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Evaluation of Screening Methods to Detect Heat Stress in Diverse Cotton Genotypes

A dissertation submitted in partial fulfilment
of the requirements for the degree of
Doctor of Philosophy in Crop, Soil, and Environmental Sciences

by

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ABSTRACT

Cotton (*Gossypium hirsutum* L.) is sensitive to heat stress (HS) during reproductive development. The objective of this study was to evaluate different screening methods for identification of heat tolerance in cotton genotypes. Three growth chamber studies and four field trials were conducted from 2014 to 2017 using genotypes Arkot 9704, VH260, DP 210 B2RF and DP393. Measurements were made of membrane leakage (ML), chlorophyll fluorescence (ChlF), glutathione reductase (GR), and sucrose concentration. In the growth chambers, measurements were made at 30 and 40°C and at 2, 4 and 6 hours of HS, as well as 3 and 7 days after HS and 7 days after recovery. Both ML and ChlF were decreased at 40°C and genotypic difference were detected, with DP393 the least affected indicating heat tolerance. Arkot 9704 was affected the most indicated sensitivity to HS. The small genotypic responses to HS was related to modern genotypes having less tolerance to HS than older obsolete genotypes and wildtype cotton. Glutathione reductase was increased by HS and VH260 and DP393 increased the most in the growth chamber but not in the field studies. Sucrose concentrations were decreased by HS with no genotypic differences. Analysis of the fluorescence transient after HS was imposed showed that maximum fluorescence intensity, plant performance index (PI_{ABS}) and electron transport flux (ET/CS) provided more intrinsic quantitative measurements of the effect of HS on PSII function. For both ML and ChlF, for a one day heat stress period, measurements could be made at 2 hours, but for a longer heat stress, parameters should be measured 7 days after stress. The method of measuring genotype response to HS in the field by comparing cool versus hot days was not sufficiently accurate. A new method of comparing early morning cool 6.00 AM measurements versus hot midday measurements, showed genotypic increases in ML,

but for ChlF only on clear, high radiation days. Differential genotypic responses to HS can be detected by ML and particularly by ChlF for ease of use and accuracy, with an analysis of the fluorescence transient responses to HS providing a clear means of differentiating between genotypes for thermotolerance.

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INTRODUCTION

Upland cotton (*Gossypium hirsutum* L.), a member of the Malvaceae family, is considered to be the most important textile fiber crop in the world, providing roughly half of the global fiber requirement. Cotton is produced worldwide under a wide range of environmental conditions and is therefore exposed to numerous abiotic and biotic stresses. Temperature is a primary controller of the rate of plant growth, development, reproduction, and fruit maturation. High temperatures can have both direct inhibitory effects on growth and yield, and indirect effects due to high evaporative demand causing more intense water stress. Crops have vastly different temperature optima, indicating that some fundamental biochemical process in their makeup have differing sensitivity to temperature. The optimum temperature for cotton photosynthesis, growth and development is 30°C, and boll growth virtually ceases above 35 °C. Furthermore cotton is particularly sensitive to high temperature during reproductive development.

Elevated temperatures due to climate change are projected to cause substantial losses in crop productivity. Sensitivity of reproductive tissues to high temperature has been identified as a major reason for the disparity between actual and potential yields in crops, and more information is needed on the physiological effects of high temperature during flowering. This information is essential in the development of techniques to screen genotypes for temperature tolerance for improved performance and optimum sustainable yields. Much of the previous research on techniques and screening for high temperature tolerance in cotton has been conducted under controlled environmental conditions and, as such, does not necessarily reflect the reliability of these techniques under more natural but variable field conditions. The onset of high temperature

stress in cotton production systems may be minimized by selecting higher yielding cotton genotypes under high temperature stress. Understanding plant response to high temperature will permit the use of the response for selection of thermo-tolerant genotypes, and also provide the knowledge to formulate strategies for ameliorating the deleterious effects of high temperature stress.

HYPOTHESIS AND OBJECTIVES

Hypothesis

It is hypothesized that high temperatures cause physiological responses in cotton leaves that affect growth and yield, and that these responses can be used to screen for temperature tolerant genotypes.

Objectives

- To ascertain the effect of high temperature stress on select physiological functions.
- To use these measurements to screen cotton genotypes for tolerance to high temperature, and determine the most practical and accurate screening technique.

REVIEW OF LITERATURE

Cotton (*Gossypium hirsutum* L.) is grown on more than 32 million hectares worldwide and is one of the world's major fiber crops (Singh *et al.*, 2007). Out of 50 *Gossypium* species, four have been domesticated (Rahman *et al*., 2011). These four species *Gossypium hirsutum* L., *G barbadence*, *G arboreum* and *G herbaceum* are widely planted in over 76 countries, including the US, China and India (Zhang *et al*., 2007). Three major components affect cotton yields, namely, genotype, environment and management practices (Oosterhuis, 1999). Genotype decision and management practices can be influenced by the cotton producer, but only limited control can be exerted over the daily environment of the cotton crop during the growing season. Adverse weather, especially temperature and drought, are some of the main deterrents to high yields in cotton. Water and heat stress are the most important environmental variables affecting cotton growth and development (de Ronde *et al*., 2000). Oosterhuis (1999) concluded that although cotton originates from hot climates, it does not necessarily grow the best at excessively high temperatures. Reddy *et al.* (1991) reported the ideal temperature range for cotton from 20 to 30°C, and concluded that growth decreases once temperatures reach about 35 °C. The optimum temperature for photosynthesis was reported by Burke *et al*. (1988) to be 28 °C, with a thermal kinetic window where optimum metabolic activity takes place of between 23.5 to 32 °C.

Taxonomy

The botanical classification of cotton according to de Kock (1994) is as follows: Division: Angiospermae, Class: Dicotyledonae, Subclass: Dilleniidae, Order: Malvales, Family: Malvaceae Tribe: Gossypieae, and Genus: Gossypium.

Morphology

Rehm (1991) stated that all cotton species are potentially perennial, even though they are normally grown for only one year in modern agriculture. The cotton seedling, with its fast-growing radicle and gland-studded stem (hypocotyl), which lifts the two big cotyledons and the growing point out of the soil, develops from the seed (van Heerden, 1978). Cotton plants form a strong taproot, which develops even at the seedling stage, and which can reach a depth of 3 m (Rehm, 1991).

A cotton plant has a single ascending main stem that bears a leaf at each node and usually has one branch. Vegetative branches (monopodia) tend to be produced lower down on the plant, while reproductive (sympodia) branches are produced higher up or on the monopodia. Sympodia are generally short and terminate in a flower bud (Bennett, 1991). Cotton leaves are large, palmately lobed (three, five or seven lobed) and covered with multicellular stellate hairs (Kochhar, 1981). Plants in the genus Gossypium have showy flowers, each with five sepals united into a cuplike calyx and five petals of whitish or yellowish color that turn pink with age (Wolfe, 1959). Pollination usually occurs in the morning. By late afternoon the corolla begins

to change color, first becoming a faint pink and later a deep red-mauve. At the same time, the bracts close around the ovary. At this stage, the bud is termed a square. As the square develops, the fruit increases in size and protrudes beyond the bracts. The fruit or boll is a 3 to 5-locular, dehiscent capsule, each locule containing approximately nine seeds (Figure 1.). These seeds produce the lint fibres as well as the short fuzz (Bennett, 1991).

Figure 1. Fruit formation. a. Flower bud; b. Flowers; c. Unripe boll; d. Mature boll

Importance of high temperature stress in plant growth.

Above optimum temperatures and temperature extremes during critical stages of plant development, are major factors limiting crop production (Hall, 1992). According to the fourth assessment report of the Intergovernmental Panel on Climate Change (IPPC, 2007), the eleven years during 1995 to 2006 ranked among the warmest years since 1850 of global surface temperature. This report stated the increase in temperature is widespread over the globe and greater in northern latitudes (IPPC, 2007). Global surface temperature has increased by approximately 0.6 °C since the late 19th century and is projected to increase by 1.4 to 5.8 °C by the end of the current century (Houghton *et al*., 2001). Numerous climatic studies are projecting future increases in temperatures. For example, global temperature models show an increase in mean annual temperatures of between 1.5 and 6 °C by 2100 (IPCC, 2013). Increases in frequency, duration and severity of high temperatures (i.e., heat waves) will also be more likely (Dai *et al*., 2001). Emission of greenhouse gasses (GHG) such as carbon dioxide (CO_2), methane (CH_4) and nitrous oxide (N_2O) from agricultural systems are some of the sources that contribute to the global increase in temperature (Shah, *et al*., 2011). These authors also concluded that the increase in temperature has and will expose most of the world's crops to heat stress during some stages of their life cycle. Reddy *et al*. (2002) and Peng *et al*. (2004) concluded that investigations regarding the effect of climate change on crop yield suggest a major loss of productivity due to projected surface temperature increases by the end of the 21st century. Schlenker and Roberts (2009) found that yields increased with temperature up to 29 °C for corn (*Zea mays*), 30 °C for soybeans (*Glycine max*) and 32 °C for cotton (*Gossypium* hirsutum), and that temperatures above these thresholds were very detrimental. Blanc (2012)

concluded that over the 21st century, temperature is predicted to increase under all five atmosphere-ocean general circulation model scenarios. Jarvis *et al.* (2010) concluded that for cotton the temperature threshold extends to 33.0 °C. These authors also mentioned that with the lowest scenario of climate change, losses of up to 21 % for soybean and 19 % for cotton are projected by 2030.

Abiotic stress conditions cause extensive losses to agricultural production worldwide and stress conditions such as drought, salinity or heat have been the subject of intense research (Mitler, 2006). Different crops have different temperature optima. For cotton, the thermal kinetic window (TKW) for enzyme activity is between 23.5 to 32 °C (Burke *et al*., 1988) and this strongly correlates with the optimal temperatures for general metabolism and growth for various species (Ferguson and Burke, 1991; Burke and Oliver, 1993). The reported temperature optima for cotton enzyme function, germination, seedling growth, root development, shoot development, flowering, and lint production provide a range of optimum temperatures centred around 28°C ± 3°C (Burke and Wanjura., 2010). Because typical daily high temperatures in cotton growing areas are often in excess of the optimum range during the growing season, high temperature represents a major limitation to crop development and productivity (Snider, 2010). High temperatures of above 35 °C throughout the growing season are common in cotton production areas and exceed the thermal kinetic window for which metabolic activity is most efficient in cotton plants, thereby limiting growth development and yield (Hodges *et al*., 1993).

Effects of temperature on cotton.

Growth and yield

Reddy (1996) stated that weather is one of the most important factors that affects crop growth and yields, and with cotton, temperature controls crop development and indirectly, water requirements. Heat stress occurs when plants are exposed to above-optimum temperatures, and when the stress lasts long enough to cause irreversible damage to plant growth and development (Wahid *et al.*, 2007). Cotton yields are negatively affected by rising temperatures (Crafts-Brander and Salvucci, 2000; Oosterhuis, 2002; Oosterhuis and Snider, 2011; Snider *et al.,* 2009; Snider, 2010). According to Oosterhuis (2002) and Bibi (2005) high temperatures during the reproductive development of cotton in Arkansas reduced yield, and Oosterhuis (2002) showed a strong correlation between high temperature and reduced yield, where high temperatures during the flowering period of cotton resulted in lower yields.

Temperature is one of the major factors affecting crop growth and yield. During the growing season of cotton, sensitive processes such as the flowering and boll development occur simultaneously with temperatures that are too high for optimum functioning (Snider, 2010). Temperatures above 35°C occur frequently during the reproductive stage of cotton and leads to a decrease in boll growth (Reddy *et al*., 1999). The most sensitive process of cotton development is boll retention with the upper limit for boll survival being 32 °C (Reddy *et al*., 1999). These authors also found that fiber length was at a maximum when plants were grown between 15 to 21 °C and fiber fineness and maturity increased up to 26 °C but decreased at 32 °C. Burke and

Wanjura (2010) stated that temperatures above 34 °C reduce production of squares and may induce flower sterility. White flowers, the stage when anthesis, pollination and fertilization occur, never shed, but is considered a critical time in the development of the crop with regard to temperature requirements, as above optimum temperatures leads to non-viable pollen and decreased pollen growth (Snider *et al*., 2009). Waraich *et al*. (2012) reported that both low and high temperatures affect plant development and growth at the whole plant, tissue and cell level and even at the sub-cellular level. High temperatures (>35°C) throughout the growing season may adversely affect growth and ultimately yield and quality of cotton (Hearn and Constable, 1984). Heat and drought can result in drastic losses in cotton yield and fiber quality (Sekmen *et al*., 2014). Heat stress has been reported as one of the most important causes of reduction in yield and dry matter production in many crops, including wheat (*Triticum aestivum L.*); rice (*Oryza sativa L.*); millet (*Pennisetum glaucum L.*) (Al-Khatib and Paulsen, 1999); maize (*Zea mayz L.*) (Crafts-Brandner and Salvucci, 2002; Lobell *et al*., 2013); soybean (*Glycine max L.*) (Djanaguiraman *et al*., 2013) and cotton, (*Gossypium hirsutum* L.) (Burke *et al*., 1988; Rahman *et al*., 2011, Reddy *et al*., 1991; Oosterhuis, 2002). High temperature is predominant among the primary environmental factors that determine crop growth and productivity in cereals (Al-Khatib and Paulsen, 1999). Cotton growth, development and yield are responsive to changes in environment, and management adjustments must be designed to optimize the environment (Kerby *et al*., 2010).

Physiology

As plants cannot move, they defend themselves from heat stress through metabolic and structural adjustments (Yamanouchi *et al*., 2002). Plants overcome high temperature stress by adopting several physiological and biochemical mechanisms such as excess heat dissipation through evaporative cooling (Kheir *et al*, 2012). The most readily observable response to heat stress is the induction of heat shock proteins (HSPs) and HSPs appear to be co-ordinately expressed when the plant tissue is under heat stress (Chen *et al*., 1990). The optimal induction for HSPs is a drastic temperature upshift from 39 – 42 °C, however, these proteins are also induced with a gradual temperature rise of 2.5 °C, which often occurs in the field (Altschuler and Mascarenhas, 1982).

