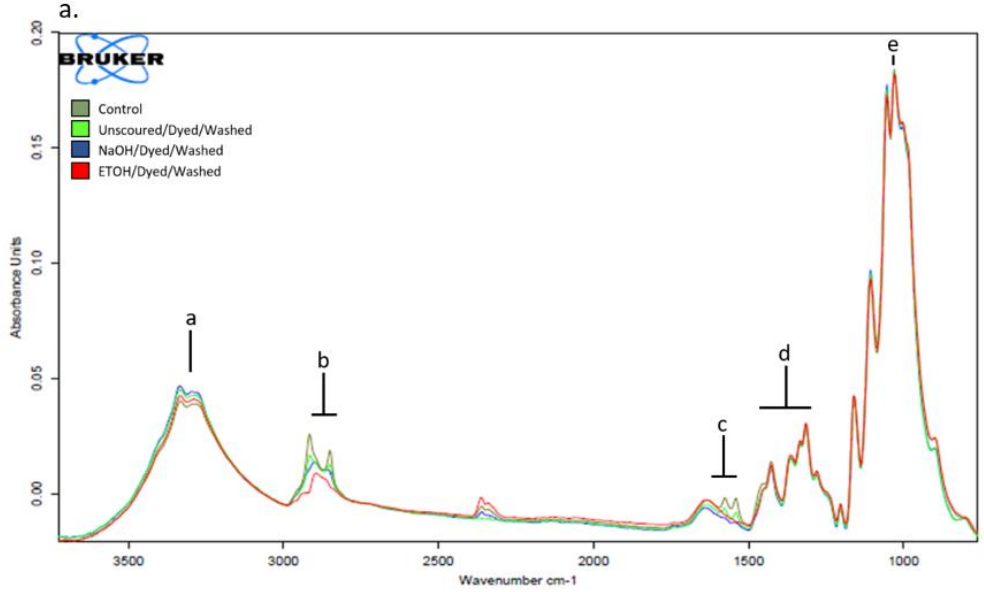
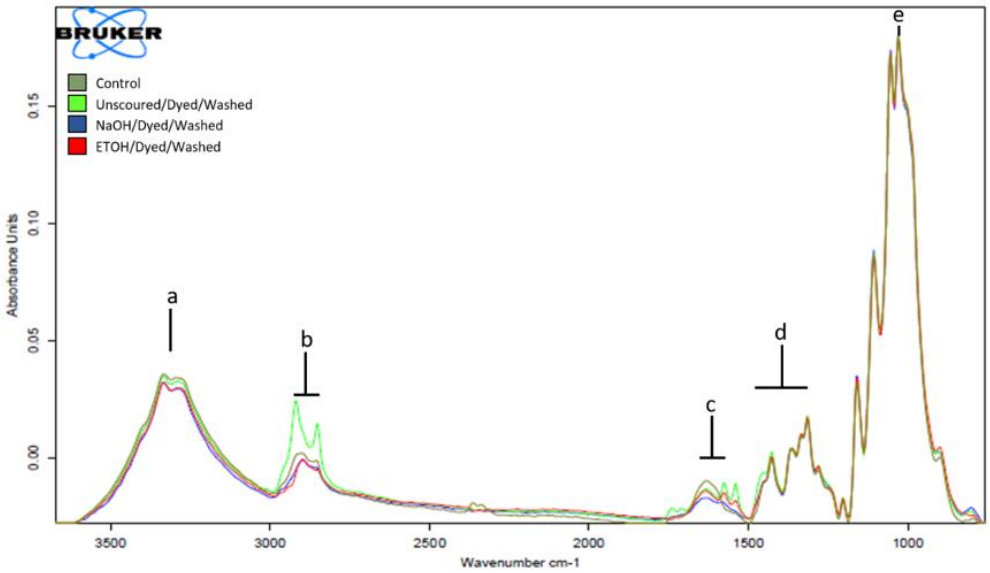


Figure 5.3. Comparison of scouring efficiency on fabrics using traditional NaOH and hot ETOH scouring. Total percentage of fabric weight lost by scouring using either traditional caustic sodium hydroxide (NaOH) scouring or hot ethanol (ETOH) scouring. Average values reported with error bars representing an L.S.D of 5%.

a.


BRUKER
Control
Unscoured/Dyed/Washed
NaOH/Dyed/Washed
ETOH/Dyed/Washed
Absorbance Units
Wavenumber cm-1
a
b
c
d
e

b.


BRUKER
Control
Unscoured/Dyed/Washed
NaOH/Dyed/Washed
ETOH/Dyed/Washed
Absorbance Units
Wavenumber cm-1
a
b
c
d
e

c.

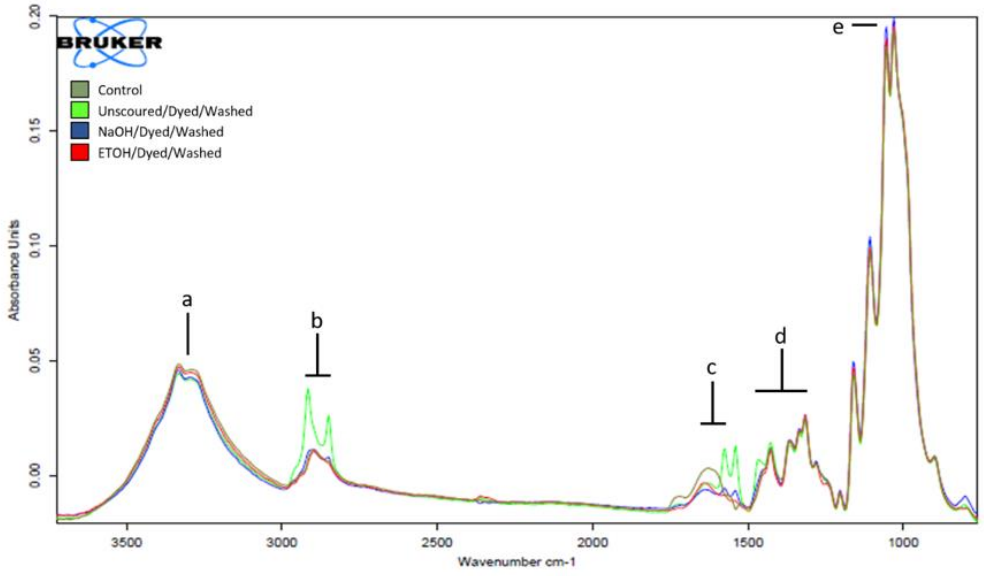

BRUKER
Control
Unscoured/Dyed/Washed
NaOH/Dyed/Washed
ETOH/Dyed/Washed
Absorbance Units
Wavenumber cm-1
a
b
c
d
e

Figure 5.4. FTIR analysis of ground white cotton fabrics. FTIR analysis of (a) white cotton fabrics, (b) brown cotton fabrics, and (c) green cotton fabrics showing spectra from 1000-3500 cm^{-1} used to identify the wax content of the fabrics. Individual peaks labelled at approximately 3000 cm^{-1} for (a) alkynes, 2850 cm^{-1} for (b) alkanes, 1640-1680 cm^{-1} for (c) alkenes, 1350-1470 cm^{-1} for (d) alkanes, and 1000 cm^{-1} for (e) alkenes.

unscoured dyed fabric showing greater peak heights compared with the NaOH and ETOH scoured and dyed fabrics. Similarly, we saw increased relative abundance of Alkenes at 1640-1680 cm^{-1} (Figure 5.4a, c) in both the raw control and the unscoured dyed fabric compared with both the NaOH and ETOH scoured fibres.

In the brown fabric there were no observable differences in either the alkenes at 1000 cm^{-1} (Figure 5.4b, e) and the alkanes at 1350 cm^{-1} (Figure 5.4b, d) between treatments however increased relative abundance of alkanes were observed at 2850 (Figure 5.4b, b) for both the raw control and unscoured and dyed fabrics when compared with both the NaOH scoured and ETOH scoured fabrics with the greatest relative abundance measured in the unscoured and dyed brown fabric. Unlike the white there were slight increases in the relative abundances of alkynes at 3300 cm^{-1} (Figure 5.4b, a) in both the raw control and the unscoured and dyed brown fabrics when compared with the NaOH and ETOH scoured fabrics. Again, like the white fabric increased relative abundances of alkenes at 1640-1680 cm^{-1} (Figure 5.4b, d) were found in both the raw control and the unscoured and dyed fabrics compared with both the NaOH and ETOH scoured and dyed fabrics.

In the green fabric there were no observable differences between the treatments for either the alkanes at 3300 cm^{-1} (Figure 5.4c, a) or the alkenes at 1000 cm^{-1} (Figure 5.4c, e). Differences in the amount of alkanes at 2850 cm^{-1} (Figure 5.4c, b) were measures in the unscoured and dyed fabric when compared to each of the other 3 treatments. An increased relative abundance of alkanes at 1350-1470 cm^{-1} (Figure 5.4c, d) were also measured for both the raw control and the unscoured and dyed fabrics when compared with both the NaOH and ETOH scoured and dyed samples with the greatest relative abundance observed in the unscoured and dyed fabric.

When comparing the different fabric types, there were observable differences in the relative abundance of alkanes at 2850 cm^{-1} (Figure 5.4a-c, b) in both the brown and green unscoured and dyed fabrics which had greater proportional amounts when compared to the white with the greatest relative abundance shown in the green fabrics. No other observable differences were seen at the 2850 cm^{-1} alkanes for any other treatments.

There were also no observable differences in the relative abundance of alkanes at 1350-1470 cm^{-1} (Figures 5.4a-c, d) between any of the fabric types. When comparing the relative abundance of alkenes at 1000 cm^{-1} (Figures 5.4a-c, e) the highest concentration was seen in the green fabric for all four treatments followed by the brown and the white. There were no observable differences in total relative abundance of alkynes at 3300 cm^{-1} (Figures 5.4a-c, a) between any of the fabric types. There was a slightly increased relative abundance of alkenes at 1640-1680 cm^{-1} (Figures 5.4a-c, c) in the green fabrics when compared to the white and brown for which there were no observable differences.

5.3.6 Measurement of dye fastness of dyed fabrics treated using varying scouring methods.

Visual assessment of colour change

Scouring treatment alone did not appear to change the colour of the white fabric. Following dyeing, the ethanol scoured and dyed fabric appeared to be different from the unscoured and NaOH scoured fabrics with more of a green hue apparent. Following the post-dye commercial wash treatment, no obvious colour differences were apparent (Figure 5.5).