Membrane leakage

Membrane leakage has been considered a symptom of stress-induced membrane damage and deterioration (Peng *et al*., 2003; Melkonian *et al*., 2004). Sulivan (1971) developed a heat tolerant test that determined ML through measuring the amount of membrane leakage from leaf discs bathed in de-ionized water after exposure to heat stress. Rahman *et al*. (2004) used membrane leakage (ML) as a method to determine high temperature tolerance in cotton. These authors found that high temperature modifies the composition and structure of cell membranes by weakening the hydrogen bonds and electrostatic interactions between the polar groups of proteins within the aqueous phase of the membrane. Disruption and damage to membranes alters their permeability, and results in the loss of electrolytes. Buchanan *et al*. (2009) concluded that

for membrane fluidity to cope successfully with the problem of elevated temperature, plants alter the composition of their membranes to optimize fluidity for a given temperature. Wang (1988) stated that plants experiencing high temperature stress have their membrane structures altered, with membrane permeability increases, electrolyte or ion leakage increases, and eventually cell death. Asha and Lal Ahamed (2013) investigated 40 genotypes of cotton, eliciting information on heat tolerance using ML. The mean relative electrical conductivity values showed gradual increase from 32.06 (S/m) at 25 ^{0}C to 84.15 (S/m) at 50 ^{0}C indicating that higher temperatures had a direct effect on the leakage of electrolytes from the cells and higher levels of cell injury. Bibi *et al.* (2008) observed that membrane leakage in cotton significantly increased when temperature exceeded 33 to 35 ^{0}C. Rana *et al.* (2011) evaluated twelve cotton genotypes for thermo tolerance, using membrane leakage and found 3 out of the 12 genotypes to be tolerant to heat. Membrane leakage is a widely used method for assessing heat tolerance or susceptibility in crops, with the only disadvantage being that it is a time consuming measurement.

Chlorophyll fluorescence

Chlorophyll fluorescence (ChlF) is a rapid, non- destructive method to quantify heat stress developed by Kitajima and Butler (1975), and is today one of the most widely used stress tests in crop production (Baker and Oxborough, 2004; Resco *et al*., 2008; Wu *et al*., 2011). ChlF is one of the most popular techniques in plant stress physiology because of the ease of gaining detailed information on the state of Photosystem II. It has a major role in understanding the fundamental mechanisms of photosynthesis, the responses of plants to environmental change and genetic variation (Murchie and Lawson, 2013). ChlF takes place in the chlorophyll, where light energy

is absorbed by pigments present in the photosynthetic antenna molecules in the thylakoid membranes (Misra *et al.*, 2012). ChlF is light re-emitted by chlorophyll molecules during return from non-excited states and used as indicator of photosynthetic conversion in higher plants. ChlF intensity is an indication of the absorbed photons that is not used for photosynthesis. Light energy absorbed by chlorophyll molecules in a leaf can undergo one of three fates, namely a) drive photosynthesis, b) dissipate excess energy as heat, or c) it can be re-emitted as light (ChlF). These three processes are in competition with each other, such that the increase in efficiency of one will lead to a decrease in the yield of the other two (Misra *et al*., 2012, Strasser *et al*., 2004). ChlF is defined as the loss of partial exit energy after the antennae has absorbed the chlorophyll light. This happens in Photosystem II (PSII) through the radiation of red light with a wavelength of 680 nm.

Antioxidants

Exposures of plants to high temperature increased the production of reactive oxygen species (ROS) such as singlet oxygen, superoxide, hydrogen peroxide and hydroxyl radical. This major response of heat stress (increased ROS) leads to oxidative stress. Plants alter their metabolism by producing compatible solutes that are able to organize proteins and cellular structures, maintain cell turgor by osmotic adjustment, and modify the antioxidant system to re-establish the cellular redox balance and homeostasis (Hasanuzzaman *et al*., 2013). A major hydrogen peroxide detoxifying system in plant cells is the ascorbate-glutathione cycle, in which, ascorbate peroxidase (APX) enzymes play a vital role catalysing the conversion of hydrogen peroxide into water, using ascorbate as a specific electron donor (Caverzan, *et al*., 2012). Hydrogen peroxide

(H_2O_2) production is an early response to heat stress (Dat *et al*., 1998). Pre-treatment with H_2O_2 or menadione can lead to an increase in thermo tolerance in Arabidopsis (Larkindale & Huang, 2004). These findings suggest that some active oxygen species (AOS) play a role in heat stress-signalling, possibly inducing heat shock proteins (HSPs). In addition to H_2O_2, several other chemicals seem to be involved in the heat stress responses. Plants actively produce reactive oxygen intermediates (ROI's) as signalling molecules to control processes such as programmed cell death, abiotic stress responses, pathogen defence and systematic signalling. Under normal conditions, the production of ROI's in cells is low, but under stress conditions, ROI's increases because stress disrupts the cellular homeostasis of cells. These include drought stress and desiccation, salt stress, chilling, heat stress, heavy metals, ultraviolet radiation, air pollutants, nutrient depravation, pathogen attack, and high light stress (Mittler, 2002). The production of ROI's during these stresses results from pathways such as photorespiration, from the photosynthetic apparatus, and from mitochondria respiration. The enhanced production of ROI's can cause a threat to cells, but it can also acts as signals for the activation of stress response and defence pathways (Mittler, 2002). Bibi *et al*. (2005) reported that antioxidant enzyme activities increase in vegetative tissues of cotton under heat stress, thereby enhancing thermo tolerance. Increases in antioxidant activity in leaves have been used as indicators of both high and low temperature stress in many species (Gong *et al*., 1998; Iba, 2002; Anderson and Padhye, 2004). The ability of a cotton plant to withstand abiotic stress is closely related to the plants ability to increase antioxidant enzyme activity.

Carbohydrates

Snider (2010) reported that high temperature resulted in significant decreases in total soluble carbohydrate concentrations in cotton pistils and that this decline was primarily attributed to a decrease in sucrose concentration under heat stress. Loka and Oosterhuis (2013) showed that high night temperatures had a significant effect on ovary and bract carbohydrate content. Ovary glucose, fructose and sucrose content of heat-stressed plants significantly increased compared to a control. Leaf photosynthetic rates of heat- stressed plants were decreased and in combination with increased respiration resulted in marked decreases in leaf carbohydrate content (Loka and Oosterhuis, 2016).

Photosynthesis

Photosynthesis of leaves is effected by many stresses including drought, flooding, salinity, chilling, high temperature, soil compaction and inadequate nutrition, and many of these stresses have common symptoms, for example, decreases in stomatal conductance and the rate of assimilation of CO_2 (Farquhar *et al*., 1989). High temperature inhibits photosynthesis (Ogren, 1984; Brooks and Farquhar, 1985; Bibi *et al*., 2008). Sharkey (2005) reported that photosynthesis is particularly sensitive to heat stress and that even a moderate heat stress can reduce the photosynthetic rate to near zero. Crafts-Brandner and Salvucci (2002) evaluated the sensitivity of components of the photosynthetic apparatus of maize (*Zea mays*) to high temperature stress and concluded that net photosynthesis was inhibited at leaf temperatures above 38°C, and the inhibition was much more severe when the temperature was increased

rapidly rather than gradually. Bibi (2008) observed that the optimum temperature for photosynthetic carbon fixation of cotton was approximately 32 °C and that photosynthesis in cotton decreased significantly at temperatures of 36 °C and above. The primary cause for this is the increased thylakoid membrane ionic conductance and Rubisco (ribulose-1,5-bisphosphate carboxylase/oxygenase) deactivation (Crafts-Bradner and Salvucci, 2000). High temperature caused an increase in thylakoid permeability at temperatures as low as 36°C and reduced photosynthetic efficiency by stimulating photorespiration as well as by damaging the photosynthetic apparatus.

Respiration

Loka and Oosterhuis (2010) found increased respiration when they evaluated high night temperature regimes on cotton. They evaluated a short-term (2 hours of high night-temperature) and a long-term heat stress (four weeks of high night-temperature). In the short term experiments, they found that the 27.0°C and 30.0°C temperatures caused a significant increase in respiration rates by 49.0 % and 56.0 %, respectively, compared to the control temperature of 24.0°C. In the long term experiment, they again found respiration to increase significantly by 39 % and 21 %, respectively, during the second and fourth week of measurements at the 30/28°C temperature.

Sensitive stage of crop development to heat stress

High temperatures during the growing season of cotton can affect all stages of development, but cotton seems particularly sensitive to high temperatures during the reproductive (flowering) stage (Oosterhuis, 2002). The flowering stage in crops is generally the most sensitive to high temperature (Ferris, 1998; Snider, 2010). This was also found in other crops such as rice (*Oryza sativa L.*), Matsui and Omasa, 2002; tomato (*Solanum lycopersicum*), Peet *et al*., 1998; Lohar and Peat, 1998); maize (*Zea mays* L.) Sinsawat *et al*., 2004 and wheat (*Triticum aestivum* L.) (Stratonovitch and Semenov, 2015). In cotton, the development of flowers up the main stem decreases with increasing temperatures, with abscission of squares, flowers and young bolls at temperatures of above 35°C (Hodges *et al*., 1993). Reddy *et al*. (1999) found boll growth increased with temperatures up to 25 °C, and then declined at higher temperatures and conclude that boll retention was the most sensitive part of cotton growth. During cotton flowering, the stage that is most vulnerable to temperatures higher than 33°C was immediately after meiosis of the microspore mother cells had occurred (Meyer, 1966). Jain *et al*. (2007) concluded that depending on the time, duration and severity of the heat stress, fertilization can be inhibited by male and female gametophyte development in grain sorghum. Pollen germination and pollen growth of cotton are also negatively affected by high temperatures, with optimum temperatures for pollen germination of 28 to 37°C (Burke *et al*., 2004; Kakani *et al*., 2005; Liu *et al*., 2006; Snider *et al.,* 2011).

Thermo tolerance

Heat tolerance is generally defined as the ability of the plant to grow and produce economical yield under high temperature (Wahid *et al.*, 2007; Shah *et al.*, 2011; Vignjevic *et al.*, 2015). When plants are subjected to environmental stress conditions such as temperature extremes, drought, herbicide treatment or mineral deficiencies, the balance between the production of ROS and the quenching activity of the antioxidants is upset, often resulting in oxidative damage. Plants with high levels of antioxidants have been reported to have greater resistance to this oxidative damage (Gossett *et al.*, 1994; Snider *et al*., 2010). Heat stress affects development of growth by opening of stomata which results in enhanced respiration and cooler plants (Loka and Oosterhuis, 2010). Heat stress also decreases the rate of carbon assimilation in cereals (Barnabas, 2008). Plants, like most organisms, respond to an elevation in temperature by synthesizing heat shock proteins (Al-Whaibi, 2011; Vierling, 1991).

Remediation of heat stress

Genotype selection

It is generally accepted that the most important and economic way to overcome the negative effects of heat stress is to develop heat-tolerant cultivars (Singh *et al.,* 2007), however, little success has been achieved as although substantial genotypic variation exists, it has not been exploited in breeding programs (Oosterhuis *et al.*, 2009). Bibi *et al*. (2010) found that wild type cotton was significantly more heat tolerant than commercial cultivars which emphasized the need

to introduce wild germplasm in breeding programs. Brown and Oosterhuis (2010) reported that newer cultivars were less tolerant to heat than older obsolete cultivars. Constable *et al*. (2001) concluded that breeding programs have principally relied on yield and fiber quality as screening tools in environments and that screening for thermo tolerance has been largely incidental. Breeders have improved yield in Pima cotton (*Gossypium barbadence* L.) by increasing high temperature tolerance (Kittock *et al*., 1988), however little has been done to improve high temperature tolerance in Upland cotton (*G. hirsutum* L.). Plant physiologists from Phoenix, USA, are working on the inclusion of a gene for rubisco activase from a desert scrub into cotton, that they hypothesize will alter cotton enabling greater tolerance of heat, producing higher yields with less water use (Salvucci and Crafts-Brandner, 2002). The development of heat tolerant cotton cultivars started in the 1950's in Phoenix Arizona and a number of cultivars were developed and released for commercial use, the first being Pima S-2 (Singh, 2007). In Pakistan (Cotton Research Institute, Faisalabad, the cultivar CRIS-134 has been developed that is capable of producing 32 bolls in 75 days at average maximum temperatures of 41 °C (Soomro, 1998). Zhang (2013) evaluated two heat tolerant Pakistani cotton cultivars, VH260 and MNH456 compared to two heat-susceptible cultivars ST213 and ST4288B2F, originating from the Mississippi Delta Region and found no obvious differences in photochemical efficiency of photosystem II in the four lines, however the heat susceptible cultivars showed greater ML after heat treatment as compared to the heat tolerant lines.

Plant growth regulators

Plant growth regulators (PGR's) were originally divided into five classes: Auxins, gibberellins, cytokines or kinins, abscisic acid and ethylene (Gardner *et al*., 2003). Numerous synthetic PGR's have been developed and used on cotton production to influence growth and yield. Some of these PGR's have shown a potential for counteracting periods of higher temperatures during the growing season. One example is Pix (mepiquat chloride), a PGR used to control vegetative growth, that has also shown potential for alleviating stress. At elevated temperatures (55°C) cotton plants previously treated with mepiquat chloride showed increased heat resistance compared to the untreated control (Huang and Gausman, 1982). The mepiquat chloride-treated leaves had larger starch grains in their chloroplasts than control leaves, which suggest a difference in photosynthetic activity (Reddy *et al*., 1996). Exogenous application of the polyamine putrescine to cotton partly ameliorated the negative effects of extreme temperatures and significantly increased the total seed number (Bibi *et al*., 2008). Other potentially useful treatments include xeathin that might be expected to strengthen thylakoid membranes by inducing high levels of zeaxanthin (Havaux *et al*., 1996) or providing isoprene (Sharkey & Loret, 1993) to protect photosynthesis from moderately high temperatures. This opens the possibility that the deactivation of Rubisco is an adaptation or protective mechanism in response to high-temperature sensing by the thylakoid membrane.