Scouring treatment alone appeared to cause a slight colour change in the brown fabrics with less red tinge apparent following both scouring treatments. There were no obvious

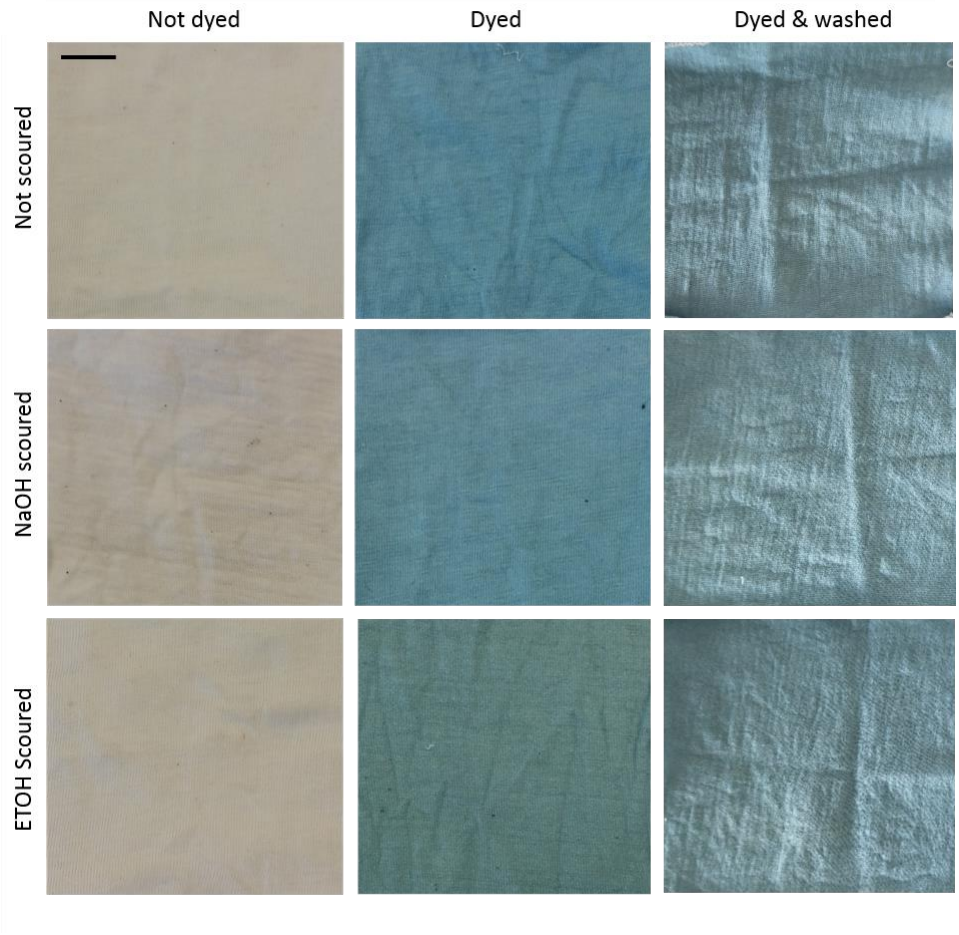


Figure 5.5. Comparison of Fabrics made using *G. hirsutum* Upland Standard II white cotton pre and post scouring and dyeing. Photos show raw white fabrics, fabrics following scouring and dyeing treatment and fabric after the post dye wash Scale bar = 1cm

differences in colour between the unscoured and the ethanol scoured brown fabrics following dyeing however the NaOH scoured fabric appeared to look different from the unscoured and ethanol scoured fabric following dyeing. The same was true following a post-dyeing commercial wash treatment (Figure 5.6).

In the green fabric, both scouring treatments appeared to cause a change in colour compared with the unscoured fabric with a greater change in colour seen following NaOH scouring. The same was true following dyeing. After the post-dye commercial washing there were apparent differences in colour between all three fabrics however the ethanol scoured fabric appeared to have retained the most dye (Figure 5.7)

Spectroscopic analysis of colour change

Spectroscopic analysis of the colour change of white fabrics (DEcmc) showed that the type of scouring treatment did not have an observable effect (DEcmc >1.0) on the colour of fabric following dyeing where changes were measured for the unscoured (20.45 ± 0.29), NaOH scoured (21.07 ±0.33), and ethanol scoured (20.66 ± 0.28) fabrics compared to the raw fabric (Table 5.7).

The colour change of white fabrics showed that scouring treatment did not have an observable effect on the final fabric colour of the white fabrics following a post-dyeing commercial wash treatment where changes were measured for the unscoured (19.86 ± 0.60), NaOH scoured (19.68 ± 0.20), and ethanol scoured (19.79 ± 0.10) fabrics following dyeing and washing compared to the raw fabric (Table 5.7). For all three scouring treatments a colour change of less than 1 was seen as a result of the commercial wash.

Figure 5.6. Comparison of Fabrics made using *G. hirsutum* var. Brown cotton pre and post scouring and dyeing. Photos show raw brown fabrics, fabrics following scouring and dyeing treatment and fabric after the post dye wash Scale bar = 1cm

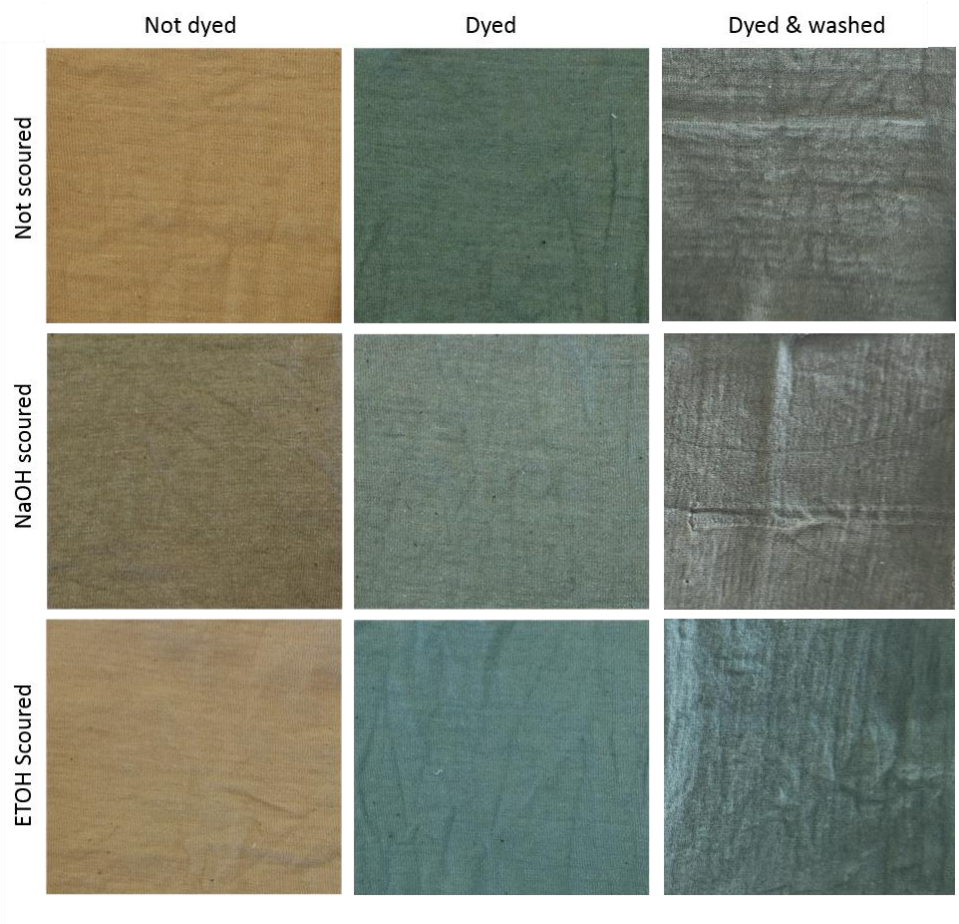


Figure 5.7. Comparison of Fabrics made using *G. hirsutum* var. Green cotton pre and post scouring and dyeing. Photos show raw green fabrics, fabrics following scouring and dyeing treatment and fabric after the post dye wash Scale bar = 1cm

Table 5.7. Spectroscopic analysis of colour change (DEcmc) of fabrics made from *G. hirsutum* white fibre following, scouring, washing and dye treatments. Average DEcmc values reported ± standard deviation. Values greater than 1.0 indicate observable differences discernable by the human eye. Raw = unscoured fabric, NaOH= sodium hydroxide, ETOH = ethanol, S = scoured, D = dyed, W = post-dye washing.

	Raw	**NaOH-S**	**ETOH-S**	**Raw D**	**NaOH-S D**	**ETOH-S D**	**Raw D W**	**NaOH-S D W**	**ETOH-S D W**
Raw	0								
NaOH-S	0.12 ± 0.08	0							
ETOH-S	0.24 ± 0.11	1.92 ± 0.30	0						
Raw D	20.45± 0.29	-	-	0					
NaOH-S D	21.07 ± 0.33	20.81 ± 0.33	-	0.62 ± 0.34	0				
ETOH-S D	20.66 ± 0.28	-	20.53 ± 0.22	0.48 ± 0.13	0.50 ±0.49	0			
Raw D W	19.86 ± 0.60	-	-	0.01± 0.03	-	-	0		
NaOH-S D W	19.68 ± 0.20	19.57 ±0.45	-	-	0.11 ±0.48	-	0.45 ± 0.31	0	
ETOH-S D W	19.79 ± 0.10	-	19.80 ±0.10	-	-	0.01 ± 0.04	0.48 ± 0.31	0.19 ± 0.23	0

Following dye and wash treatments a hue shift from yellow to blue and an increase in the intensity of darkness of colour for all three treatments was observed (Figure 5.8).

Spectroscopic analysis of the colour change of brown fabrics showed that traditional NaOH scouring had an observable effect (DEcmc >1) on the colour of fabric following dyeing whilst ethanol scouring did not. Changes were measured post dyeing for the unscoured (4.78 ± 0.31), NaOH scoured (5.34 ± 0.31), and ethanol scoured (5.34 ± 0.11) fabrics compared to the raw fabric (Table 5.8). Following the post-dyeing commercial wash treatment to assess colour fastness, spectroscopic analysis of the colour change of brown fabrics showed that there was no observable difference between final fabric colours of the unscoured and the ethanol scoured and dyed fabrics following wash treatment, however an observable difference was measured for the NaOH scoured fabrics compared to the other treatments. DEcmc values were measured for the unscoured (5.83 ± 0.25), NaOH scoured (7.52 ± 1.06), and ethanol scoured (5.62 ± 0.16) fabrics following dyeing and washing respectively compared to the raw fabric (Table 5.8). For both the NaOH and ethanol scoured fabrics a colour change of less than 1 was measured as the result of the commercial wash, however a change of 1.94 ± 3.12 was seen for the unscoured fabric indicating that more dye had been washed off compared with the other fabric treatments. Following dye and wash treatments a hue shift from yellow/red towards yellow/green hue and an increase in the intensity of colour for all three treatments was observed although there was greater difference in the final colour result of the NaOH scoured fabrics (Figure 5.9). The total change in intensity of colour was less than the change found for both the white and green fabrics.