1-Methylcyclopropene (1-MCP) is an anti-ethylene compound that counteracts ethylene production under stress. Kawakami *et al*. (2010) found that plants treated with 1-MCP exhibit higher maximum quantum efficiency of Photosystem II, decreased activity of antioxidant

glutathione reductase, increased cotton boll weight, and that overall, the detrimental effects of heat stress on plant growth was decreased. The effect of 1-MCP was evaluated on a heat susceptible-wheat cultivar (*Triticum aestivum* L.) and was found to enhance wheat tolerance to high temperature conditions (Hays *et al*., 2007). Although growth regulators have been used to induce or enhance protective functions in plant cells (Zhang *et al*., 2003; Horvath *et al*., 2007), when plants are subjected to more severe stress, these protective mechanisms may be inadequate.

Crop Management to Alleviate Heat Stress

Some of the adaptive measures to help relief yield reductions due to high temperatures include: replacement of heat-sensitive cultivars with heat-tolerant ones, adjustment of planting time, choosing cultivars with a growth duration allowing avoidance of peak stress periods and adapting irrigation practices, as well as the application of exogenous plant hormones (Shah *et al*., 2011). Bange *et al*. (2016) recommended that in regions where there is a significant risk of high temperature stress, cultivars that demonstrate resilience to these stresses should be considered. Brown and Oosterhuis (2010) showed that obsolete cultivars were more resilient to heat stress than modern commercial cultivars. Reddy *et al*. (1996) concluded that with warmer temperatures early in the season, shorter periods of growth might not be able to support high fruit loads because reproductive development will be quicker. Proper cultivar selection and management will be required to avoid “cutout” which will reduce yield. Irrigation is important in helping the plant mitigate the negative effects of high temperature as crop’s capacity to moderate tissue temperature through transpirational cooling is dependant upon adequate moisture supply (Bange *et al*., 2016).

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CHAPTER I

Evaluation of Screening Methods in Growth Chamber Studies

To Detect Heat Stress in Cotton Genotypes

ABSTRACT

Heat stress (HS) has become an important factor affecting cotton (*Gossypium hirsutum* L.) growth and yield. Worldwide cotton crops experience periods of high temperatures during flowering and boll development, which leads to decreased performance. The objective of this study was to assess the effect of HS on select physiological processes and to screen cotton genotypes Arkot 9704, VH260, DP393 and DP 210 B2RF for high-temperature tolerance at a 40°C heat stress and a 30°C control in three growth chamber studies. Measurements were made of membrane leakage (ML), chlorophyll fluorescence (ChlF), glutathione reductase (GR) and sucrose contents of leaves. Measurements were made at 2, 4 and 6 hours after the HS was applied, and at 3 and 7 days after HS and 7 days after recovery. Membrane leakage was increased by the 40°C heat stress compared to the 30°C control in all studies. Increases in ML from HS could be detected 2 to 6 hours after the heat stress was started and the effect was still detectable 7 days after stress and at 7 days after recovery. Genotypic differences in ML response to HS were found with DP393 being the least affected indicating heat tolerance and Arkot 9704 the most affected indicating heat sensitivity. Decreased chlorophyll fluorescence F_v/F_m values from the 40°C HS were recorded for all four genotypes in all three studies. Although genotypic difference in response to HS was variable in the three studies, DP393 had the lowest percentage

decrease in F_v/F_m compared to the 30°C control indicating tolerance to HS, and Arkot 9704 the lowest F_v/F_m during HS, showing sensitivity to HS. GR was increased by heat stress and genotypes DP393 and VH260 showed significant increases as a tolerant response to HS. There was no clear genotypic response in sucrose concentrations to HS. Genotypic differences in heat tolerance were clearly recorded with both ML and ChlF measurements, but the ChlF technique was preferable due to the ease of use, rapid measurements with immediate results, and more precise measurments.

Abbreviations. HS = heat stress; ML = membrane leakage; ChlF = Chlorophyll fluorescence; GR = glutathione reductase.

INTRODUCTION

Elevated CO_2-induced climate change will affect cotton production practices due to more frequent occurrence of heat waves (Oosterhuis, 2013). Warmer temperatures caused by global warming will have a negative effect on sustainable crop production (Bange *et al*., 2016). Heat stress has been reported as one of the most important causes of reduction of yield in cotton (Burke and Wanjura, 2010; Cottee, 2009; Crafts-Bradner *et al*., 2000; Oosterhuis, 1999; Snider, 2010; Rahman., 2006; Reddy *et al*., 1992), and a strong negative correlation has been reported for high temperature and cotton yield (Oosterhuis, 2002; Rawson, 1992; Hodges *et al*., 1993; Singh *et al*., 2007). Heat stress is defined as where temperatures are hot enough for sufficient time that they cause irreversible damage to plant function or development (Hall, 1992). Plant physiological functions during reproductive stages are affected negatively with elevated above

optimum temperatures. A better understanding of the impact of heat stress on physiological and morphological development of cotton would help in understanding the adverse effects and in developing reliable field-screening tools.

Bibi *et al.* (2008) found that with cotton high day temperatures above 36 °C caused significant decreases in the efficiency of photosystem II and showed decreases in chlorophyll fluorescence when temperature was increased to 40 °C, indicating high-temperature stress. The principle of using chlorophyll fluorescence to measure plant stress was summarized by Misra *et al.*(2009) who said that light energy that is absorbed by chlorophyll in a photosynthetic system undergoes three fates: a) to drive photosynthesis, b) dissipated as heat, or c) re-emitted as fluorescence. These three processes occur in competition and any increase in the efficiency of one process will result in a decrease in the yield of the other two. Therefore, determining the yield of ChlF will give information about changes in the efficiency of photochemistry and heat dissipation (Misra *et al.*, 2009). Karademir *et al.* (2012) found a positive relationship between seed cotton yield and ChlF when evaluating 15 upland cotton cultivars under field conditions, and concluded that increasing fluorescence measurements was a practical tool for improving seed cotton yield in large breeding trials.

Plant responses to high temperature vary with plant species and developmental stages. In most plants, the reproductive processes are markedly affected by high temperatures, which ultimately affect the fertilization processes leading to reduced crop yield (Snider *et al.*, 2009). Burke *et al.* (2004) reported that the reproductive phase of cotton is the most sensitive to high temperature stress, as pollen germination declined above temperatures of 37 °C. Pollen tube elongation showed temperature sensitivity above the optimal temperature range (Burke *et al.*, 2004; Snider

et al., 2009). Understanding how heat stress affects physiological processes would permit the formulation of strategies for screening cotton genotypes physiological responses to the withstanding effect of heat (Bibi *et al*., 2008). Murkowski (2001) reported that a reduction in chlorophyll fluorescence (F_v/F_m) reflects the damaging effect of HS on the structure and function of the photosynthetic apparatus. Cui *et al*. (2006) concluded that the tall fescue (*Festuca arundinacea*) cultivar with higher F_v/F_m values under HS had a less heat-susceptible photosynthetic apparatus. Willits and Peet (2001) did research on tomatoes (*Lycopersicon esculentum* Mill.) and concluded that ChlF was useful in identifying germplasm that demonstrates apparent heat tolerance under controlled conditions. Bibi *et al*. (2008) reported ML and ChlF as suitable methods to screen cotton for high temperature tolerance.

The objective of this study was to evaluate the effect of HS on select physiological processes that could be used as screening methods on four diverse cotton genotypes grown in growth chambers. It was hypothesized that high temperatures cause physiological responses in cotton leaves, and that these responses can be used to screen for temperature tolerant genotypes.

MATERIAL AND METHODS

Three growth chamber studies were conducted during May 2013 and 2014, and June 2015 at the Altheimer Laboratory, University of Arkansas, Fayetteville, with four diverse genotypes selected on the basis of previous reported plant responses to elevated temperatures (Bibi, *et al*., 2008; Snider *et al*., 2010; Karadimer *et al*., 2012). The pedigrees of the three genotypes Arkot

9704, VH260, DP393 and cultivar DP 210 B2RF are given in Table 1. Arkot 9704 was chosed because of its performance in the national cotton variety trials (http//rbtn.cottoninc.com/files – 2006 results). VH260 was chosen as it was identified as heat tolerant by Zhang (2013). DP393 gave good yields in Dr Bourlands trials, and DP210 B2RF had unknown tolerance to heat, and is planted as a commercial cultivar in South Africa. In each study, sixty 2 L PVC pots were filled with Sun-Gro potting mix (Sun-Gro Horticulture Distribution Inc., Bellevue, WA) and planted in two growth chambers (Model PGW36, Conviron, Winnepeg, Canada). Plants were watered daily with half-strength Hoagland solution (Hoagland and Arnon, 1950). During each of the studies, two heat treatments were compared namely a control of 30/20 °C (day/night) temperature and a heat stress treatment of 40/20 °C (day/night) temperature. The growth chambers were maintained at 30/20 °C, 80% relative humidity, 12 hour photoperiod, and 600 µmol m^{-2} s^{-1} photosynthetically active radiation (PAR). At approximately one week before flowering, half the plants were randomly selected and transferred from one growth chamber to the other. Measurements started at first flower when temperatures were elevated in 2°C increments in one of the two growth chambers to reach 40 °C by 12.00 PM. In study 1, measurements of ML, ChlF, GR and leaf sucrose were taken 3 days and 7 days after the heat stress (DAS) was applied and again at 7 days after the heat stress was removed (DAR). In study 2 and 3, measurements were taken at 2, 4 and 6 hours after heat stress and included ML and ChlF and leaf sucrose content. All ML measurements after autoclaving was cooled down to room temperature and then measured.

Table 1. Pedigree information for the genotypes used in growth chamber studies.

Genotypes	**Area of origin**	**Parent lines**
VH260	Pakistan genotype grown at temperatures of 45 °C (Zhang, 2013)	S12 x H1692 VH55 XLRA5166
Arkot 9704	Arkansas Agricultural Experiment Station (Bourland and Jones, 2009)	Ark 9108 x M331RKN
DP393	USA, Deltapine & Pineland & Co.	PVP 200400266
DP 210 B2RF	South Africa, Monsanto	DP560BGIIx2[B1][B2]/ COKER312[R2].

Membrane leakage

Membrane leakage (ML) was measured using the method of Sullivan (1971) and FitzSimons (2016). According to this method, ML was determined by sampling three 10 mm discs per plant at first flower with a cork borer. Ten plants per replicate were sampled at 11.00 AM. The samples were taken from the youngest fully expanded main-stem leaf of a plant and main and secondary veins were avoided. Leaf discs were placed in separate test tubes with 10 mL de-ionized water and rinsed three times to remove excess electrolytes. The samples were placed in the dark for 24 hours, after which electrical conductivity (EC) was measured with an EC meter (Primo 5, HANNA Instruments, USA) and recorded as the initial ionic leakage. Tubes were capped and autoclaved for 20 minutes to dissociate all cellular cytosols into solution. After cooling to room temperature, the EC was again measured as total ionic leakage. Calculations

were performed as an injury index percentage (eq. 1)   at 100 °C, and the final EC measurements were taken after cooling down to room temperature.

$$1 - \left(\frac{Final - Initial}{Initial}\right) * 100 \qquad \text{(eq. 1)}$$

Where final and initial are the EL measurements at that time.

Chlorophyll fluorescence

Chlorophyll fluorescence of attached leaves was measured with a modulated chlorophyll fluorometer OSI-FL (Opti-Sciences, Tyngsboro, MA). With this instrument, chlorophyll is excited by a 660 nm solid-state white source with filters blocking radiation longer than 690 nm. The average intensity of the modulated white light was adjusted to 1 μE. Detection was in the 700-750 nm range using a PIN silicon photodiode. All measurements were taken on the youngest fully developed mature leaf. Ten leaves of four genotypes were harvested at dawn and transported to the laboratory and stored in ziploc bags in the dark in the laboratory. Leaves were cut into 5 cm discs and measured at 5 minute intervals at temperatures of 20, 25, 30, 35 and 40 °C in Study 1, with the new Leaftech instrument (Plate 1). During study 2 and 3, measurements were conducted at 2, 4 and 6 hours after heat stress was applied.

LeafTech Measurement of Plant Response to Elevated Temperature

To quantify in situ differences in actual quantum yield (ΦPSII) temperature responses of leaves in different treatments (i.e. genotypes, fertilizer, water stress, position in canopy etc.). Heating or cooling is accomplished by placing leaves on moist filter paper in contact with 40 mm X 44 mm

X 3.3 mm thermoelectric cooler (All Electronics Corporation, Van Nuys, CA, USA) powered by a 12V battery. The bottom side of the thermoelectric device is held at ambient temperature by an off-the-shelf CPU fan/heat sink combination. When varying current is applied to the thermoelectric element, the top side rapidly changed temperature with respect to the bottom because of the Peltier effect. Temperature changes are continually monitored with a digital thermometer attached to a fine wire thermocouple (Type K) pressed against the abaxial surface of the leaf. Leaves should be continually illuminated at 500 μmol photons m-2 s-1 of growth chamber irradiance, and initially maintained at 15°C for 10 min (preliminary experiments determined that 10 min of illumination was sufficient for ΦPSII to reach a maximum value under direct and continuous irradiance). Subsequently, leaf temperature should be increased in 5°C increments up to 50°C, and ΦPSII determined after 5 min of incubation at each temperature (5 min was a sufficient period of time for ΦPSII to stabilize at a given temperature). Both the temperature at which ΦPSII is maximal (Topt) and the temperature at which ΦPSII declines 15% from Topt (T15PSII), can be determined from a best fit curve for each treatment (e.g. genotype) of ΦPSII versus leaf temperature data (Sigma Plot 10; Systat Software Inc., San Jose, CA). Fv/Fm and ΦPSII were calculated according to the equations given in Maxwell and Johnson (2000). T15PSII was used as an indication of heat stress and is comparable to the method of Froux et al. (2004), where the temperature causing a 15% decrease in Fv/Fm from a maximum value in dark-adapted leaves was considered a threshold temperature for photochemical efficiency of PSII. Representative curves illustrates how Topt (the temperature at which the highest quantum efficiency was obtained for a given leaf) and T15PSII (the temperature causing a 15% decline in ΦPSII from the value at Topt) are determined for a given cultivar or treatment.

Plate 1. Leaftech instrument with digital thermometer, leaf clip holder and cotton plants and fluorometer.

Glutathione reductase assay

Glutathione reductase activity (GR) was measured using the method of Anderson *et al*. (1992). Three leaves per pot were sampled after 7 days of heat stress and immediately placed in liquid nitrogen and transported to a -80 °C freezer. Leaf tissue was homogenized using a mortar and pestle in an ice-cold extraction solution comprised of 50 mM Pipes (1,4-Piperazine diethanesulfonic acid) buffer (pH 6.8), 6mM cysteine hydrochloride, 10mMd-isoascorbate, 1mM ethylenediaminetetraacetic acid, 0.3% Triton X-100, and 1 % (w/v) soluble Polyvinylpyrrolidone (PVP). Solutions were further blended for 1 minute in a tube containing 0.25 g insoluble PVP and 1 drop of antifoam A emulsion using a homogenizer (Model Polytron; Brinkman Instruments Inc., Palo Alto, CA). Subsequently, samples were centrifuged at 21 000 g for 20 minutes (4 °C) and the supernatants were stored at -80 °C for further determination of glutathione reductase content according to Shaedle and Bassham (1977) with modification. To

each well of a 96-well micro titration plate, a 15.7 µl aliquot of enzyme extract from each ample was added to a 300 µl reaction solution containing 50 mM Tris-HCL buffer (pH=7.5), 0.15 mM reduced nicotinamide adenine dinucleotide phosphate (NADPH), 0.5 mM oxidized glutathione, and 3 mM MgCL2. Oxidation of NADPH was determined as the decrease in absorbance at 340 nm during a 1 min reaction time using an Ascent Multiscan microplate reader (Molecular Devices Corporation, Sunnyvale, CA), and glutathione reductase activity was expressed as GR units/g fresh weight.

Sucrose

Measurements of the fourth main-stem leaf from the terminal was used to determine non-structural sucrose concentration according to the Hendrix (1993) protocol with modifications by Zhao *et al*. (2010). Five leaves per genotype were harvested after 7 days of heat stress and 7 days after recovery of the heat stress and oven dried for three days at 50 °C before analyses. Forty mg of ground leaf tissue were extracted 3 times with 80 °C aqueous ethanol (800 ml ethanol/L) and the samples were centrifuged after each extraction at 5000 rpm and finally the fraction were pooled. Active charcoal was then added to the pooled fractions in order to remove substances that could interfere with the carbohydrate measurements and the samples were centrifuged again at 3500 rpm. The supernatant was immediately stored at – 80°C for further determination of sucrose and hexose (fructose and glucose) with a Multiscan Ascent Microplate Reader (Thermo Fisher Scientific Inc., Waltham, MA).

Statistical methods

The trial design was a randomized block design with 10 replications. Comparison analysis was performed using JMP 11.1 software (SAS Institute, Cary, NC). Comparison of ML, ChlF, GR and sucrose between temperature treatments and genotypes were made using a two-way factorial and the student's t test at ($\alpha < 0.05$).

RESULTS

Membrane leakage (ML)

Growth chamber study 1

Overall when plants were heat stressed ML was significantly (P<0.05) increased compared to the control plants (Fig. 1).

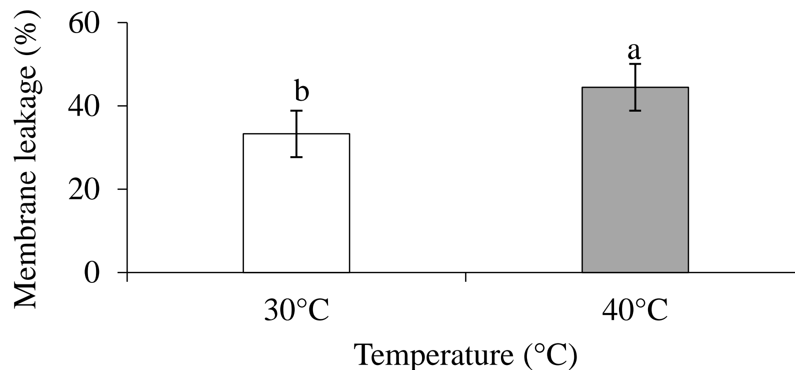

Figure 1. Membrane leakage of two heat treatments, 30°C control and 40°C heat stress in Study 1. Fayetteville, Arkansas. Treatment values not connected by the same letters are significantly different (P<0.05). Error bars indicate the standard error at $\alpha = 0.05$.

There was a significant interaction between cultivars and measuring times. Three DAS Arkot 9704 leaked the least (32 %) but not significantly different from VH260 (38.9 %). Seven DAS VH260 leaked the least (36.5 %) but not significantly different from DP210 (37.1 %) or DP393 (40.3 %). Seven days after recovery DP210 leaked the least, but not significantly different from DP393 (36.4 %), VH260 (38.2 %) and Arkot 9704 (41.0 %) (Data not shown). Measurements of ML made 3 and 7 DAS after imposing the 40°C stress at first flower showed that HS significantly increased the ML compared to the 30°C control (Fig. 2) and the effect was still maintained 7 days after relief of the HS. With a prolonged HS treatment as would occur in the field, the effects of the HS were detectable at the 3 and 7 days of the stress for all genotypes (Fig. 2). The effect of HS on ML of the three measuring times (3DAS, 7DAS, and 7DAR) meaned over the genotypes (Arkot 9704, VH260, DP393 and DP 210 B2RF) starting at first flower showed that HS increased ML of all genotypes (Fig. 3). The adverse effect of the HS was greater for Arkot 9704 than for VH260, DP393 and DP 210 B2RF.

DP393 consistently gave the lowest ML at 3 and 7 days after stress compared to Arkot 9704, VH260 and DP 210 B2RF (Fig. 4) which were similarly affected by the HS. It was suggested from these results that measurements of ML at 3 and 7 days into a HS period would gave satisfactory results. When ML was measured 7 days after stress the effects of the stress were still detectable (Fig. 2 & 4) indicating that the recovery of ML after a HS period would not provide a means of differentiating between genotypes for heat tolerance.

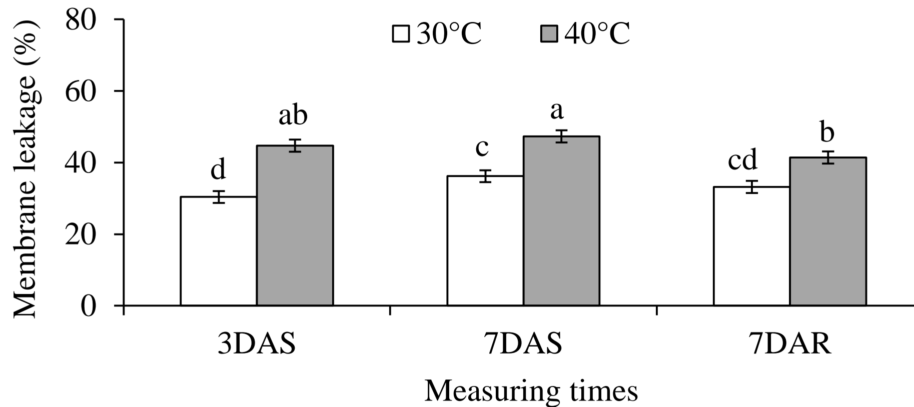

Figure 2. Membrane leakage at 3DAS, 7DAS and 7DAR measured at two temperatures, 30°C and 40°C in Study 1. Fayetteville, Arkansas. Treatment values not connected by the same letters are significantly different ($P<0.05$). Error bars indicate the standard error at $\alpha = 0.05$.

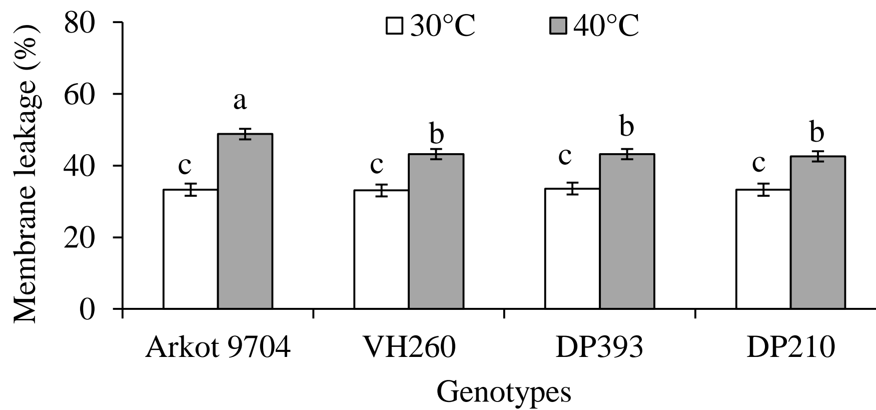

Figure 3. Membrane leakage of the four genotypes measured at two temperature regimes, 30°C and 40°C heat stress, meaned over the 3 measuring times (3DAS, 7DAS, and 7DAR) in Study 1. Fayetteville, Arkansas. Treatment values not connected by the same letters are significantly different ($P<0.05$). Error bars indicate the standard error at $\alpha = 0.05$.

The percentage increase in ML from the HS compared to the control treatment (Fig. 4) showed that at 3 DAS, DP393 showed the smallest increase in ML of 18.8% over the control, and Arkot 9704, VH260 and DP 210 B2RF were similar with larger increases in ML with HS. A smaller change in ML with heat stress shows less effect of the HS and indicates that genotype DP393 showed the most tolerance to the HS. At 7 DAS, DP393 again had the lowest % change in ML of 5.0 %. Genotype Arkot 9704 was the most affected with a 23.0% increase in ML. At 7 DAR, Arkot 9704 had the highest % change in ML, a 29.3% increase over the control, whereas VH260, DP393 and DP 210 B2RF had the lowest % increases in ML of 12.3%, 19.8% and 12.1%, respectively, indicating that Arkot 9704 had recovered less than the other three genotypes seven days after the HS was removed. DP393 consistently gave the lowest ML at 3 and 7 days compared to Arkot 9704, VH260 and DP 210 B2RF (Fig 4) which were similarly affected by the HS.

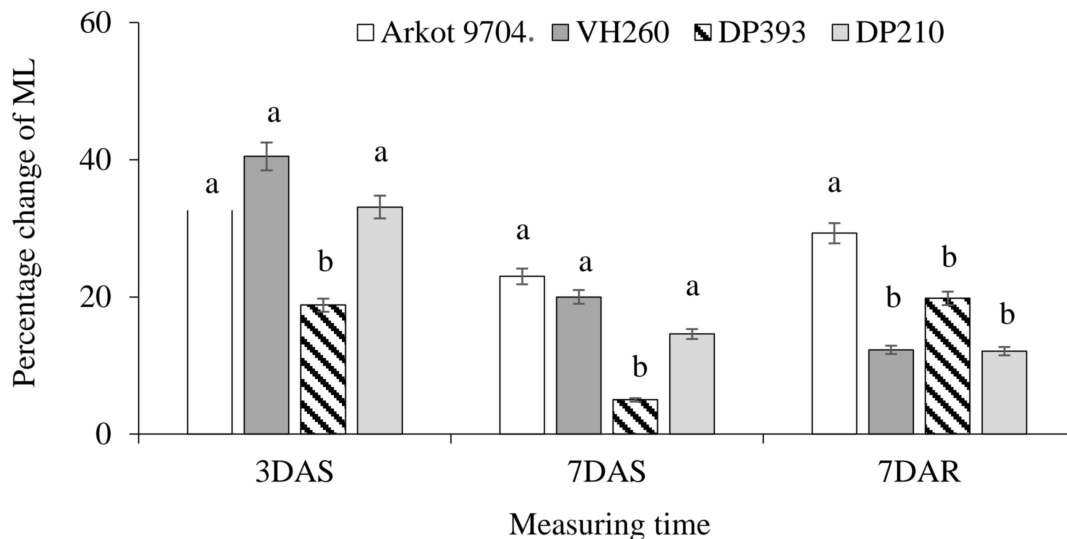

Figure 4. Percentage change in membrane leakage of the heat stress treatment compared to the control in Study 1 of four cotton genotypes, Arkot 9704, VH260, DP393 and DP 210 B2RF, at three measuring times, 3DAS, 7DAS and 7DAR. Treatment values for each measuring time not connected by same letters are significantly different ($P<0.05$). Error bars indicate the standard error at $\alpha = 0.05$.

Growth chamber study 2 and 3

There were inherent genotypic differences in Study 2 in ML at 30°C (Fig. 5A) as may be expected with DP 210 B2RF exhibiting the highest ML. At 40°C ML was significantly increased in Arkot 9704 and VH260, but not in DP393 and DP 210 B2RF (Fig. 5 A). In study 3 (Fig. 5B) Arkot 9704 and VH260 again showed sensitivity towards HS with significantly increased ML from 30°C to 40°C compared to DP393 and DP 210 B2RF which were not significantly affected by the HS.