Spectroscopic analysis of the colour change of green fabrics showed that both traditional NaOH scouring and ethanol scouring had an observable effect on the colour of fabric

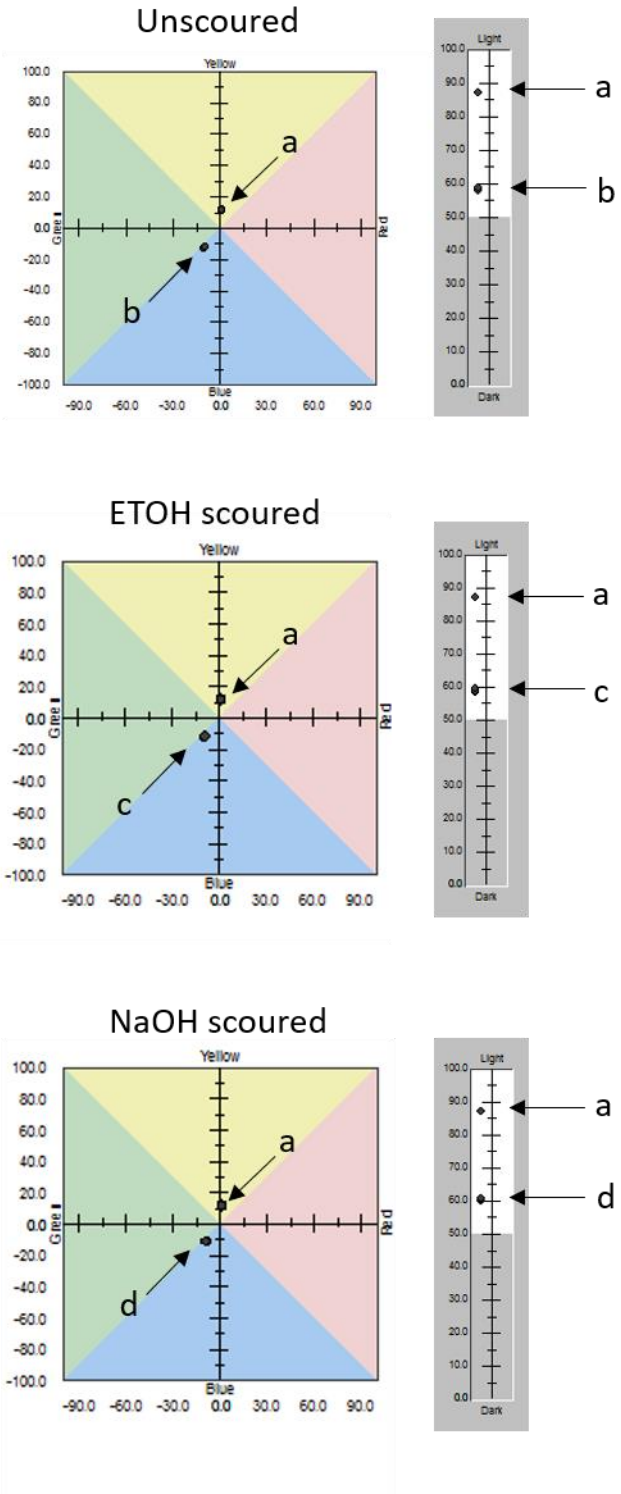


Figure 5.8. CIELAB Spectral plots demonstrating hue differences and colour intensity for white (Upland Standard II) fabrics. Figure shows the colour space spectral plots (left) and the intensity of colour from dark to light (right) before and after dyeing for (a) Raw control fabric, (b) Unscoured, dyed and washed fabric, (c) Ethanol (ETOH) scoured, dyed and washed fabric, (d) Sodium Hydroxide (NaOH) scoured, dyed and washed fabric. For all treatments n=3

Table 5.8. Spectroscopic analysis of colour change (DEcmc) of fabrics made from *G. hirsutum* brown fibre following, scouring, washing and dye treatments. Average DEcmc values reported ± standard deviation. Values greater than 1.0 indicate observable differences discernable by the human eye. Raw = unscoured fabric, NaOH= sodium hydroxide, ETOH = ethanol, S = scoured, D = dyed, W = post-dye washing.

	Raw	**NaOH-S**	**ETOH-S**	**Raw D**	**NaOH-S D**	**ETOH-S D**	**Raw D W**	**NaOH-S D W**	**ETOH-S D W**
Raw	0								
NaOH-S	5.81 ± 0.36	0							
ETOH-S	0.30 ± 0.42	5.81 ± 0.58	0						
Raw D	5.14 ± 0.28	-	-	0					
NaOH-S D	4.78± 0.31	17.16 ± 0.33	-	0.37 ± 0.58	0				
ETOH-S D	5.34± 0.11	-	5.37 ± 0.15	0.20 ± 0.33	0.56 ± 0.26	0			
Raw D W	5.83 ± 0.25	-	-	1.94 ± 3.12	-	-	0		
NaOH-S D W	7.52 ± 1.06	7.50 ±1.02	-	-	0.29 ± 1.30	-	1.69 ± 1.22	0	
ETOH-S D W	5.62 ± 0.16	-	5.59 ± 0.18	-	-	0.02 ± 0.03	0.2 ± 0.31	1.98 ± 1.98	0

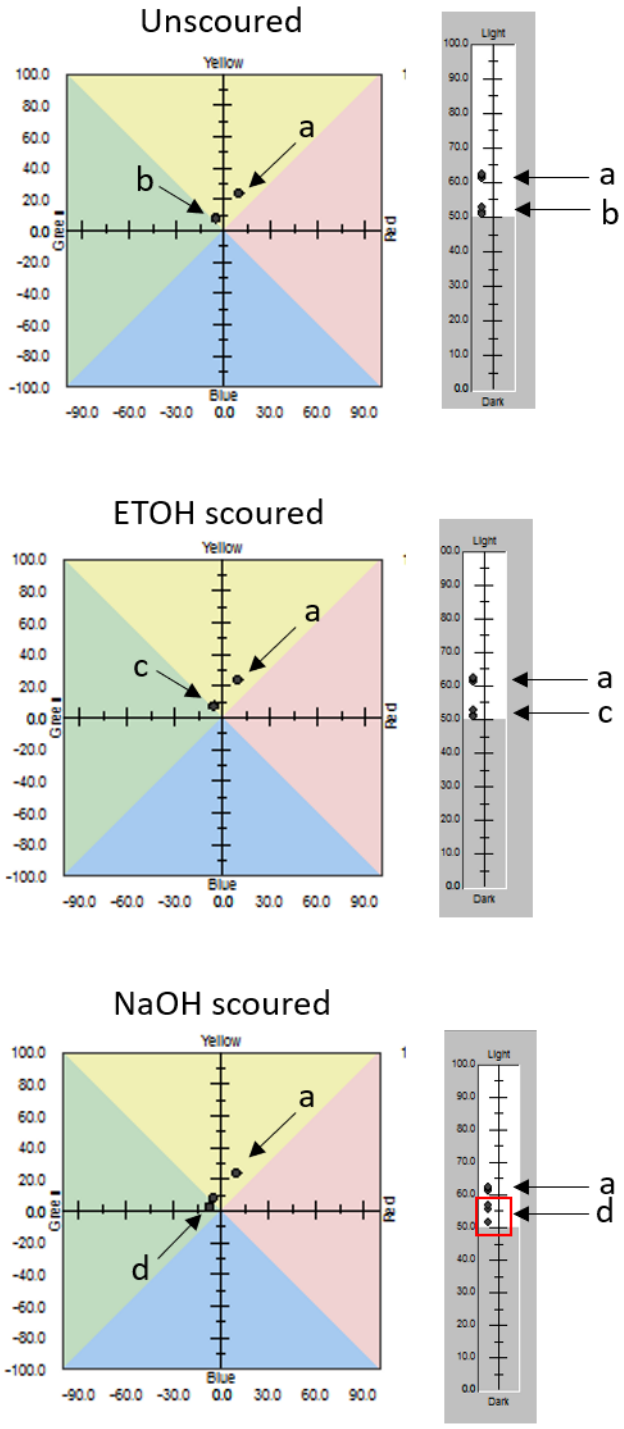


Figure 5.9. CIELAB Spectral plots demonstrating hue differences for naturally coloured brown cotton fabrics. Figure shows the colour space spectral plots (left) and the intensity of colour from dark to light (right) before and after dyeing for (a) Raw control fabric, (b) Unscoured, dyed and washed fabric, (c) Ethanol (ETOH) scoured, dyed and washed fabric, (d) Sodium Hydroxide (NaOH) scoured, dyed and washed fabric. For all treatments n=3

following dyeing with a greater colour change seen for the ethanol scoured fabric. There was no observable difference in the unscoured fabric following dyeing. Decmc values were measured for the unscoured (0.51 ± 0.17), NaOH scoured (3.53 ± 0.25), and ethanol scoured (8.60 ± 0.77) fabrics respectively compared to the raw fabric (Table 5.9). Following the post-dyeing commercial wash treatment to assess colour fastness, spectroscopic analysis of the colour change of green fabrics showed that there was observable differences between the final fabric colours of all three fabric treatments. DEcmc values were measured for the unscoured (2.51 ± 0.18), NaOH scoured (6.20 ± 1.02), and ethanol scoured (9.40 ± 0.68) fabrics following dyeing and washing respectively compared to the raw fabric (Table 5.9). For both the NaOH and ethanol scoured fabrics a colour change of less than 1 was seen as a result of the commercial wash, however a change of 2.54 ± 0.12 was seen for the unscoured fabric indicating that more dye had been washed off the unscoured fabric compared with the other fabric treatments. Following dye and wash treatments a hue shift from close to the central yellow axis towards green hues and an increased intensity of darkness of colour for all three treatments was observed although there was a greater degree of colour shift and a greater difference in the intensity of colour seen for the ethanol scoured syed and washed fabric (Figure 5.10).

Table 5.9. Spectroscopic analysis of colour change (DEcmc) of fabrics made from *G. hirsutum* green fibre following, scouring, washing and dye treatments. Average DEcmc values reported ± standard deviation. Values greater than 1.0 indicate observable differences discernable by the human eye. Raw = unscoured fabric, NaOH= sodium hydroxide, ETOH = ethanol, S = scoured, D = dyed, W = post-dye washing.

	Raw	**NaOH-S**	**ETOH-S**	**Raw D**	**NaOH-S D**	**ETOH-S D**	**Raw D W**	**NaOH-S D W**	**ETOH-S D W**
Raw	0								
NaOH-S	0.38 ± 0.08	0							
ETOH-S	2.71 ± 0.40	7.14 ± 0.70	0						
Raw D	0.51 ± 0.17	-	-	0					
NaOH-S D	3.53 ± 0.25	3.60 ± 0.19	-	2.96 ± 0.39	0				
ETOH-S D	8.60 ± 0.77	-	8.63 ± 0.95	8.03 ± 0.81	5.07 ± 0.75	0			
Raw D W	2.51 ± 0.18	-	-	2.54 ± 0.12	-	-	0		
NaOH-S D W	6.20 ± 1.02	6.30 ± 1.26	-	-	0.7 ± 0.06	-	3.74 ± 1.15	0	
ETOH-S D W	9.40 ± 0.68	-	9.38 ± 0.69	-	-	0.01 ± 0.07	6.94 ± 0.83	3.2 ± 0.38	0