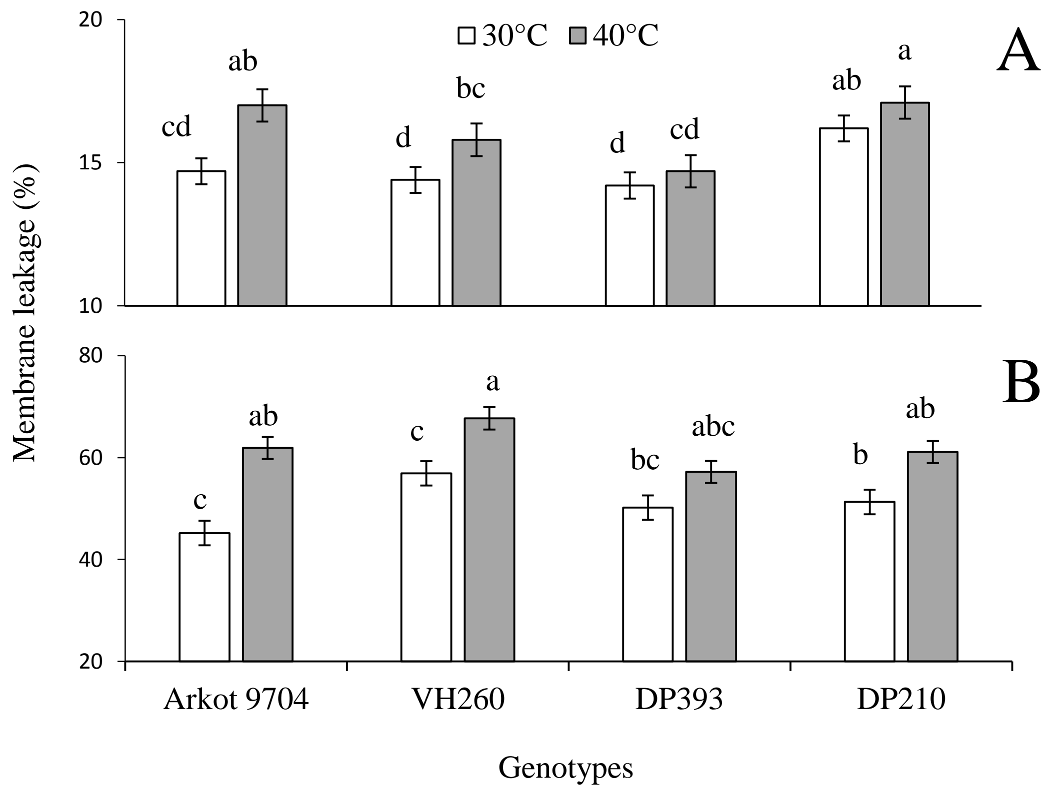

Figure 5. Membrane leakage in (A) Study 2 and (B) Study 3 of four cotton genotypes, Arkot 9704, VH260, DP393 and DP 210 B2RF, at two temperatures, control 30°C and heat stress 40°C, meaned over measuring times, 2, 4 and 6 hours. Treatment values not connected by same letters are significantly different ($P<0.05$). Error bars indicate the standard error at $\alpha = 0.05$.

When HS was applied in the growth room and the effect on ML measured the same day at 2, 4 and 6 hours after the start of the HS, there were significant increases in ML at all the measuring times (Fig. 6&7). All four genotypes showed increased ML with HS. There were, however some variation in genotypic response. DP393 had significantly lower ML than Arkot 9704, VH260 and DP 210 B2RF after 2, 4 and 6 hours, whereas Arkot 9704 generally gave the highest ML of the genotypes. In Study 3, the effects of the heat stress were variable at 2 hours, but showed similar trends at 4 and 6 hours (Fig. 7). It was concluded that ML should be measured at least 6 h after the imposition of the HS for more reliable and consistent results, as the longer period of HS showed the most damage.

In Study 2 (Fig. 6) percentage change showed that DP393 had the lowest change in ML from the control treatment (30°C) compared to the HS treatment (40°C). Percentage change in ML in Study 3 (Fig. 7) showed that after HS was applied for 2 hours, VH260 (5.1%) gave the lowest change in ML, but after 4 hours and 6 hours of HS, DP393 had the lowest percent change in ML of 6.6% and 7.4 %, respectively. These results indicate that DP393 exhibited more tolerant behaviour towards high temperature after 4-6 hours of heat stress.

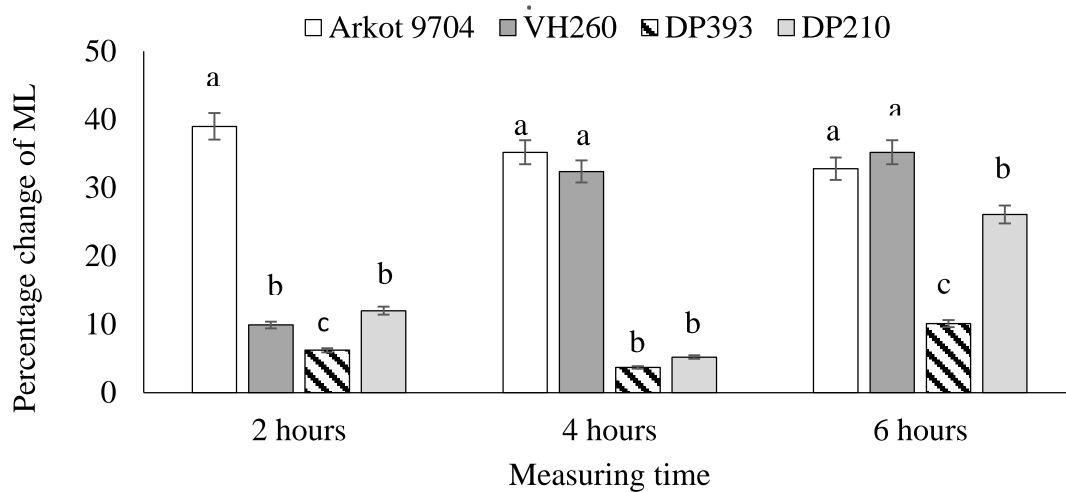

Figure 6. Percentage change in membrane leakage (ML) of the heat stress treatment compared to the control in Study 2 of four cotton genotypes, Arkot 9704, VH260, DP393 and DP 210 B2RF, at three measuring times, 2, 4 and 6 hours after the start of the 40°C heat treatment. Treatment values for each measuring time not connected by same letters are significantly different (P<0.05). Error bars indicate the standard error at α = 0.05.

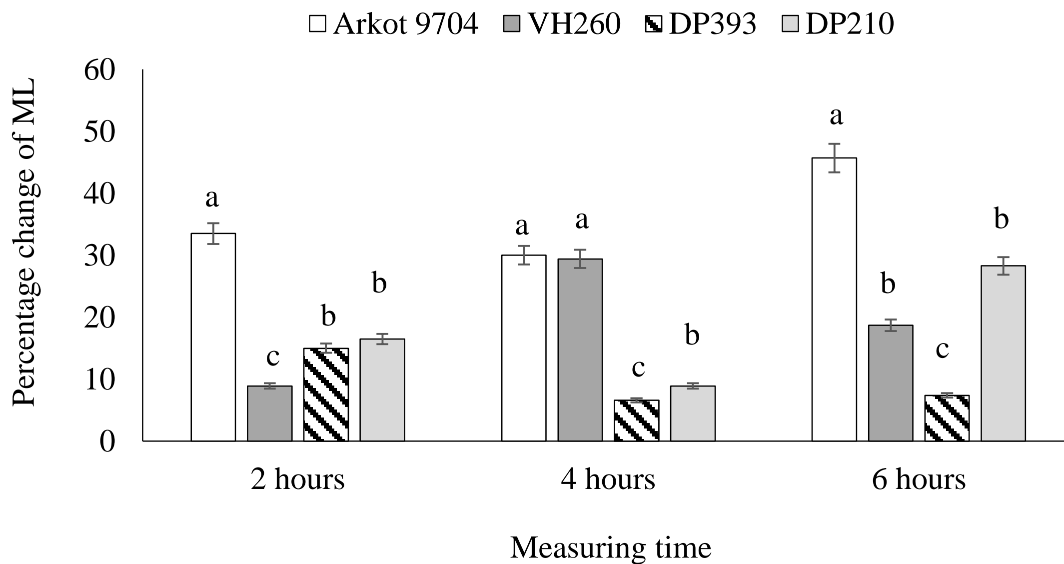

Figure 7. Percentage change in membrane leakage (ML) of the heat stress treatment compared to the control in Study 3 of four cotton genotypes, Arkot 9704, VH260, DP393 and DP 210 B2RF, at three measuring times, 2, 4 and 6 hours after HS. Treatment values for each measuring time not connected by same letters are significantly different (P<0.05). Error bars indicate the standard error at α = 0.05.

Chlorophyll fluorescence

Chlorophyll fluorescence (F_v/F_m) of leaves of four genotypes was measured with the Leaftech instrument in 5°C increments from 20 to 40°C. Chlorophyll fluorescence increased from 20 to 25°C, and was similar at 25 and 30°C, but decreased significantly at 35°C and at 40°C (Fig. 8). The use of 30°C in a control and 40°C as the heat stress treatment was adopted in all other experiments in these studies.

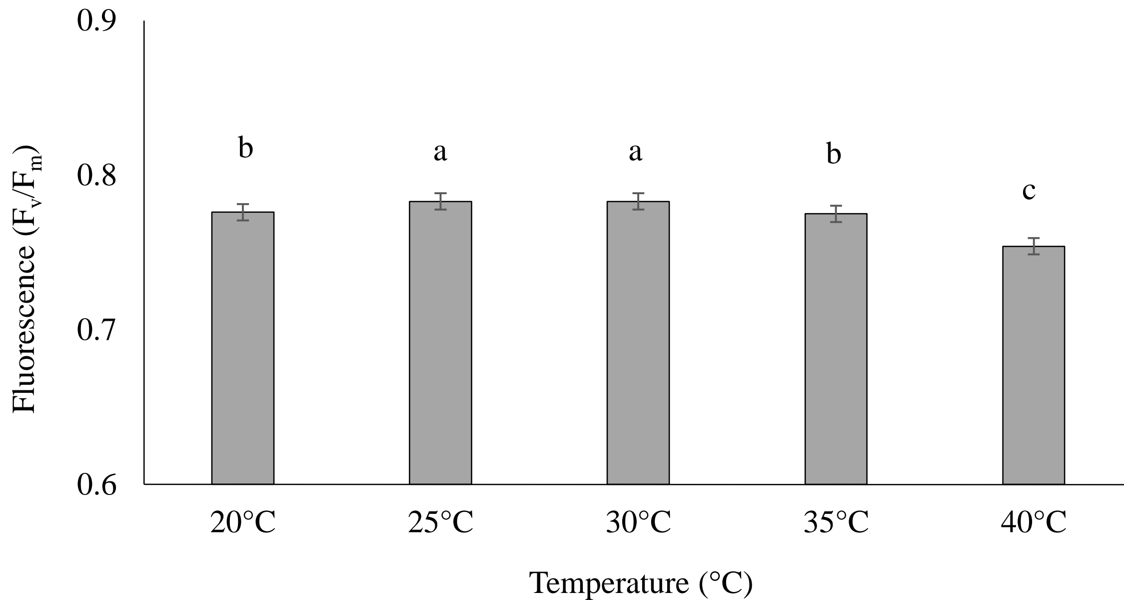

Figure 8. Chlorophyll fluorescence (F_v/F_m) measured in Study 1 at temperatures 20, 25, 30, 35 and 40°C measured on leaves meaned over genotypes. Treatment values not connected by same letters are significantly different ($P<0.05$). Error bars indicate the standard error at $\alpha = 0.05$.

Chlorophyll fluorescence decreased significantly for all four genotypes from the 30°C control to the 40°C heat stress (Table 2). The ratio of F_v/F_m in a healthy plant ranges from 0.78 to 0.84 (Bjorkman & Demmig 1987). Arkot 9704 decreased in F_v/F_m values from 0.773 to 0.750,

VH260 decreased from 0.760 to 0.737, genotype DP393 decreased from 0.755 to 0.737, and cultivar DP 210 B2RF decreased from 0.764 to 0.741. Changes in F_v/F_m (Table 2), between the 30°C control and the 40°C HS indicate that DP393 (2.44%) had the lowest percentage change in F_v/F_m compared to Arkot 9704 (3.07%), VH260 (3.12%) and DP 210 B2RF (3.10%) indicating that DP393 was the least effected by the heat stress.

Table 2. Chlorophyll fluorescence (F_v/F_m) of four cotton genotypes at five different temperatures and the change in fluorescence from 30 to 40°C in Study 1. Fayetteville, Arkansas.

Temperature	Chlorophyll fluorescence (F_v/F_m)			
(°C)	Arkot 9704	VH 260	DP393	DP 210 B2RF
20	0.781a[1]	0.769bc	0.765b-e	0.748gh
25	0.774ab	0.756d-g	0.758c-g	0.761cde
30	0.773ab	0.760c-f	0.755d-g	0.764b-e
35	0.765bcd	0.754d-g	0.754efg	0.754e-g
40	0.750fgh	0.737i	0.737i	0.741hi
% Change (30-40°C)[2]	-3.07a	-3.12a	-2.44b	-3.10a

[1] The same letters for each genotype at each temperature in a row indicates no significant difference between treatments ($P < 0.05$). [2] % Change values with the same letters in the row do not differ significantly ($P < 0.05$).

When measured at 2, 4 and 6 hours after the heat treatment was imposed, F_v/F_m was significantly decreased with the lowest F_v/F_m value after 6 hours (0.630) of HS, compared to the 0.790 for the 30°C control (Fig. 9), indicating that the longer the plants were stressed, the more damage occurred in PSII efficiency. Chlorophyll fluorescence of the control and HS treatments were similar at 3DAS, but decreased significantly at 7DAS (Fig 10) indicating that the most damage to PSII occurred after 7 days of high temperature. At 7DAR the F_v/F_m was similar showing that the

fluorescence had recovered to control levels by 7 days after the plants were returned to the 30°C control temperature.

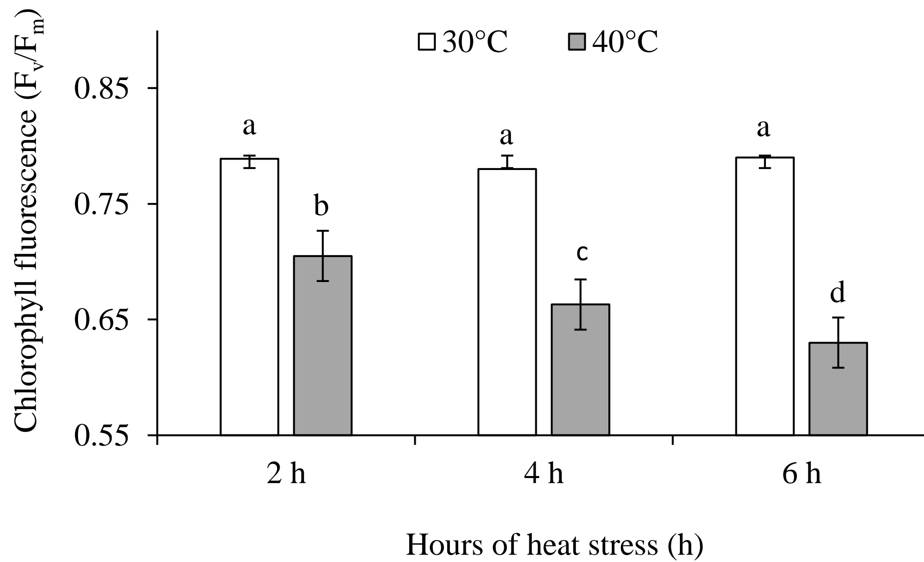

Figure 9. Chlorophyll fluorescence (F_v/F_m) in Study 3 at three measuring times; 2, 4 and 6 hours after application of the heat treatment at first flower, meaned over genotypes. Treatment values not connected by same letters are significantly different (P<0.05). Error bars indicate the standard error at α = 0.05.

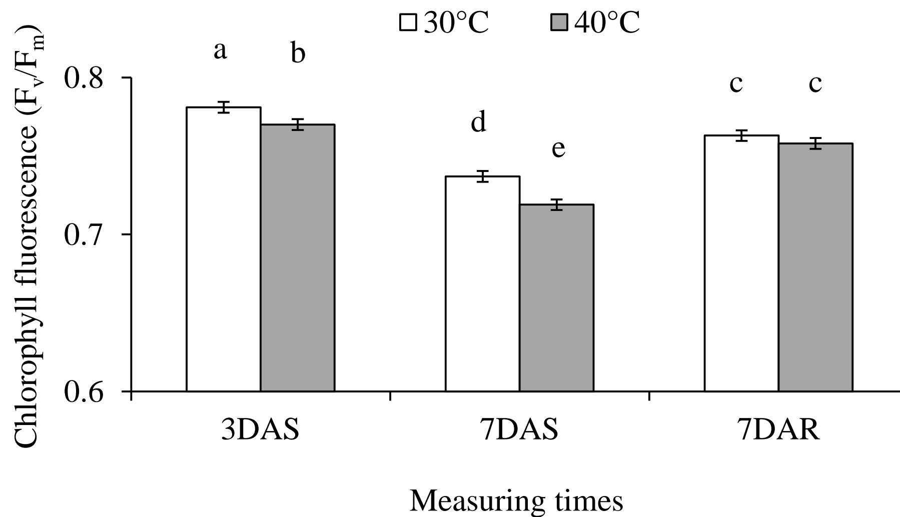

Figure 10. Chlorophyll fluorescence (F_v/F_m) in Study 1 for heat treatment x measuring times after applying the heat stress meaned over genotypes. Treatment values not connected by same letters are significantly different (P<0.05). Error bars indicate the standard error at α = 0.05.

In Study 2, (Fig. 11A) when the four genotypes was evaluated at the control (30°C) and the HS (40°C) treatment, Arkot 9704 resulted in the lowest F_v/F_m ratio of 0.628 at 40°C, showing sensitivity to HS. This was not significantly lower than DP393 (0.659), but significantly different from VH260 (0.691) and DP 210 B2RF (0.686) (Fig. 11A). In Study 3, (Fig. 11 B) when genotypes were heat stressed, all four genotypes resulted in significant decreases in F_v/F_m ratios. Arkot 9704 had the highest percentage change of -35.8%, showing sensitivity to the high temperature treatment. DP393 decreased only -6.6%, indicating tolerance to the 40°C heat treatment (Fig. 11B). The interaction genotypes x measuring times differed significantly with the least damage of HS at 4 hours after HS at DP210 (0.720) and 2 hours of HS at genotypes DP393 (0.701) (Data not shown).

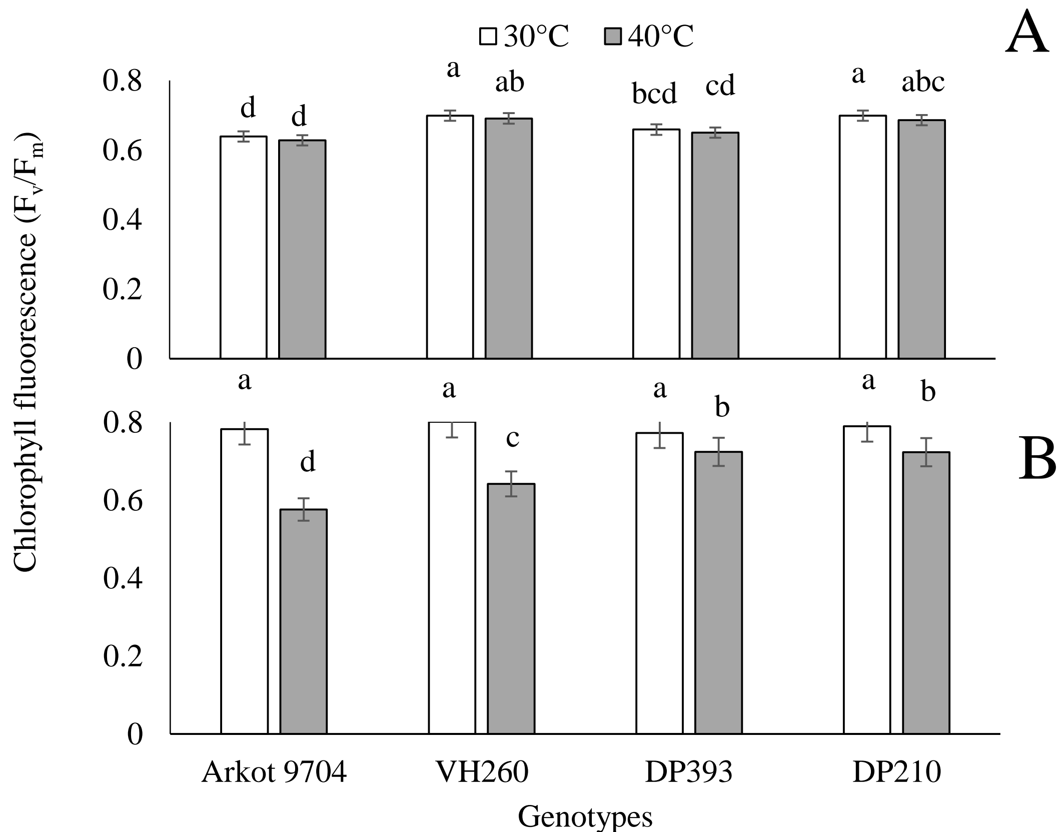

Figure 11. Chlorophyll fluorescence (F_v/F_m) in (A) Study 2 and in (B) Study 3 for four genotypes (Arkot 9704, VH260, DP393 and DP 210 B2RF) at control (30°C) and heat stress (40°C) treatments, meaned over measuring times, 2, 4, and 6 hours. Treatment values not

connected by same letters are significantly different (P<0.05). Error bars indicate the standard error at α = 0.05.

In Study 3, genotypes x heat treatments x measuring time differed significantly (Table 3). Two hours after HS, DP 210 B2RF (0.779), DP393 (0.754) and VH260 (0.729) significantly outperformed Arkot 9704 (0.558) (Table 5). After 4 hours of HS DP 210 B2RF (0.753) and after 6 hours DP393 (0.695) performed the best with the highest F_v/F_m values indicating that it had the least damage to PSII efficiency. Percentage change after 2 hours was the lowest at DP 210 B2RF (-0.5%), but after 4 and 6 hours of HS, DP393 consistently had the lowest % change of -3.7%, and -10.1%, indicating tolerance to the 40°C heat stress (Table 5).

Table 3. Chlorophyll fluorescence of four cotton genotypes at two temperature treatments and three measuring times. Study 3, Fayetteville, Arkansas.

Measuring	Heat	Chlorophyll fluorescence (F_v/F_m)			
Time (hours)	Treatment	Arkot 9704	VH260	DP393	DP 210 B2RF
2	Control	0.776ab[1]	0.801a	0.801a	0.775ab
	Heat stress	0.558c	0.729b	0.754ab	0.779ab
	% Decrease[2]	-39.0a	-9.9b	-6.2b	-0.5c
4	Control	0.783a	0.793a	0.750ab	0.792a
	Heat stress	0.579c	0.599c	0.723b	0.753ab
	% Decrease	-35.2a	-32.4a	-3.7b	-5.2b
6	Control	0.786a	0.807a	0.765a	0.803a
	Heat stress	0.592d	0.597cd	0.695b	0.637c
	% Decrease	-32.8a	-35.2a	-10.1c	-26.1b

[1] The same letters for each genotype in each study and at every heat treatment in a row indicates no significant difference between treatments (P < 0.05). [2] The same letters for percentage change in a row for each measuring time do not differ significantly (P < 0.05).

Glutathione reductase

Measurements of GR were done in Study 1 after 7 days of HS. There were significant difference between heat treatments, with the HS treatment (40°C) resulting in the highest GR activity of 49.2 Units g^{1} FW compared to the 11.0 Units g^{1} FW at the control (30°C) (Fig. 12). These results are in accordance with research done by Snider *et al*. (2010) who also found increases in GR with heat stressed cotton genotypes.

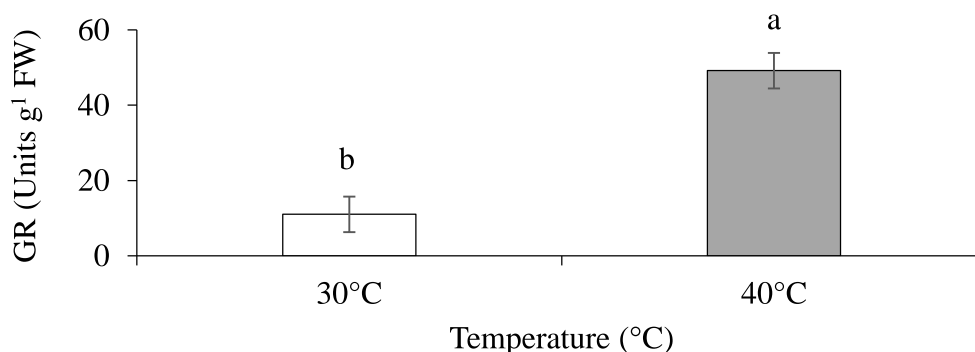

Figure 12. Glutathione reductase activity in Study 1 of two heat treatments, 30°C and 40°C, after 7 days of HS. Treatment values not connected by the same letters are significantly different ($P<0.05$). Error bars indicate the standard error at $\alpha = 0.05$.

The interaction heat treatment x genotype differed significantly (Fig. 13) with DP393 at the 40°C showing the highest GR activity and the largest increase in GR with HS compared to the other three genotypess. Genotypes differed significantly with DP393 that resulted in the highest GR activity (93.8 Units g^{1} FW), compared to Arkot 9704 (49.6 Units g^{1} FW), VH260 (45.9 Units g^{1} FW) and DP 210 B2RF (7.4 Units g^{1} FW). This shows that DP393 had the best ability to accumulate the antioxidant GR to protect its cells from heat damage. High levels of GR activity

is seen as a general feature of enhanced oxidation within a tissue (Foyer and Noctor, 2005). Increased antioxidant levels have been attributed to increased protection from the damaging effects of both biotic and antibiotic stresses (Wahid *et al*., 2007) and Snider *et al*. (2010) for cotton.

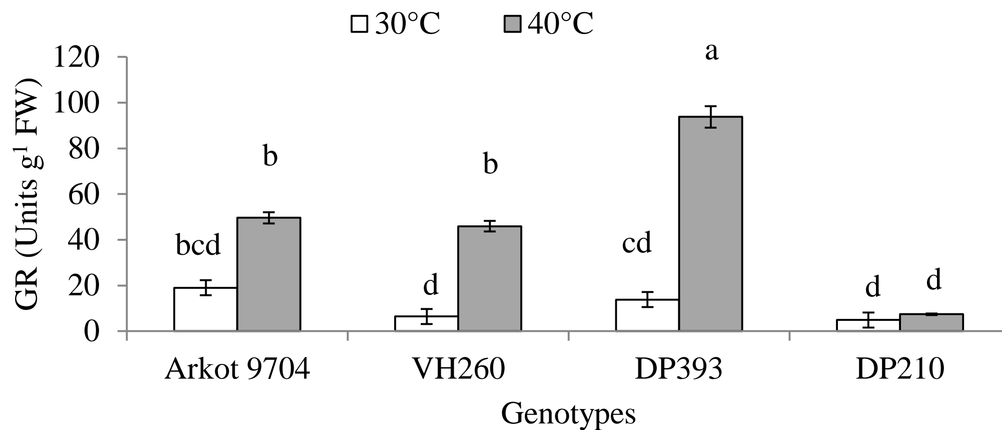

Figure 13. Glutathione reductase (GR) activity in Study 1 of four genotypes at two temperature regimes, 30°C and 40°C heat stress, after 7 days of heat stress. Treatment values not connected by the same letters are significantly different ($P<0.05$). Error bars indicate the standard error at $\alpha = 0.05$.

Sucrose

Non-structural sucrose were measured in study 1. Sucrose concentrations in the 30°C control did not differ significantly among genotypes (Fig. 14A). However after 7 days of heat stress at 40°C sucrose levels were significantly enhanced in genotypes Arkot 9704 but not in VH260, DP393 and DP 210 B2RF. Harsh *et al*. (2016) also found increases and contrasting decreases in 5 genotypes in total sugar content in 37 genotypes of moth bean (*Vigna aconitifolia*) and concluded that the increment in total sugars may be due to inhibition of sucrose synthase or invertase activities. This over accumulation of sucrose is regarded as a basic strategy for the

protection and survival of plants under abiotic stress (Chen *et al*., 2007). After 7 days after recovery (Fig. 14B), when the heat treatment plants were allowed to recover by returning them to the 30°C control, sucrose levels in all genotypes had declined below the control levels (Fig. 14B), although DP393 had the lowest percentage difference from the control, re affirming previous results that DP393 showed heat tolerance by the ability to adjust carbohydrate levels more rapidly in response to the heat stress and return to pre-stress levels upon relief of the stress. Increases in sucrose levels with heat stress was explained by Goldschmidt (1992) and FitzSimons (2016), that sucrose import deficiencies may be a plausible reason for increased sucrose levels. Snider, (2010) found in research with cotton that either cell wall invertase or the apoplastic sucrose importer mechanisms may be impaired by high temperature. FitzSimons (2016) further found steep increases in sucrose concentrations during HS at anthesis in cotton that suggested that high temperature places constraints on proper carbon partitioning. Sucrose appears to have been used and depleted from leaves after relief of the heat stress. These variable responses in sucrose concentration of the genotypes to heat stress are difficult to explain but suggests different genotypic responses in carbohydrate metabolism and partitioning with stress. Measurement of sucrose levels in leaves does not seem to offer a mean of detecting heat tolerances in genotypes.