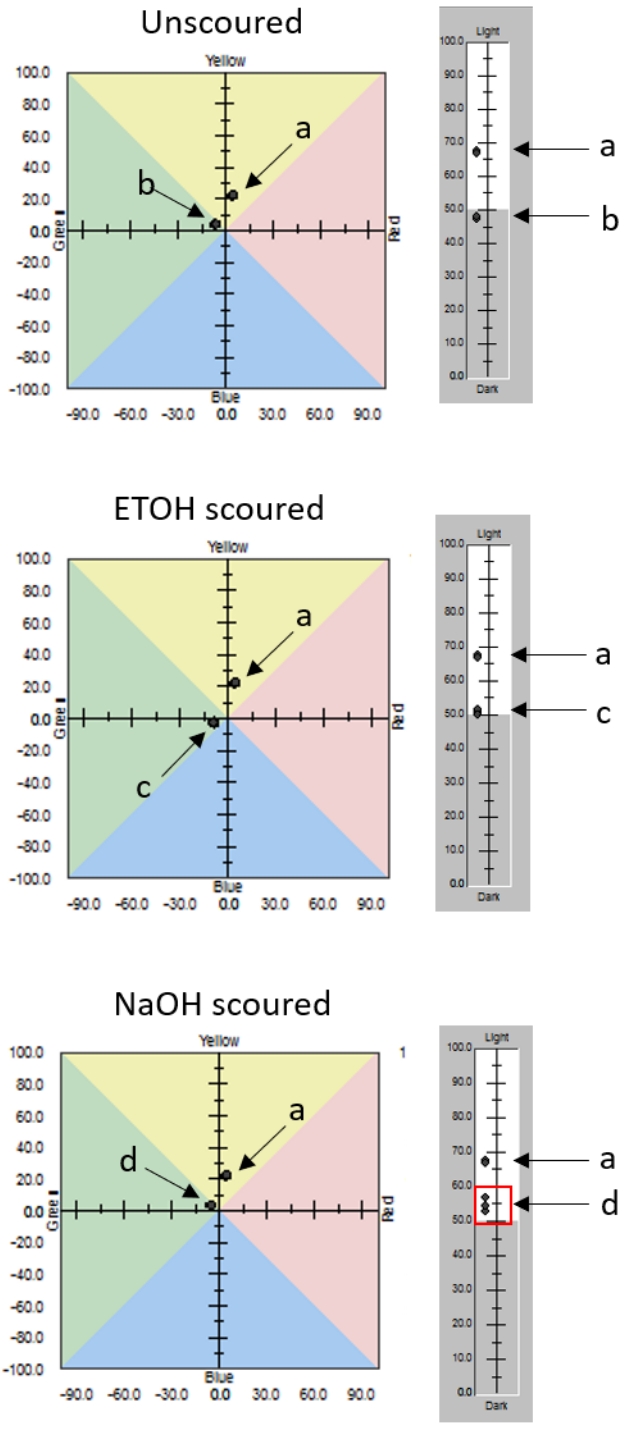


Figure 5.10. CIELAB Spectral plots demonstrating hue differences for naturally coloured green cotton fabrics. Figure shows the colour space spectral plots (left) and the intensity of colour from dark to light (right) before and after dyeing for (a) Raw control fabric, (b) Unscoured, dyed and washed fabric, (c) Ethanol (ETOH) scoured, dyed and washed fabric, (d) Sodium Hydroxide (NaOH) scoured, dyed and washed fabric. For all treatments n=3

5.4 Discussion

Fabrics made from three different naturally coloured upland cottons were scoured using either traditional NaOH scouring or ethanol scouring, then dyed and washed, to determine the effect of varying scouring methods on the dyeability and colourfastness of fabrics made from cottons with varying cuticular wax content. Although the dyeability and colour fastness of commercial white cottons has been established to my knowledge there have been no published studies investigating the efficacy of traditional scouring on naturally high wax cottons. It was hypothesised that following dyeing, fabrics that were not scoured would have inferior colour fastness following a standard fabric wash test and the effect was expected to be more prominent for fabric made from the higher wax content coloured cottons. Although dyeability varied between fabrics, likely due to alkane wax component of the total wax, NaOH adequately disrupted the hydrophobic fibre cuticle allowing for colourfast dyeing regardless of the wax content of the fabrics.

5.4.1 For high wax cottons, ethanol scouring was more efficient at removing the total cuticle component of the fibre compared with traditional NaOH scouring method.

Traditional NaOH scouring method has long been the accepted practice for scouring of fabrics made from white cottons, whilst fabrics made from naturally coloured genotypes typically are not scoured (Murthy, 2001). This is due to the natural pigment of the fibre which makes them an attractive choice for fabrics manufacturing because they do not require dyeing and therefore do not require scouring to remove the hydrophobic waxy cuticle (Murthy, 2001, Tang et al., 2013). Because naturally coloured cottons are not typically scoured and dyed, the efficiency of traditional scouring on naturally coloured cottons as it refers to dyeability has not been studied,

however the range of wax present on naturally coloured genotypes makes them a good model for studying the effect of increased wax on the efficiency of the traditional NaOH scouring method.

The increased amount of total fibre cuticle on both the naturally coloured brown and green fibre in comparison to the white was in line with current literature (Conrad, 1944, Pan et al., 2010). Despite the higher overall total fibre cuticle , the wax portion of cuticle on the brown fabric which is typically 1-2% of the total fibre weight in comparison to the 0.4-0.7% found on white fabrics was not significantly larger than the white, however the increased wax content of the green fibre (7.88%) was consistent with previously reported studies (Conrad, 1944, Pan et al., 2010).

Here a comparison of traditional scouring and hot ethanol scouring using the Conrad method (Conrad, 1944) showed that for high wax green genotypes NaOH scouring removed significantly less total cuticle material (51% less removed) (Figure 5.3) compared to the hot ethanol scouring method, which indicates large amounts of hydrophobic material are being left behind on the fibre using traditional methods which could have a significant effect on the dyeability of the fabric. The removal of the wax component of the cuticle is a crucial key factor for the successful scouring of cotton fibre (Agrawal et al., 2007). The Conrad method has been shown to efficiently remove the total wax component of fibre after 6hrs regardless of the amount of wax present, although approximately 70% of the total wax component is removed after the first hour of scouring (Conrad, 1944). Whilst traditional NaOH scouring is a much faster process, typically around one hour of scouring time is used to prepare fibre for dyeing, it appears to be sufficient for low wax fabrics but not for high wax fabrics. The ability to remove sufficient wax is especially important

considering the results in Chapter 3 where it was shown that increased abiotic stress led to significantly increased wax deposition on (white) fibre as high 1.9 times that of the control.

The significantly higher ability of the ethanol scouring method to remove total cuticle on the higher wax green variety compared with traditional scouring methods is an indication of the potential for future problems with the NaOH scouring method if the wax content of commercially grown fibres increase due to abiotic stress possibly related to climate change.

5.4.2 Wax content influences the dye-uptake of cotton fabrics.

There were no apparent differences in dye uptake for either scouring method for the white cottons which is likely due to its relatively small cuticle component (Conrad, 1941). Traditional NaOH scouring method resulted in the greatest dye uptake for the brown fabric which had which had significantly more total fibre cuticle than the white but no difference in total wax indicating that cuticular components other than wax were responsible for the difference. For the naturally high wax green fabric, ethanol scouring resulted in the greatest dye uptake. These differences indicate that the wax may not be the only influencing factor in dye uptake as NaOH scouring and ethanol scouring target different cuticle components. The presence of suberin in green fibre, a wax found in the cuticle layer and also between the cellulose layers of the secondary cell wall, that is not present in white or brown fibre may also be responsible for the differing scour results due to its polymeric nature (Richards et al., 1999, Elesini et al., 2002) which, like the alkane waxes also present, makes it more difficult to scour (Church and Woodhead, 2006).This however does highlight the importance of removing the waxy cuticle before dyeing when wax concentrations are high and also showed that for higher wax genotypes, ethanol scouring is more efficient than

traditional scouring. The importance of disrupting the wax portion of the cuticle has been adequately reviewed and it is commonly acknowledged that scouring is required prior to fabric dyeing (Gordon and Hsieh, 2006, Lewis, 2011a), however to my knowledge no work has been done to determine the effectiveness of scouring on naturally coloured cottons which may have altered total fibre cuticle and are known to have higher wax content compared with commercial white cottons. Further investigation into the differing cuticle makeup and the influence this has on scourability is warranted.

5.4.3 Regardless of wax content in the different genotypes, for all fabrics, NaOH adequately disrupted the hydrophobic fibre cuticle allowing for colourfast dyeing.

Although differences in the amount of dye uptake were observed following the application of varying scouring techniques, there were no observable differences (DEcmc > 1) in the colour of the fabrics following the post-dye washing compared to the pre-wash fabric colour. This indicates that NaOH scouring was able to adequately disrupt the cuticle resulting in colour fast dyeing regardless of the wax or total cuticle component of the fabric. Although it did not remove as much total cuticle component on the higher wax fabrics, this traditional NaOH scouring method was able to remove or disrupt the cuticle to a great enough degree that no observable differences in colour fastness were measured between high and low wax fabrics.

5.4.3 Alkane waxes influence the dyeability of cotton fabrics.

FTIR analysis of fabrics identified the presence of Alkanes, alkenes and alkynes (Figure 5.4, 5.5, and 5.6) this is consistent with the literature where alkanes, alkenes and alkynes have been shown to be a major component of fibre cuticular wax (Hartzell-Lawson and Hsieh, 2000) (Chung

et al., 2004, Church and Woodhead, 2006). The highest proportion of these waxes for each of the fabric types were the long chain alkenes followed by the alkanes. Scouring appeared to have little effect on the alkenes but caused a reduction in the amount of alkanes present on the fabric with ETOH unsurprisingly being the most efficient method of reducing the alkanes. There were observable differences in the amount of alkanes present on the raw fibres (Figure 5.4, 5.5, and 5.6) with more alkane waxes present on the coloured fibres compared to the white. Interestingly despite not having significantly different total wax concentrations, there were more alkane waxes present on the brown fibre compared with the white. The highest proportion of alkane waxes was seen on the green fibre which is unsurprising given it had a significantly greater total wax content.

The differences in the dyeability of the fabrics was related to the amount of alkane waxes found on the fibre and this is consistent with the results of previous studies involving high-performance thin-layer chromatography (HPLC) studies of white cottons (Church and Woodhead, 2006). When scoured with ethanol we saw the greatest colour change after dyeing and the smallest colour change following the post dye washing indicating the fabric was best prepared for dyeing following ETOH scouring and was best able to retain the dye. This indicates that for both the raw and NaOH scoured fabrics, some of the colour change measured after dyeing may be due to dye that was simply sitting on the surface of the fibre that had not penetrated the fibre and was able to be washed off. The results show that fabrics with higher remaining alkane waxes were not dyed as well as those with the alkane waxes removed to a greater degree. This supports the hypothesis that increased concentrations of alkane waxes may be influencing the dyeability of fibre.

5.4.4 Scouring of low wax cottons may not be necessary

Interestingly in the white cotton which has a low percentage of wax in the cuticle when compared to the naturally coloured fibres, no significant difference in the end dye results was seen between any of the treatments indicating that in some cases, scouring may not be necessary for adequate dyeing. This result was unexpected due to the current understanding of the hydrophobic nature of the waxy cuticle and its effect on dye penetration (Chung et al., 2004, Agrawal et al., 2007, Lewis, 2011b, Lewis, 2011a). It may be that because of the low wax percentage measured for the white fibre (0.55%) adequate wax removal occurred simply due to the dye process itself which allowed dye to penetrate the cellulose. The dyeing process involved holding the fabrics in the dye liquor which was at a temperature of 80°C. The melting point of the majority of the wax components known to be on cotton fibre have been shown to be around 80°C (Agrawal et al., 2007) so it is possible that this high temperature liquid aided in melting and removal of the waxes on the cotton fibre. It also may be due to the use of a dark navy coloured dye which has a greater colour difference compared to white fibre when compared to lighter coloured dyes such as yellows meaning even a small amount of dye uptake provides for intense colour on the fabrics. Further work with differing concentrations of dyes may show differences in dye uptake in the raw fibres but at a commercially acceptable dye concentration scouring had no effect on the end colour result in the white fibres.

5.4.5 Concluding remarks

Increased temperature has been shown to cause increased wax deposition on fibre (Chapter 3) and this high wax impacts on the ability to dye fabrics. Despite the risk of increased wax deposition on commercial cottons from increased abiotic stress because of climate change, it

appears that the traditional caustic NaOH scouring method will remain an adequate method for disrupting the wax layer to allow for dyeing. There are indications that cuticle components other than cuticular waxes may be influencing dyeability and further research is warranted.