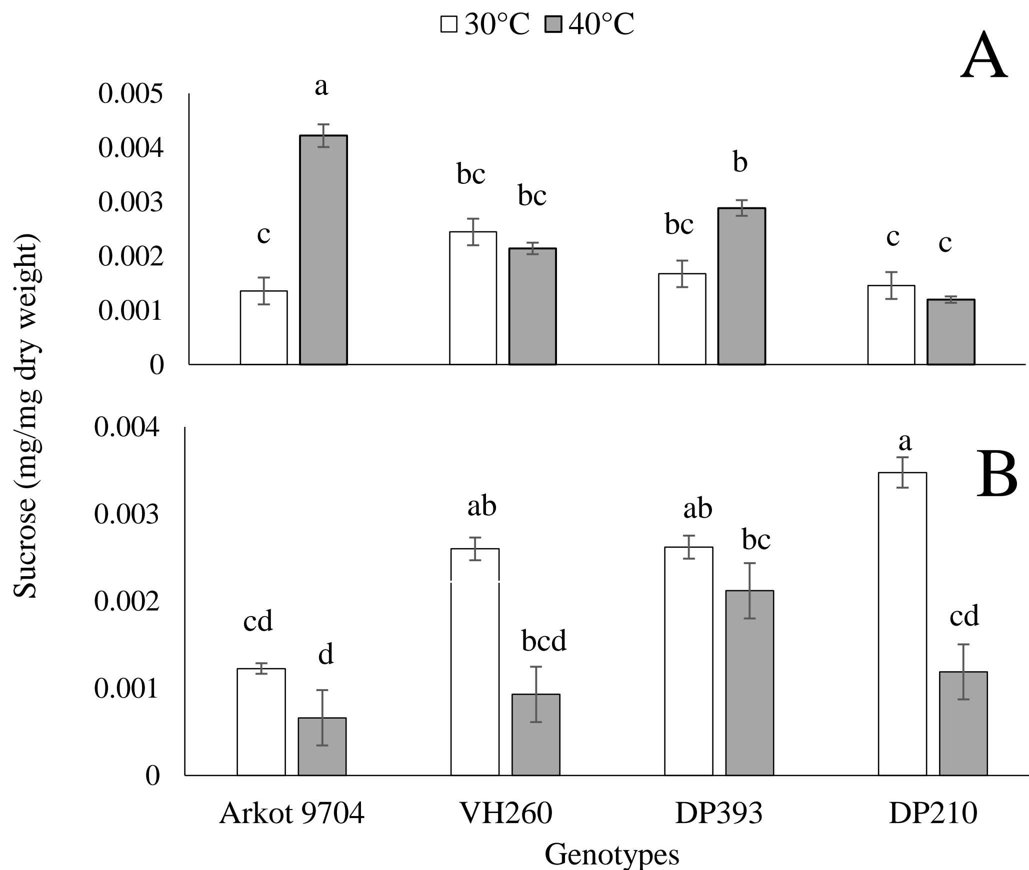

Figure 14. Sucrose concentration at (A) 7 days after heat stress, and (B) 7 days after relief of the stress of four cotton genotypes at two temperature regimes, 30°C control and 40°C heat stress. Treatment values not connected by same letters are not significantly different ($P < 0.05$). Error bars indicate the standard error at $\alpha = 0.05$.

DISCUSSION

High temperature has been considered one of the most important environmental factors that affect growth and development of plants (Mohamed & Abdel-hamid, 2013). Rising global temperatures from global warming are resulting in heat stress for various agricultural crops in traditional growing regimes limiting growth and metabolism, and leading to significant loss of

yield potential worldwide (Kaushal *et al*., 2016). Future cotton production is likely to occur under an increased prevalence of multiple abiotic stresses, including extreme and prolonged high temperature (Dabbert and Gore, 2014). Cotton has been shown to be sensitive to high temperatures, particularly during the flowering stage (Oosterhuis, 1999; Snider, 2010, Reddy *et al*., 1992) resulting in fruit abscission, smaller bolls and decreased yields (Reddy, 1999). Quantitative measurements of physiological functions would provide information permitting the ability to screen genotypes for temperature tolerance. Various physiological measurements to evaluate genotypic tolerance in crops have been studied including ML, molecular response, ChlF, antioxidants and pollen viability (Bibi *et al*., 2008; Cottee *et al*., 2012; Wu, 2013; Fitzsimons 2016; Saadalla *et al*., 1990, Burke *et al*., 2004) with varying success. Of these measurements, the two that offer the best means of detecting differential responses of cotton genotypes to heat stress appear to be ML (Bibi *et al*., 2008; Azhar *et al*., 2009; Rahman *et al.,* 2006) and ChlF (Bibi *et al*., 2008; Wu, 2013; Zhang, 2013).

The membrane leakage method developed by Sullivan (1971) has been used to detect heat stress in crops such as wheat (Sadaalla *et al*., 1990); rice (Tripathy *et al*., 2000); soybean (Martineau *et al.,* 1979); cowpea (Thiaw and Hall, 2004) and cotton (Azhar *et al*., 2009). Saadalla *et al*., (1990) reported heat tolerant wheat genotypes with low ML out-yielded sensitive genotypes by 19% under field conditions, and Bibi *et al*. (2008) reported that ML was an easy and practical method that could be used to screen for heat tolerance in cotton genotypes. Azhar *et al*. (2009) also found a strong negative association of ML with fiber length and micronaire which further verifies the utility of this trait for selecting for heat tolerant cotton. Although several studies has shown a positive association between ML and yield in cotton (Rahman *et al*., 2006), other

studies did not show a strong relationship between ML and reproductive traits (Kakani *et al*., 2005).

In the current study, HS (40°C) increased the ML of all four genotypes at all three measuring times, 3 and 7 DAS and 7DAR, compared to the 30°C control. At 7DAS and 7DAR, genotype Arkot 9704 consistently gave higher ML than VH260, DP393 and DP 210 B2RF, indicating sensitivity to HS (Fig. 4). DP393 gave significantly lower ML than Arkot 9704, VH260 and DP 210 B2RF after 2, 4 and 6 hours of HS as well as after 6 hours of HS in Study 3 (Fig. 6). When HS was applied and measurements of the four genotypes made at 2, 4 and 6 hours after applying the HS, DP393 showed the least ML at all three measuring times in study 2, but only at 6 hours in study 3. It was concluded measurement of ML should be made at least 6 hours after the imposition of the HS in order to get the desired effect of the HS on plant damage.

There was in an indication that DP393 showed some heat tolerance by a smaller change in ML compared to the other genotypes, but Arkot 9704, VH260 and DP 210 B2RF did not show any appreciable and consistent protection of membranes, i.e. smaller increase in ML under heat stress (Fig. 4). The lack of response in ML of genotype VH260 was unexpected and disappointing as it is a genotype that was developed in Pakistan to grow in warm environments (i.e. >40°C). Another reason why ML results in the growth chambers were variable could be because of the very short duration of the heat stress (2-6 hours) which may not have been sufficient for a significant plant response to be manifested. There was some indication of a recovery or acclimation 6 hours after the start of the heat stress, as was suggested by FitzSimons (2016).

This study showed that ML did indicate the damage from high temperature stress and could be used to differentiate between genotypes for heat tolerance. However, some variability and inconsistency of the results of ML under HS was observed. This could be due to inadequate sample size because of limited genotype replication in controlled environment chambers for significant responses to HS. This is in accordance with Srinivasan *et al*. (1996) who did research on ML on groundnut, soya bean, pigeon pea and chickpea, and found ML to be a sensitive test to evaluate heat tolerance but recommend that high replication was necessary in order to achieve a small standard error and that a minimum of eight discs per leaf needed to be sampled to reduce variability within the genotype. Martineau *et al*. (1979) also found with research on soybean that the ML technique required large numbers of replication to achieve a sufficiently small standard error, but then concluded that ML showed promise as a screening method. Abro *et al*. (2015) evaluated 58 cotton genotypes including a standard check genotype Sadori and concluded that ML was a useful technique in identifying heat tolerant genotypes. Roy and Basu (2009) reported that heat tolerant plant species tend to have a higher percentage saturated fatty acids in membranes and that ML measured as the conductivity of electrolytes leaking from leaf disks at HS can be used as a technique for selecting heat-tolerant genotypes. These results indicate that measurements should be made after 6 hours of the imposition of the HS treatment and start 3 days after a prolonged heat treatment. An advantage of using membrane leakage as screening technique is that it is easy and inexpensive, and it could be used to measure larger populations for heat tolerance.

Chlorophyll fluorescence (F_v/F_m) has been reported to be a rapid and reproducible method to measure for stress symptoms (Srinivasan *et al*., 1996). In my study heat treatment (40°C)

significantly decreased F_v/F_m compared to the control. Increases in variable fluorescence in heat-stressed samples was attributed to a decrease of electron transport between the OEC and the reaction centers of PSII (Srivastava & Strasser, 1997). The 40°C temperature significantly decreased F_v/F_m ratio compared to 25, 30 and 35°C (Fig. 8). A temperature of 30°C has been shown to be an optimum for photosynthesis (Reddy *et al*., 1999), and my study showed that 40°C provided a suitable high temperature treatment for a significant effect on F_v/F_m compared to the optimal (control) temperature of 30°C. Bibi *et al*. (2008) also showed significant effects >35°C on chlorophyll fluorescence, as did Brown and Oosterhuis (2010) at >38 °C. My results (Fig. 8) confirm that a high temperature of 40°C was sufficient to elicit a significant response. The temperature of 40°C was used as the HS treatment in all the studies reported in this thesis.

When F_v/F_m was measured on the same day that the HS occurred at 2, 4 and 6 hours after the initial imposition of the HS, significant decreases in F_v/F_m occurred at all three times compared to the control (Fig. 9), with increasing severity of the stress from 2 to 6 hours. However, when HS was applied for a longer period, the effect on F_v/F_m was clearly detectable after 7 days after stress (Fig. 10). At 7 days after recovery there were no detectable differences between the two treatments, indicating that a full recovery of cell integrity had occurred. These findings suggest that for a short one day HS period, measurements of F_v/F_m could be made at 2 to 6 hours, but for a longer heat stress, F_v/F_m should be measured at 7 days after stress.

Decreases in ChlF were obtained when genotypes were subjected to HS (Fig. 11, Table 3). This is in agreement with research in cotton by Bibi *et al*. (2008); Wu, (2013) and Zhang (2013) who recorded genotypic differences in Fv/F_m in response to HS. Bibi *et al*. (2008) found that an

increase in temperature from 30.0°C to 33.0°C did not affect F_v/F_m significantly, however, at 36°C and above, F_v/F_m decreased significantly. They have identified two Acala genotypes, Rex, and ST474 that were not significantly affected by high temperature, indicating greater tolerance to heat. Wu (2013) found that based on selection by F_v/F_m measurements, it was clear that wild accessions of cotton were more tolerant to heat than a set of random accessions and check genotypes in a growth chamber and concluded that ChlF is a broadly based, high throughput method capable of assaying the physiological status of heat tolerance and as such may be a useful screening tool for identifying useful stress tolerant resources. Zhang (2013) evaluated two heat tolerant Pakistani cotton genotypes, VH260 and MNH456 compared to two heat-susceptible genotypes ST213 and ST4288B2F, originating from the Mississippi Delta Region and found no obvious differences in photochemical efficiency of photosystem II in the four lines, however the heat susceptible genotypes showed greater ML after heat treatment as compared to the heat tolerant lines. Wilson & Greaves (1990) suggested that a large number of 10 – 12 measurements per replicate, and 5 replicates per measurement was required for ChlF analysis to reduce variability and to adequately detect genotypic variation. Sharma (2012) screened 1274 rice genotypes and found the control plants to have a high F_v/F_m of 0.82, but that F_v/F_m gradually decreased with severity and duration of HS. In my study, we found consistent decreases in F_v/F_m after HS with genotype Arkot 9704, and this genotype showed some heat sensitivity. DP393 was not affected as much by HS as the other three genotypes, as it showed consistently lower percentages change in F_v/F_m, showing it is a more heat tolerant genotype in agreement with yield results from Arizona in national variety trials.

Glutathione reductase activities in cotton have been shown to increase with high temperature (Bibi *et al*., 2005). Heat stress (40°C) resulted in increased GR activity compared to the control (30°C) (Fig. 12). Genotypes differed significantly with DP393 having the highest GR activity compared to Arkot 9704, VH260 and DP 210 B2RF (Fig. 13). This showed that DP393 had the better ability to accumulate the antioxidant GR to protect its cells from heat damage (Snider *et al*., 2010). However as a screening tool, measuring of GR is very time consuming and expensive and cannot be recommended as a practical screening tool.

Paupiere *et al*. (2014) reported in a review that sucrose increases when plants are heat-stressed. Sucrose concentrations in the 30°C control did not differ significantly between genotypes (Fig. 14A). However after 7 days of heat stress at 40°C sucrose levels were enhanced in genotype Arkot 9704 but not in VH260, DP393 and DP 210 B2RF. After 7 days after recovery (Fig. 14B), when the heat treatment plants were allowed to recover by returning them to the 30°C control temperature, sucrose levels were decreased in all genotypes. Sucrose is an energy source required for plant function and has a role in maintaining osmotic balance, stress signalling and in protecting membranes (Paupiere *et al*., 2014). However, in my studies although sucrose concentration was increased by HS in leaves, there were no significant differences between the genotypes studied, and therefore measurements of sucrose did not provide a means of detecting heat tolerance in cotton genotypes.

My results show some limited differences in heat tolerance in the four genotypes studied. This may be because of a lack of inherent genotypic variation in the genotypes studied. It has been suggested that there does not appear to be sufficient genotypic differences in the current upland

cotton breeding trials grown in the US Cotton Belt for exploitation by plant breeders for improved thermotolerance (Oosterhuis *et al*., 2009). Three of the four genotypes used in the present study were developed in the USA (Table 1) and although researchers have documented genotypic thermotolerance in cotton (Cottee *et al*., 2007; Taha *et al*., 1981; Brown and Zeiher, 1998), this was not clearly observed in the current study. Researchers have shown that modern cultivars have less thermotolerance compared to obsolete, i.e. > 30 years old cultivars (Brown and Oosterhuis, 2010) and wildtype cotton (Bibi *et al*., 2008). Modern cultivars have increased variability in yields in years with extreme temperatures, especially when the stress occurs during reproductive development (Oosterhuis, 1999). However, modern cultivars have greatly increased yields which also contributes to higher variability.