Chapter 6. The effect of scouring on the polysaccharide content of three naturally coloured cottons and the resulting impact on dyeability and colour fastness.

Abstract

The novel assessment of cotton polysaccharide content by GC-MS of three fabrics made using one commercial white cotton genotype and two naturally coloured cotton genotypes enabled a different perspective to be obtained on the dyeability of cottons with different cuticle makeup compared to standard commercial cotton. Following the work in the previous chapter where fabrics were scoured using either the traditional caustic NaOH method or hot ethanol scouring which more specifically targets the waxes, before being dyed, washed and analysed spectroscopically to determine dyeability and colour fastness, fabrics were recovered for analysis of their polysaccharide content. Green fabric had been shown to have significantly greater wax content than either the white or brown between which no significant differences were measured. Differences in polysaccharide content were measured between all three raw fabrics with increased concentration of non-cellulosic polysaccharides found on the two naturally coloured cottons compared to the white with the greatest concentration of each measured non-cellulosic polysaccharide found on the green fabrics. There was also a disproportionately higher concentration of pectin on the green fabric compared to the white and brown. It was shown that despite the differences in overall concentration, NaOH scouring was able to remove non-cellulosic polysaccharides on naturally coloured cottons to a similar degree as on commercial white cottons, and as expected the ethanol scouring was less efficient at removing non-cellulosic

polysaccharides. The impact of non-cellulosic polysaccharides present on cotton on dyeability was less than that of the waxes present.

6.1 Introduction.

In addition to the cuticular waxes present on cotton fibre which are known to influence fabric dyeability, there are other non-cellulosic fibre components including hemicelluloses and pectins (Caffall and Mohnen, 2009, Broxterman and Schols, 2018). Hemicelluloses are a class of polymers which include heteroxylan, xyloglucan, heteromannan, Type I and II arabinogalactan and arabinan (Caffal and Mohnen, 2009). Pectins are complex polysaccharides that include homogalactonuran (HG), xylogalacturonan (XGA), apiogalacturonan (AGA), rhamnogalacturonan II (RG-II) and rhamnogalacturonan I (RG-I) (Caffal and Mohnen, 2009). The most abundant non-cellulosic polysaccharides in the primary cell wall of cotton fibre are the pectic polysaccharides (Caffal and Mohnen, 2009). Pectins are hydrophobic molecules embedded primarily in the epicuticular layer of the fibre and in the outer cuticle (Wakelyn et al., 2006). Waxes and pectins have been shown to be most responsible for the hydrophobicity of raw cotton fibres (Hartzell-Lawson and Hsieh, 2000). In the previous chapter it was shown that when dyeing cotton fabrics made from naturally coloured cottons with varying wax content both ethanol scouring and traditional NaOH scouring were adequate when assessing the colour fastness of the dyed fabrics using a standard commercial wash test. This was true regardless of the amount of wax present on the fabrics. There were however differences in the dyeability between the different fabrics following the different scouring techniques prior to this washing. In that study NaOH scouring lead to greater dye uptake by naturally coloured brown fabrics whilst ethanol scouring resulted

in the greatest dye uptake on the naturally coloured green fabrics. No differences in dye uptake were found for either scouring method on the low wax commercial white cotton fabrics.

Using recovered fabrics from the experimental work in chapter 5, assessment was undertaken of the ability of traditional NaOH scouring and hot ethanol scouring to remove non-cellulosic polysaccharides from fabrics made from three naturally coloured cotton genotypes with varying cuticular components. Further, the impact these non-cellulosic polysaccharides had on fabric dyeability was evaluated. It was hypothesised that non-cellulosic polysaccharides present in the fibre cuticle, and in particular pectins, would negatively affect dye uptake and therefore fabric dyeability and were responsible for the differences in dyeability measured between the naturally coloured cotton fabrics.

6.2 Materials and methods.

6.2.1 Experimental cotton bales

Three bales of *Gossypium hirsutum* upland cotton were sourced from the CSIRO cotton processing facility and a commercial farm (Bidstrupp farming) and used to produce the fabrics described in chapter 5. Bales had been produced by Australian commercial growers. One bale was a standard high yielding common commercial white cultivar. The other two were less common naturally coloured upland cultivars, one brown and one green.

6.2.3 Fabric experiments

Experimental fabrics were generated and treated then analysed for wax content, dyeability and colour fastness as per chapter 5 before being recovered for analysis of the polysaccharide content. Raw untreated fabrics were also recovered for use as the control.

6.2.4 Analysis of the polysaccharide content of fabrics by GC-MS

Experimental fabrics were cut into small pieces and ~100mg ground in 20mL Stainless Steel jars with a 20mm Stainless Steel ball at 30Hz, 60 secs using a Qiagen Tissue-lyser. Cell walls from replicates of each sample were collected as an alcohol insoluble residue (AIR) by extracting the ground material with three successive washes of 70% (v/v) ethanol, one chloroform: methanol (50:50, v: v), one 100% methanol and finally 100% ethanol before air drying. Samples were carboxyl reduced for the detection of uronic acids with 125mg/ml 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide-metho-*p*-toluene sulphonate in MES pH 4.75 for 3h at 28C. 4M imidazole pH 7.0 was added to increase the pH and sodium borodeuteride added to 0.7M and incubated at RT for 3h. Excess reductant was destroyed with acetic acid and polysaccharides recovered by precipitation with 3 volumes of acetone. The precipitate was twice washed with 70% acetone followed by ethanol.

Relative polysaccharide composition was deduced from monosaccharide linkage composition based on partially methylated alditol acetates prepared by methylation, hydrolysis, reduction and acetylation of the pre-reduced fabric alcohol insoluble residue according to Pettolino et al., (2012).

5.2.7 Statistical analysis

Statistical analysis was performed using Microsoft Excel for basic descriptive statistics. GenStat Version 16 (Lawes Agricultural Trust, IACR. Rothamsted, UK) was employed for Analysis of Variance (ANOVA) of data. ANOVA was conducted as a two-way model with genotype and treatment being the two factors. Mean values were reported with preference given to significant

two factor interaction, which was reported in bar chart format. Otherwise mean values for main effects with or without statistical significance were tabled where appropriate. The degree of the statistical significance of the ANOVA tests were indicated using standard star symbol convention, being when P values were either <0.05*, <0.01**, or <0.001***. Least significant difference (LSD) (5%) values were reported alongside significant ANOVA results to assist in mean value separation. The statistical analysis design for the ANOVA can be seen in table 6.1.

Table 6.1. Analysis of Variance Table. This table details the degrees of freedom (d.f.) assigned to each component of the single factor model used to analyse the polysaccharide content data.

Source of variation	Degrees of freedom
Block	2
Genotype	2
Treatment	3
Genotype x Treatment	6
Residual	22
Total	35

6.3 Results.

6.3.1 Relative percentages of cellulose on fabrics before and after scouring treatment.

Of the raw fabrics, the white fabrics had the greatest cellulose content, followed in order by brown and then the green fabric which had the lowest total cellulose content by total weight of the fabric (Figure 6.1). After dyeing and washing alone, there were significant increases in the relative cellulose percentages for the brown and green fabrics but no effect on the white fabrics was measured, indicating the dyeing and washing were removing some remaining cuticle component. Following NaOH scouring, washing and dyeing, the relative percentage of cellulose significantly increased for all three fabrics and again there were no significant differences in the amount of cellulose in the three fabrics following treatment. Ethanol scouring, washing and dyeing caused a significant increase in the relative percentage of cellulose in all three fabric types. The white and brown fabrics remained significantly different in percentage cellulose content from one another following treatment, however the green was no longer significantly different in content to the brown fabric.

6.3.2 Relative percentages of non-cellulosic polysaccharides on fabrics before and after scouring.

Two-way interactions were measured between fabric type (white, brown and green) and treatment for cellulose (Figure 6.1) and all measured non-cellulosic polysaccharides (Figure 6.2) except for callose and heteroxylan for which significant main effects were shown and reported (Table 6.2).

Arabinans

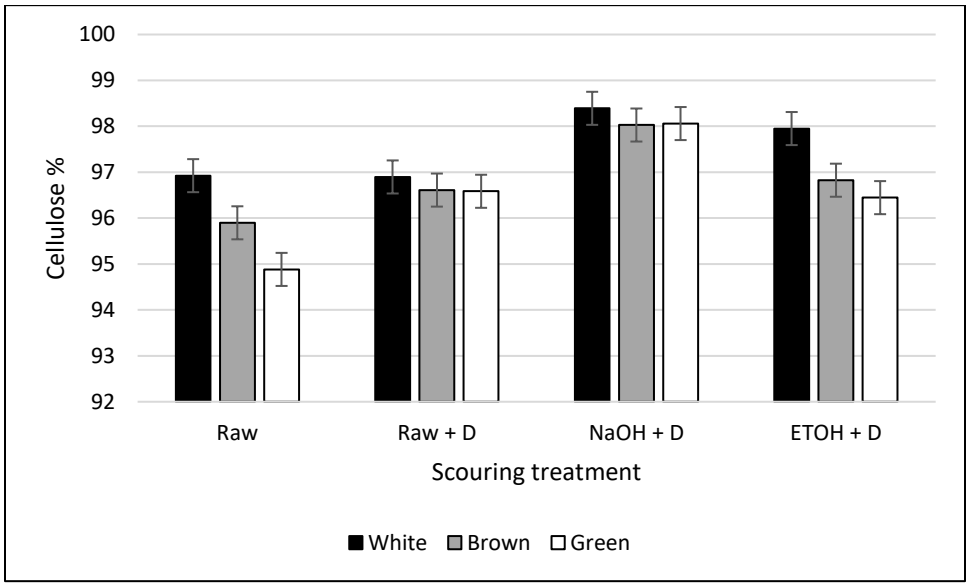


Figure 6.1. Cellulose content of dyed and washed fabrics made from naturally coloured fibre. Graph illustrates the relative percentage of cellulose content in scoured, dyed and washed fabrics. ANOVA of raw values showing two–way interaction of scouring treatment and fabric type. Mean values shown with error bars indicating significance using L.S.D. of 5%. Raw = Untreated fabric; Raw + D = Unscoured, dyed and washed fabrics; NaOH + D = Sodium hydroxide (NaOH) scoured dyed and washed fabrics; ETOH + D = Ethanol (ETOH) scoured, dyed and washed fabrics.

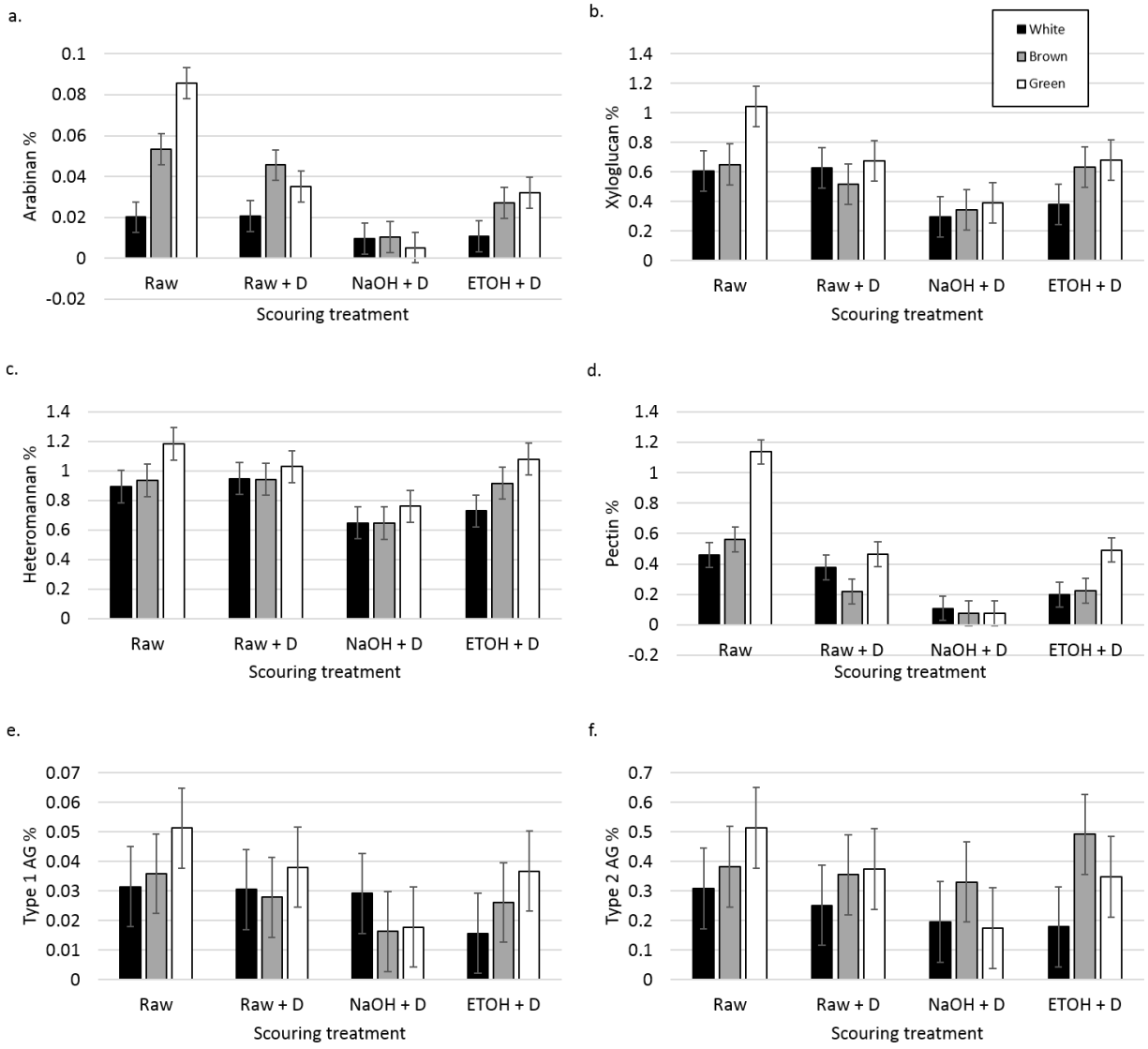


Figure 6.2. Non-cellulosic polysaccharide content of dyed and washed fabrics made from naturally coloured fibre. Graph illustrates the relative percentages of non-cellulosic polysaccharide content in scoured, dyed and washed fabrics. (a) Arabinan, (b) Xyloglucan, (c) Heteromannan, (d) Pectin, (e) Type 1 Arabinogalactan (AG), (f) Type 2 arabinogalactan. ANOVA of raw values showing two–way interaction of scouring treatment and fabric type. Mean values shown with error bars indicating significance using L.S.D. of 5%. Raw = Untreated fabric; Raw + D = Unscoured, dyed and washed fabrics; NaOH + D = Sodium hydroxide (NaOH) scoured dyed and washed fabrics; ETOH + D = Ethanol (ETOH) scoured, dyed and washed fabrics. Fabrics underwent commercial wash for colourfastness following dye treatment.

Table 6.2. Polysaccharide content of fabrics made from naturally coloured fibre. The table illustrates the main effects of fabric type and treatments on the relative percentages of polysaccharides present in fabric for (a and c) callose; and (b and d) heteroxylan. Mean values reported with lower case letters indicating significance using L.S.D of 5%. P values represented where P ≤ 0.05 = *, P ≤ 0.01 = **, P ≤ 0.001 = ***. Raw = Unscoured; Raw + D = Unscoured, dyed and washed fabrics; NaOH + D = Sodium hydroxide (NaOH) scoured dyed and washed fabrics; ETOH + D = Ethanol (ETOH) scoured, dyed and washed fabrics. Fabrics underwent commercial wash for colourfastness following dye treatment. Lettering system indicates numbers that are the same or different where matching letters are assigned to numbers for which no significant difference is measured.

a.

White	Brown	Green	$L.S.D^{P}$
0.228a	0.638b	0.257c	0.144^{***}

b.

Raw	Raw + D	NaOH + D	ETOH + D	$L.S.D^{P}$
0.533a	0.431a	0.212b	0.322b	0.166^{**}

c.

White	Brown	Green	$L.S.D^{P}$
0.210a	0.233a	0.285b	0.032^{***}

d.

Raw	Raw + D	NaOH + D	ETOH + D	$L.S.D^{P}$
0.308a	0.260b	0.168c	0.233b	0.037^{***}

On the raw fabric, significant differences in arabinan concentrations were measured for each of the fabric types (Figure 6.2 a). The highest concentration was measured for the green fabric followed by the brown and then the white which had the lowest concentration. For the unscoured fabrics, dyeing and washing alone did not affect the arabinan concentration on the white and brown fabrics but a significant reduction was found for the green fabrics. NaOH scouring, dying and washing treatment had the greatest impact on arabinan content of any treatment. Here, arabinan concentration was significantly reduced in all three fabrics and no significant differences were seen in the resulting arabinan concentration of the three fabrics. The ethanol scouring, dyeing and washing treatment did not affect arabinan concentration of the white fabric, but significant reductions were measured for both the brown and the green fabrics.

Xyloglucans

On the raw fabric no significant differences in xyloglucan concentration was measured for either the white or brown fabrics, however the green fibre had a significantly higher xyloglucan concentration (Figure 6.2 b). On the unscoured fabrics, dyeing and washing did not affect the xyloglucan concentration on the white and brown fabrics, but a significant reduction was seen for the green fabrics. NaOH scouring, dying and washing treatment had the greatest impact on xyloglucan content of any treatment. Here, xyloglucan concentration was significantly reduced in all three fabrics and no significant differences were seen in the resulting xyloglucan concentration of the three fabrics. Ethanol scouring dyeing and washing treatment did not affect xyloglucan concentration of the white fabric, but significant reductions were measured for both the brown and the green fabrics.

Heteromannans

On the raw fabrics no significant differences in heteromannan concentration were seen for the white and brown fabrics, however the green fabric has a significantly higher concentration (Figure 6.2 c). On the unscoured fabrics, there was no effect of the dyeing and washing alone on heteromannan concentration. NaOH scouring, dyeing and washing treatment led to a significant reduction of heteromannan on all three fabrics. There was also no effect of the ethanol scouring, dyeing and washing treatment on heteromannan concentration for any of the fabrics.

Pectins

No significant differences in pectin concentration on raw fabrics were observed between the white and brown fabrics however the green fabric had a significantly higher pectin concentration (Figure 6.2 d). On the unscoured fabrics, dyeing and washing treatment alone significantly reduced pectin concentration on both the brown and green fabrics but did not have a significant effect on the pectin concentration of the white fabric. NaOH scouring, dying and washing treatment had the greatest impact on pectin content of any treatment. Pectin concentration was significantly reduced in all three fabrics and no significant differences were seen in the resulting pectin concentration of the three fabrics. Ethanol scouring, dyeing and washing treatment also significantly reduced the pectin concentration of all three fabrics although to a lesser degree than the NaOH scouring treatment and the concentration of pectin following treatment remained significantly greater on the green in comparison to the white and brown fabrics.

Type 1 arabinogalactans

No significant differences were found in the concentration of type 1 arabinogalactan (Type 1 AG) of the white and brown fabrics, however the concentration of type 1 AG on the raw green fibre was significantly higher than on the white (Figure 6.2 e). On the unscoured fabrics, there was no effect of dyeing and washing alone on the type 1 AG concentration for any of the fabrics. The NaOH scouring, dyeing and washing treatment significantly reduced the concentration of Type 1 AG present on the brown and green fabrics but did not have an effect on the Type 1 AG concentration of the white fabrics. Ethanol scouring, dyeing and washing treatment significantly reduced the concentration of Type 1 AG on the white fabrics to a greater degree than the reduction seen from the NaOH scouring. Ethanol scouring, dyeing and washing treatment did not have an effect on the Type 1 AG concentration.

Type 2 arabinogalactan

No significant differences were seen in the concentration of type 2 arabinogalactan (Type 2 AG) of the white and brown fabrics, however the concentration of type 2 AG on the raw green fibre was significantly higher than on the white (Figure 6.2 f). There was no significant effect of any treatment on the Type 2 AG content of the white and brown fabrics, however the NaOH scouring, dyeing and washing treatments significantly reduced the Type 2 AG concentration of green fabrics.

Callose

Significant main effects of fabric type and treatment were seen for callose (Table 6.2 a). There were significant differences in callose concentration between all three fabrics with the lowest concentration measured for the white fabrics followed by the green and then the brown which

had the highest callose concentration. There were no significant effects of dyeing and washing alone on callose concentration compared to the raw fabric however both NaOH scouring, dyeing and washing and ethanol scouring, dyeing and washing treatments caused significant reductions in callose concentration.

Heteroxylans

Significant main effects of fabric type and treatment were also seen for heteroxylan (Table 6.2 b). Green fabric had a significantly greater concentration of heteroxylan compared with the white and brown fabrics which were not significantly different from each other. Significant differences were seen in heteroxylan concentration for all three treatments compared to the raw fabric. A significant reduction was seen for both the dyeing and washing alone and the ethanol scouring, washing and dyeing treatments although these changes were not significantly different from each other. The greatest reduction in heteroxylan concentration was seen following the NaOH scouring, dyeing and washing treatment.

6.4 Discussion.

Three cottons, a white, and naturally coloured brown and green genotypes were used to make fabric which was then scoured, dyed and washed using either ethanol or NaOH scouring methods. Novel assessment of polysaccharide content using GC-MS showed differences in polysaccharide content between all three fabrics before and after scouring, with increased concentration of non-cellulosic polysaccharides found on the two naturally coloured cottons compared to the white. The greatest concentration of each measured non-cellulosic polysaccharide was found on the raw green fabrics. NaOH scouring was able to remove non-cellulosic polysaccharides on naturally

coloured cottons to a similar degree as on commercial white cottons, and as expected the ethanol scouring was less efficient at removing non-cellulosic polysaccharides although it did have some impact. The influence of non-cellulosic polysaccharides present on fabric dyeability was assessed and found to be of less consequence than that of the waxes present.

6.4.1 Hot ethanol scouring reduced the non-cellulosic polysaccharide content of fabrics but to a lesser degree than traditional NaOH scouring.

Generally, the process of dyeing and washing the fabrics alone did not remove non-cellulosic polysaccharides from fabrics and scouring with either NaOH or hot ethanol was required. There were a few exceptions seen for the coloured cottons where dyeing and washing alone significantly reduced the pectin concentration of brown fabrics and the pectin and xyloglucan concentration of the green fabrics. In all cases this reduction was less than the reduction measured following either scouring method. It is likely that due to the larger cuticle component on the coloured fibre, the hot dyeing and washing process disrupted the total fibre cuticle to a small extent and is responsible for the reduced concentrations of non-cellulosic polysaccharides. This was confirmed by the relative proportion of cellulose measured following dyeing and washing which showed that more total fibre cuticle was removed from the fabrics during this process thereby increasing the relative proportion of the cellulose. This was only seen for the brown and green fabrics which are known to have a greater cuticle component (Conrad, 1941, Conrad, 1944, Pan et al., 2010).

Hot ethanol scouring was included in this study primarily as a method of removing the total wax portion of the cuticle. This was done to aid in the FTIR analysis of wax components which was

then used to determine the effect of wax on fabric dyeability, as discussed in chapter 5. It was not expected that ethanol scouring would remove a significant portion of the non-cellulosic polysaccharides. Despite this, ethanol souring did significantly reduce a few non-cellulosic polysaccharides including arabinan, pectin, callose and heteroxylan. However, in all cases the reduction was less than that of the traditional NaOH scour and with the exception of pectin, for which reductions were seen on all three fabrics, affected only the naturally coloured fabrics which had a higher total amount of cuticle compared to the white. For heteroxylan, a polysaccharide that coats cellulose (Broxterman and Schols, 2018), the reduction following ethanol scouring was not different than the degree of reduction following the dyeing and washing alone for either of the coloured fabrics. This was expected as xylans are typically strongly hydrogen bonded to cellulose chains and are known to require exposure to alkaline solvents such as NaOH to solubilize them from the cellulose (Caffall and Mohnen, 2009). For arabinan, the reduction in green fabric following ethanol scouring was not different than after dyeing and washing alone. The fine structure of arabinan is not well known (Broxterman and Schols, 2018) however it is thought that it may be crosslinked to other components such as xylans and xyloglucan which are known to require alkaline scouring in order to solubilize them from cellulose (Caffall and Mohnen, 2009). This explains why NaOH scouring had the greatest effect on arabinan concentration. Generally, these results indicate that it is the dyeing and washing process rather than the ethanol that is responsible for the reduction of both xyloglucan and arabinan.

NaOH significantly reduced the concentration of all measured non-cellulosic polysaccharides for all three fabric types, apart from type 1 AG which was only reduced on the brown and green and 2 AG which was only reduced on the green fabrics. The variation in the amount of total cuticle

material present on fabrics did not appear to affect the ability of NaOH scouring method to remove non-cellulosic polysaccharides which may indicate that a shorter scouring time or a reduced concentration of scouring liquor could be utilised to adequately scour commercial white cottons.

6.4.1 NaOH scouring does not remove all cotton polysaccharides.

Whilst the impact of traditional NaOH scouring on cellulose has been studied, little work has been done to assess the fate of other polysaccharides present in fibre. One recent study of traditional scouring of commercial white cotton showed that most pectins and arabinose-containing polysaccharides are able to be removed using traditional scouring, however there were some pectins that were more resistant to scouring than others (Runavot et al., 2014). It also showed that both xylan and xyloglucan were less affected by traditional scouring than other non-cellulosic polysaccharides likely due to their strong hydrogen bond with cellulose (Caffall and Mohnen, 2009) and that callose was highly resistant to traditional NaOH scouring (Runavot et al., 2014). In this study, although a significant reduction was observed for all three fabrics following NaOH scouring, 52%, 47%, and 62% of the xyloglucan remained on the white, brown and green fabrics respectively indicating that like the above study on white cottons, xyloglucans on coloured cottons were also resistant to scouring. This resistance to scouring may be due to a possible covalent-crosslink which has been shown to occur between pectin and xyloglucan (Caffall and Mohnen, 2009). Conversely, while antibody testing showed no arabinogalactans present in the inner part of the secondary wall following NaOH scouring, here although significant reductions were seen on Type 1 and 2 AG concentrations for some fabrics following NaOH scouring, a large percentage of type 1 and 2 arabinogalactans remained on all three fabrics following both

traditional NaOH and hot ethanol scouring. This is consistent with a study of non-cellulosic polysaccharides and their interactions with cellulose where although the fine structure of arabinogalactans could not be deduced, it was assumed that they are covalently linked to cellulose making them difficult to remove (Broxterman and Schols, 2018). Previous studies have not assessed the specific effects of scouring on heteromannan concentration, so comparisons were not able to be made to the naturally coloured cottons in this experiment.

6.4.2 For fabrics made from naturally high wax cottons, waxes had a greater impact on dyeability than polysaccharides.

In general, the polysaccharide analysis showed that scouring coloured cotton fabrics with NaOH was more efficient at removing pectins and other non-cellulosic polysaccharides compared with ETOH scouring which was more efficient at removing the waxes. Despite the resistance of some polysaccharides to scouring, in all cases NaOH scouring was more effective at removing the polysaccharides on the naturally high wax coloured cottons compared with hot ethanol scouring. This was as expected since aqueous NaOH is specifically used as a cell-wall extractant which is capable of removing not only cuticular waxes but also several non-cellulosic polysaccharides (Runavot et al., 2014), whilst ETOH is more likely to precipitate the polysaccharides and retain them on the fibre whilst acting as a solvent to remove the waxes more efficiently (Fargher and Probert, 1924, Conrad, 1944).

The actual dyeing process also appears to have affected the non-cellulosic polysaccharide content of the fabrics in the brown and green cottons, but not the white, in a similar manner to the ethanol scour. This may be due to the low total cuticular portion in the white fabric compared

with the brown and green and it is possible that the hot dyeing solutions have impacted on the fibre although the effect was minimal in comparison to scouring and not enough to adequately dye the naturally coloured cotton fabrics.

Pectins are hydrophobic molecules embedded primarily in the epicuticular layer of the fibre and in the outer cuticle (Wakelyn et al., 2006). Hydrophobic molecules, including waxes and polysaccharides, in the fibre cuticle and cell wall act as a barrier to dyeability (Runavot et al., 2014). Increased dyeability was seen on the green fabrics following ethanol scouring rather than traditional NaOH scouring which was shown to more efficiently remove polysaccharides. Because of this it was concluded that although the non-cellulosic polysaccharides clearly are impacting dyeability, for naturally high wax fabrics it is the waxes, and more specifically the alkane waxes (chapter 5), affecting dyeability rather than the pectins and other non-cellulosic polysaccharides. This is further emphasised when we consider that, for all measured non-cellulosic polysaccharides except for callose, the highest concentrations were recorded for the green fabrics. Despite this, for the green fabrics, the ethanol scouring which had the least effect on the non-cellulosic polysaccharides had the greatest impact on dyeability. The increased dyeability in the brown fabrics, following traditional NaOH scouring rather than ethanol scouring is more in line with current thinking and is likely due to its lower overall wax percentage which was not significantly greater than the white fabrics.

6.4.3 Concluding remarks.

Naturally coloured high wax cottons not only have a significantly greater amount of cuticular wax compared with commercial white cottons they also have significantly greater non-cellulosic

polysaccharide content and following scouring they dye differently compared with white fabrics. Whilst traditional scouring was able to significantly reduce non-cellulosic polysaccharide content on all fabrics and was similarly effective for dyeability of the brown cottons, the improved dyeability of the green fabrics following ethanol scouring rather than traditional scouring techniques indicates that waxes have a crucial influence on fabric dyeability and if wax content on commercial fibre increases, traditional scouring techniques may not be adequate to prepare fabrics for dyeing.

Chapter 7. General discussion

Cotton is the most commercially important natural textile fibre in the world. Australia is the third largest exporter of cotton, with 2.6 million bales of cotton being produced in Australia for the 2018-2019 season. On average the Australian cotton crop is worth AUD $2 billion dollars annually (www.cottonaustralia.com). The value of cotton varies according to the quality of the fibres produced and is determined against specific criteria as discussed in chapter 1. Understanding the factors which may affect these qualities is crucial to protecting this valuable industry against synthetic alternatives. Synthetic fibres do not have the same variability in quality or the scouring requirements that cotton does. Synthetics are also arguably cheaper, although they are now linked to micro plastic contamination (Dris et al., 2016). Nonetheless, synthetics compete effectively against cotton, with cotton's market share now at 30% (www.cottonaustralia.com).

7.1 Current issues related to abiotic stress and cotton fibre quality.

Cotton fibre quality is influenced by a number of factors including temperature and water availability. Understanding how cotton fibre attributes are affected by abiotic stress is important because these attributes directly affect the commercial value of the cotton and its ability to be successfully utilised to manufacture dyed fabrics. Previously published studies have also noted the requirement for more research to assess how abiotic stress might affect the total amount of cuticular wax present on fibre and the work in this thesis aimed to build on this knowledge. This work showed that abiotic stress influences not only standard and cross-sectional fibre properties but also the total amount of cuticular wax present on the fibre. This highlights the importance of managing these conditions where possible via intervention with increased irrigation or by the

careful selection or generation of cotton genotypes that are more resistant or tolerant to increasing abiotic stress conditions.

Understanding how abiotic stress affects fibre quality is particularly important in light of current climate change projections. Currently Australian cotton growing regions are prone to periods of extreme heat stress and drought which places stress on cotton crops and this is set to increase further. Within the cotton growing regions of Australia including the Murray Basin, the projected increase in annually averaged temperature by the year 2030 is around 0.6-1.3°C, and as high as 2.7 – 4.5°C by the year 2090 with a greater frequency of hot days predicted. Additionally, the average rainfall is predicted to decline (www.climatechangeinaustralia.gov.au). These changes indicate that cotton growing regions will be exposed to increasing ambient temperatures. These increases may be large enough to cause heat stress which will affect cotton plants. Additionally, climate change may decrease water availability which ultimately could water stress cotton crops.

7.2 Summary of thesis findings.

This research has aimed to assess the effect of abiotic stress on the standard and cross-sectional properties of cotton fibre as well as the effect on the cotton fibre cuticle. Here several cotton genotypes with differing tolerances to abiotic stress and differing cuticular components were utilised for field and glasshouse studies. Additionally, three cottons with varying cuticular wax components and amounts were used to knit fabrics which were then assessed for dyeability and colour fastness following different scouring methods. The summarised findings of this research were as follows;

1. Abiotic stress affects fibre quality on both white and naturally coloured cottons and this effect is genotype dependant. More specifically, these stresses were shown to influence cross-sectional, length and tensile properties of fibre. Both heat stress and water deficit stress were shown to affect these fibre qualities although water stress had the greatest impact.
2. Abiotic stress caused increased cuticular wax deposition on cotton fibre. Although cuticular waxes on other aerial parts of the plant such as cotton leaf and bract have been shown to be increased by abiotic stress, here a response in the amount of fibre cuticular wax was seen for the first time. This response was variety dependant and the greatest responses were seen in genotypes included for their known poor tolerance to heat stress and poor water use efficiency.
3. The traditional caustic NaOH scouring method adequately disrupted the hydrophobic cuticular layer on naturally high wax coloured cottons allowing for colourfast dyeing which was measured spectroscopically following the use of a standard wash test. Here there were no observable differences in the colour of dyed fabrics following wash testing compared to the pre-wash colour for any of the fabrics.
4. Naturally high wax coloured cottons have increased non-cellulosic polysaccharide content compared to common white cotton genotypes. The traditional caustic NaOH scouring method did not remove all non-cellulosic polysaccharides and this effect was increased for naturally coloured cottons. Non-cellulosic polysaccharides present on fibre may negatively influence dyeability by affecting the penetration of the dye into the cellulose.

7.3 Future directions

Considering these findings, there are areas where further study is warranted. These include identification of or development of abiotic stress tolerant genotypes or genotypes with reduced or altered fibre cuticular wax, the development of testing methods for the faster identification of high wax cotton fibres, and the identification of genes involved in the production of cuticular waxes in cotton.

7.3.1 Identification and development of abiotic stress tolerant varieties

Further research into the development of abiotic stress tolerant varieties through selective breeding or genetic engineering would be useful. Although the effects of abiotic stress on the fibre of a number of cotton genotypes has been studied, the timing and application of these stresses have varied between studies and limited work has been done under typical commercial growing conditions in the field. This makes it difficult to determine in a commercial setting the degree of effect that different abiotic stress levels will have on commercial crop fibre qualities including increased cuticular wax deposition. Additionally, it would be useful to compare dyeability of white fabrics made from cottons that were grown either under control conditions or following the application of abiotic stress to further understand the effect this may have on the end product. More research is needed to help answer questions such as; do small/distinct stress periods have an influence on end of season total commercial harvest of fibre or only on fibre developed during the stress period? And if so, how does this affect the dyeability of fabrics produced using these fibres? Furthermore, none of these published studies have specifically utilised cotton genotypes with varying known tolerances to abiotic stress. The increasing risk of abiotic stress from climate change makes these questions important and makes necessary the

development of commercial genotypes with greater tolerances to abiotic stress than those currently utilised to mitigate any increased negative effects on fibre quality due to climate change.

7.3.2 Faster identification of high wax cotton fibres

Although the current scouring method adequately disrupts the hydrophobic fibre cuticular layer, increases in the amount of cuticular wax and the proportion of alkane waxes which are more difficult to remove may impact on the efficiency of this scouring method. Fast identification of fibre with higher than usual wax content will be a useful tool to ensure adequate scouring can be performed prior to dyeing. Further to this, faster identification of wax content on fibre will allow the easier identification of the threshold at which fibre wax content begins to affect the ability to adequately scour fabrics. It may also help to inform the choice of scouring method used as alternative more environmentally friendly scouring methods that are currently being researched become commonly used. In light of the findings in chapter 3, this fast identification is particularly important considering the potential for increasing wax deposition on fibre in the future because of increased abiotic stress from climate change. Currently fibre wax content is not used as a determinant of sale price the way that other fibre qualities such as micronaire are, so a fast method for determining the wax content of fibre has not been developed. If wax content increases with increasing climate change, then it may become a quality that is assessed to determine fibre value. Currently, there are two common ways the wax content of cotton fibre is determined, the Conrad method (Conrad, 1944) as used in this thesis and chloroform extraction followed by GC-MS analysis; both are time consuming processes. As an example, the Conrad method takes approximately 10 days before a quantitative wax measurement can be made and

often only one fibre sample can be processed at a time depending on the availability of necessary equipment including rotary evaporators. It is not a method that is currently suited to large scale or fast identification of wax content. There are a few promising alternatives.

The first is the use of microscopy techniques including scanning electron microscopy (SEM), or brightfield microscopy using stained cotton fibres to visualise the wax and make semi-quantitative assumptions about wax content based on morphological appearances. Initial work in this thesis demonstrated altered morphological appearances on the surface of fibre with increased wax deposition. Similar morphological differences have been reported on cotton leaf tissue where increased wax deposition had been measured indicating this may be a viable fast way to identify fibre with high wax content that takes hours rather than days or weeks. If further experimentation shows that morphological differences are consistently seen for fibres with higher wax content, then further study would be required to determine the sensitivity with which this method could be used to assess wax content. This method is likely viable when large differences in wax content are present but is probably limited in its ability to distinguish fibre with smaller differences.

Additionally, the use of FTIR to analyse ground fibre samples may help to identify high wax fibre. While this method is not ideal to quantify and compare total waxes between two fibre samples, spectra could be used to identify fibres that have proportionally higher alkane wax content which is more difficult to scour than other cuticle components. This could then help to select the ideal scouring method based on cuticle differences.

7.3.3 Identification of the genes involved in the production of fibre cuticular waxes.

The current understanding of the genes controlling the production of cuticular waxes in cotton is limited and broader comparisons are typically made between cotton and other plants such as the model plant *A. thaliana*. It would be useful to gain a greater understanding of the genes and gene expression pathways responsible for the development of cotton fibre cuticular waxes so that we may apply this knowledge towards the selection or development of cotton plants that produce fibre with altered fibre cuticular wax components that might better facilitate the manufacturing of dyed fabrics through reduced scouring requirement. In particular, identification of the genes that are involved in the as yet uncharacterized decarbonylation pathway responsible for the production of VLCFA's including alkane waxes would be useful. A possible way to identify these genes is through the study of cuticle mutant plants.

Plants that have abnormalities in the cuticle due to mutations in or the absence of necessary regulatory genes have numerous morphological changes and other physiological challenges as a result. Such plants may exhibit a thinning or absence of the cuticle or changes in the composition of the cuticle leading to growth differences including curled or wrinkled leaves, small flower size and fusion of aerial parts. They may also display altered susceptibility to pathogens, necrotrophy due to increased DNA damage from UV radiation and visible signs of stress from increased water loss (Barozai and Husnain, 2014). Extensive studies have been done using Arabidopsis wax mutants. In particular, a lot of work has been done on CER mutants of which there are 89 known types (Koornneef et al., 1989). These mutants are recognised by their glossier and brighter green appearance when compared with wild type. The study of these mutants has greatly increased knowledge of the cuticle biosynthetic pathways as described in the chapter 1.

Examples of Cer mutants that have been investigated recently and which may provide interesting gene candidates for exploration in cotton include the Arabidopsis CER2 and CER26 mutants which were found to be impaired in the production of wax components longer than C28 and C30 respectively (Pascal et al., 2013) as well as the mutation of BnCER1, a gene homologous to CER1, in *Brassica napus* which showed a drastic decrease in the overall wax content as well as an increase in the proportion of aldehydes but a decrease in the amount of alkanes, ketones and secondary alcohols. These results suggest that a mutation in BnCER1 leads to the blockage of part of the Decarbonylation pathway (Pu et al., 2013). These results may help elucidate the unknown aspects of the alkane forming steps of the Decarbonylation pathway and may be important in the development of low alkane cotton varieties.

Recently, some work has been completed to develop and characterize Gossypium Arboreum leaf epicuticular wax mutants (Barozai and Husnain, 2014). This study was the first to develop and examine wax mutants in cotton. In this study three mutant varieties were developed using the chemical mutagens diethyl sulfate (DES) and ethyl methane. The three mutants *Gossypium arboreum* Wax Mutant 1 (GaWM1), *Gossypium arboreum* Wax Mutant 2 (GaWM2), and *Gossypium arboreum* Wax Mutant 3 (GaWM3) each displayed altered leaf wax morphology compared to the wild type when examined by scanning electron microscopy. The wax on wild type leaves are deposited as smooth stripy layers whilst these sharp stripy layers were not present on any of the three mutant varieties. Instead GaWM1 wax appeared as embedded thick dull layers, GaWM2 wax was deposited as irregularly dispersed patched, and GaWM3 showed a thin wax layer. In addition to the differences in the pattern of wax deposition there were also significant differences in the total amount of wax present in leaves with GaWM1, GaWM2 and

GaWM3 showing 33.21%, 40.50% and 50.71% reduction in total wax load respectively when compared with the wild type. This cotton variety is known for its strong level resistance to abiotic and biotic stress and it is though that the morphology of its wax deposition is somewhat responsible for that, therefore it was important to measure differences in the patterns of this deposition. The authors of this paper briefly discuss the components of the cuticle as measured using GS-MS. They state that whilst alkanes are the dominant class present in all 4 varieties, the wild type has more alkanes, aldehydes and alcohols compared with the mutant varieties and that GaWM2 has more acid and ester present compared with the wild type and both GaWM1 and GaWM3 (Table 7.1) (Barozai and Husnain, 2014). They do not however discuss the amount of individual components as percentages of the whole cuticle instead presenting measurements as $\mu g\ cm^{-2}$ so it is difficult to draw conclusions as to the effect off the mutations on variations in wax composition. This study was the first of its kind looking at the cuticle of cotton wax mutants and provides attractive mutant candidates for future experimentation however these measurements were limited to the leaf tissue and it would be useful to repeat this study to look more specifically at the cuticle on the fibre. This study did not attempt to discover the genetic source of the mutation leading to the different phenotypes so there is scope for future work in this area also using cotton wax mutants.

In addition to this, there have been recent advances in the study of wax biosynthesis in *A. thaliana* over-expressing lines which may provide novel candidates for study in cotton. One such example is the study of an *A. thaliana* MYB94 transcription factor over-expressing line in which the researchers were able to demonstrate an approximate 2-fold increase in the amount of total cuticular wax compared with the wild type. Here they were able to demonstrate that a novel

Table 7.1. Leaf wax concentrations in cotton wax mutants. Table reproduced from Barozai and Husnain 2014. Table 1. All cotton genotypes listed are *Gossypium hirsutum*. Average values ± S.D reported.

Cotton	Wax classes and their concentrations (μg cm^{-2})					
	Alkane	Acid	Ester	Aldehyde	Alcohol	Unknown
WILD	136.7 ± 8.98	6.4 ± 2.76	1.2 ± 0.1	0.2 ± 0.04	3.5 ± 1.21	35.7 ± 4.35
GaWM1	95.7 ± 6.83	7.4 ± 1.87	2.2 ± 0.4	0.2 ± 0.07	0.5 ± 0.35	16.7 ± 3.87
GaWM2	90.3 ± 9.32	9.7 ± 2.43	3.4 ± 0.3	0.1 ± 0.03	3.2 ± 1.08	12.6 ± 8.23
GaWM3	70.2 ± 10.32	2.4 ± 0.30	0.2 ± 0.07	0.1 ± 0.01	0.4 ± 0.02	17.4 ± 10.12

MYB94 TF is able to directly upregulate wax biosynthetic genes involved in the both the acyl-reduction pathway and the decarbonylation pathway such as KCS2, ECR, CER2, Cer1 and CER4 (Lee and Suh, 2015).The over expression of MYB94 whilst increasing total wax amounts did not otherwise affect plant growth or development making it an interesting potential candidate for study in cotton and potentially for the development of transgenic crops.

As a part of the experiment in chapter 3 of this thesis which assessed the response in the fibre of white and naturally coloured high wax cotton genotypes to continuous water deficit, plant tissue samples were harvested and stored for future analysis by quantitative real time polymerase chain reaction (QRT PCR). Here leaf, bract, boll and immature fibre samples from 3DPA and 8DPA were harvested to analyse the expression of a few identified gene candidates identified in other plant species that may be related to the production of very long chain fatty acids including alkane waxes. Due to time constraints and the absence of a response to water deficit stress on fibre cuticular wax deposition in these plants the samples were not analysed. It would be useful to take similar samples from a field experiment utilising cottons that have been shown to have altered wax deposition on fibre in response to abiotic stress to help identify the genes that control the decarbonylation pathway of VLCFA synthesis. Identification of these genes in conjunction with recent progress in identifying the genetic basis of drought tolerance in cotton (Abdelraheem et al., 2019) may aid in the development of cottons that not only produce modified wax component but also have increased abiotic stress tolerance.

7.4 Concluding remarks

More than ever it is important that high quality cotton can continue to be produced so that cotton can remain competitive within a market where other synthetic alternatives are available. Continued research into factors that can influence fibre quality and therefore the value of cotton, along with the discovery of new methods to assess fibre qualities such as wax content is crucial.

Chapter 8. References

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