In conclusion, the current study investigated the use of ML and ChlF as techniques to screen cotton genotypes for temperature tolerance. Measuring ML at a control and a high temperature did differentiate between genotype responses to HS, but results were variable probably due to inadequate sample number because of the limitation of the number of plants that could be grown in the growth chambers. ML as a screening technique for heat tolerance in growth chambers is time consuming, but practical and inexpensive. Measurement of ChlF proved to be useful in identifying genotypic responses to heat stress, but as with ML there were limitations due to the amount of plants and replications in the growth chamber. Chlorophyll fluorescence as a screening method for high temperature tolerance has the advantage of providing immediate results with the fluorometer without the need for further laboratory procedures as with membrane leakages. These studies suggest that ChlF is the preferable method for screening for high

temperature tolerance. However, constrictions in growth room studies with limitations of the number of plants per treatment for sufficient replication effects both ChlF and ML procedures.

Both ML and ChlF measurements would be more feasible in the field where higher sample number and replicates can be utilized. However the difficulty in field studies is the lack of a control to compare with the high temperature stress. It is suggested that due to the rapid response of cotton plants to a high temperature (2 to 6 hours) (Fig. 9) measurements in the field could be made early morning i.e., at sunrise (6.00 AM) when temperatures are low to provide the control, and again at six hours later, i.e., at 2.00 PM, to provide the high temperature treatment. In the current studies, DP393 was the best performing genotype with the least change in ML and ChlF with HS and was identified as having heat tolerance. This study provided valuable information regarding the techniques for identification of genotypes with better tolerance to heat stress for selection of potentially high yielding cotton genotypes. Higher temperatures adversely influence the growth, development and yield of cotton, and with the increased concern about global warming, this has focused attention on the need for enhanced thermotolerance in commercial genotypes. It is therefore essential to continue research to quantify heat tolerance in cotton in field studies with the most appropriate techniques.

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CHAPTER II

Chlorophyll *a* Fluorescence as an Indicator of Temperature Tolerance in Cotton Genotypes

ABSTRACT

Cotton (*Gossypium hirsutum* L.) is sensitive to high temperatures during reproductive development, but information is lacking on genotypic tolerance to heat stress (HS). To evaluate tolerance to heat stress in cotton, chlorophyll *a* fluorescence (ChlF) induction kinetics were investigated in four diverse cotton genotypes (Arkot 9704, VH260, DP393 and DP 210 B2RF) in a 30°C control and a 40°C heat stress in two glasshouse studies at Rustenburg, South Africa during 2016 and 2017. Heat stress measurements of functions of the fluorescence response to heat stress were evaluated including fluorescence intensity, maximum efficiency of photosystem II (F_v/F_m), performance index per absorption basis (PI_{ABS}) and (ET/CS). Plants at the pinhead square stage were subjected for 6 hours to two temperature treatments, a 30°C control and 40°C HS treatment. The transient profile of chlorophyll *a* fluorescence (ChlF) intensities with time after start of the measurement showed clear genotypic differences with DP393 being the least affected by HS of the four genotypes. Analysis of the functions within the chlorophyll transient showed that fluorescence intensity, maximum fluorescence intensity, relative variable fluorescence, PI_{ABS} and ET/CS of cotton plants subjected to 40°C showed that all functions were decreased by HS indicating the adverse effects of HS on the efficiency of Photosystem II. DP393 had the lowest change in fluorescence intensities, F_v/F_m ratios, $PI_{ABS,}$ and ET/CS,

indicating heat tolerance and Arkot 9704 had the biggest changes and showed heat sensitivity. Measurement of chlorophyll a fluorescence and the analysis of the functions within the chlorophyll transient proved to be a precise method of quantifying heat stress responses in cotton genotypes.

Keywords: Chlorophyll *a* fluorescence, Cotton (*Gossypium hirsutum*), Heat stress, Photosystem II, Temperature tolerance.

Abbreviations

ChlF - Chlorophyll *a* fluorescence; ET/CS – Electron transport flux per cross section; HS – Heat stress; OEC - Oxygen-evolving complex; PEA - Plant efficiency analyser; PI_{ABS} - Performance index on absorption basis; PSII - Photosystem II; F_O, F_v and F_m - Minimal, variable and maximum Chlorophyll fluorescence of PSII in the dark adapted state; F_v/F_m - Maximum efficiency of PSII photochemistry. V*k* – Relative variable fluorescence.

INTRODUCTION

With the current change in climate heat stress has become a major factor impacting crop yields and food security (Bahuguna *et al.,* 2015). In cotton, high temperature has been shown to adversely affect crop growth and yield (Oosterhuis, 1999; Bange *et al.*, 2016). Heat stress is defined as the rise in temperature beyond a threshold level for a sufficient period of time to cause irreversible damage to plant growth and development (Wahid *et al.*, 2007). The impacts of plant stress depends on the crops tolerance towards the timing (developmental stage), duration and

severity of stress (Niinemets, 2010; Snider and Oosterhuis, 2011). To ensure future crop productivity and food security it is of vital importance to identify crops and genotypes, which can tolerate drought and heat stress.

Cotton (*Gossypium* spp.) is produced in about 76 countries, covering more than 32 million hectares across a wide range of temperature conditions (Singh *et al*., 2007). The ideal temperature range for cotton is between 20°C to 30°C (Reddy *et al*., 1991). Burke *et al*. (1988) reported the thermal kinetic window for enzyme function in cotton to be between 23.5 and 32°C. In cotton, the most sensitive stage to heat stress is during flowering with elevated temperatures above 30°C resulting in fruit abscission (Reddy *et al*., 1992). Different screening methods for heat tolerance in cotton have been investigated including membrane leakage, chlorophyll fluorescence (Bibi *et al*., 2008; Cottee *et al*., 2010 & 2014; Wu *et al*., 2014), pollen germination and pollen tube growth (Kakani *et al*., 2005), seed number traits, (Ragsdale, 2003) and antioxidants and carbohydrate contents (FitzSimons, 2016; Snider *et al*., 2010), but chlorophyll fluorescence seems to be the best and most practical screening technique.

The process in plant cells that is the most sensitive to heat stress is photosynthesis (Sharkey and Schrader, 2006). Photosystem II (PSII) is the initial complex in the photosynthetic electron transport chain, responsible for the oxidation of water and generation of molecular oxygen (Pilon *et al*., 2016). Heat stress causes changes in the reduction-oxidation properties of PSII acceptors and reduces the efficiency of electron transport in the photosystems (Mathur *et al*., 2014). Chlorophyll fluorescence is a non-destructive method that has been used to quantify heat stress in plants. The ChlF technique was developed by Kitajima and Butler (1975), and is one of the

most widely and popular stress tests in crop production (Baker and Oxborough, 2004; Resco *et al*., 2008; Wu *et al*., 2011) because of the ease of gaining detailed information on the effects of stress on photosystem II. Florescence measurements provide an understanding of the fundamental mechanisms of photosynthesis and the responses of plants to environmental change (Murchie and Lawson, 2013). Although chlorophyll fluorescence (F_v/F_m) is the most widely used parameter in chlorophyll research, other parameters of the overall fluorescence process such as performance index on absorption basis (PI_{ABS}) and electron transport flux per cross section of the leaf (ET/CS) have been identified and used to further assess the efficiency of PSII in photosynthesis (Force *et al*., 2003).

The objective of the study was to evaluate a procedure for measuring the fluorescence response of cotton genotypes to heat stress and to investigate the applicability of various function processes, F_v/F_m, PI_{ABS} and ET/CS derived from the fast chlorophyll *a* fluorescence kinetics to evaluate heat stress responses of cotton and identify heat tolerance among four diverse genotypes.

MATERIAL AND METHODS

Four diverse cotton genotypes namely Arkot 9704, VH260, DP393 and DP 210 B2RF (Table 1), were planted in 2 litre PVC pots in two greenhouse studies at Rustenburg, South Africa (S 26° 41’ 20”, E27° 05’ 25”) in August 2016 (Study 1) and January 2017 (Study 2). The selected genotypes represented a diverse set representative of the major germplasm pools in cotton production. Details of the origin and parent lines of the four genotypes are listed in Table 1. The

pots (14 cm in diameter and 13 cm in height) were filled with soil which was composed of a 50/50% mixture of coarse sand and black clay and planted with four cotton seeds which were thinned to one cotton plant per pot a week after emergence. Plants were watered daily with half-strength Hoagland's solution (Hoagland & Arnon, 1950). Air temperature was kept at 30/20 °C (day/night). Cotton plants were grown for 5 weeks up to the pinhead square stage and then subjected to two temperature regimes, namely a 30°C control and a 40°C heat stress for 6 hours using two converted laboratory ovens (Scientific 2000, Potchefstroom, Northwest) to create the temperature treatments.

Fluorescence intensities, maximum efficiency of PSII photochemistry (F_v/F_m), (PI_{ABS}), and electron transport flux per cross section of a leaf (ET_0/CS) were taken on intact cotton leaves using a MPEA fluorometer (Hansatech Instruments, King's Lynn, Norfolk, UK) (Plate 1). Cotton plants were dark adapted for 6 hours (while subjected to heat stress) before the measurements and then illuminated with continuous light (2400 μmol m^{-2} s^{-1}, 650 nm peak wavelength) for 1 s provided by an array of six light-emitting diodes focused on a circle of 5 mm diameter of the sample surface. Six plants per genotype was evaluated from the control (30°C) and HS (40°C) and measurements were taken at three different spots on the adaxial surface of the fourth mainstem leaf from the terminal, and three plants per treatment.

Table 1. Pedigree information for the genotypes used in greenhouse studies in 2016 and 2017.

Genotypes	**Area of origin**	**Parent lines**
VH260	A Pakistan genotype that grows at temperatures of 45 °C (Zhang *et al*., 2016)	S12 x H1692 (VH55 XLRA5166)
Arkot 9704	Arkansas Agricultural Experiment Station (Bourland and Jones, 2009)	Ark 9108 x 8 M331RKN
DP393	USA, Deltapine & Pineland & Co.	PVP 200400266
DP 210 B2RF	South Africa, Monsanto	DP560BGIIx2[B1][B2]/ COKER312[R2].

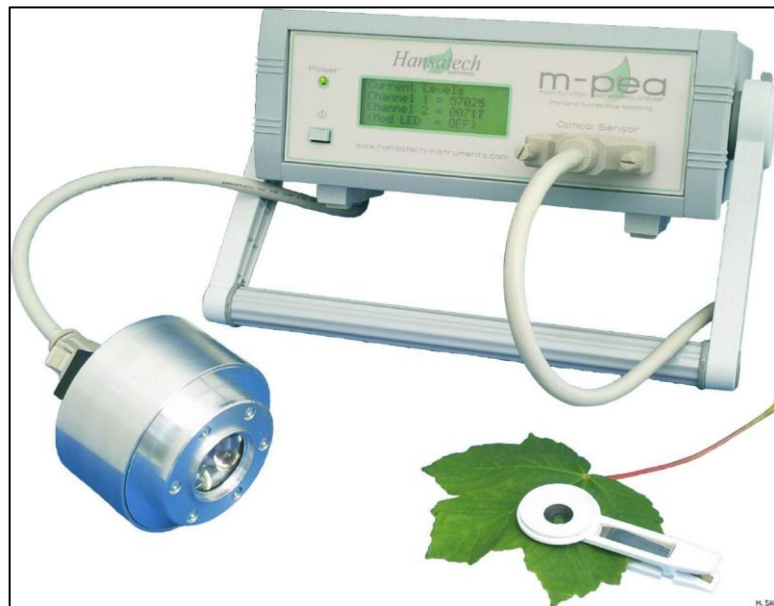

Plate 1. Instrument used to measure fluorescence showing the chlorophyll fluorescence data logger, the sensor, and the leaf clip for dark adaption.

Maximum quantum yield (F_v/F_m) is one of the most employed parameters in ChlF, as it provides evidence about the amount of light absorbed by chlorophyll in PSII for photochemical processes (Genty *et al.*, 1989). F_v/F_m only utilizes extreme values of minimal variable fluorescence (F_0) and maximal variable fluorescence (F_m) of chlorophyll fluorescence. In the current study, the ratio of variable fluorescence ($F_v = F_m - F_0$) to maximal (F_m) fluorescence of dark- adapted leaves was used as a measurement of plant stress, because it rapidly determines changes in the maximum efficiency of PSII functionality (Andrews *et al*., 1995: Fracheboud *et al*., 1999). F_v/F_m is a quantitative measurement of maximum or potential photochemical efficiency (Kitajima and Butler, 1975) and optimal quantum yield of PSII (Schreiber and Bilger, 1993), and determined as:

$$F_v/F_m = (F_m\text{-}F_0) / F_m \qquad \text{(Eq. 1).}$$

Where F_0 = minimal fluorescence, F_m = maximal fluorescence and F_v = variable fluorescence.

Performance index (PI_{ABS}) as described by Oukarroum *et al*. (2007) is a combination of three measurements, namely, (1) the amount of photosynthetic reaction centres (RC/ABS): (2) maximal energy flux that reaches the PSII reaction center (TR_0), and (3) the electron transport at the onset of illumination (ET_0). It therefore reflects the accumulation of all of PSII's responses:

$$PI_{ABS} = \frac{RC}{ABS} \cdot \frac{\varphi\text{Po}}{1 - \varphi\text{Po}} \cdot \frac{\Psi\text{o}}{1 - \Psi\text{o}} = \frac{RC}{ABS} \cdot \frac{\text{TR0}}{DI0} \cdot \frac{\text{ET0}}{\text{TR0} - \text{ET0}} \qquad \text{(Eq. 2).}$$

Where RC/ABS is the ratio of reaction centers and the absorbance (the concentration of reaction centers per chlorophyll), $\varphi_{Po}/(1 - \varphi_{Po})$ is an expression related to primary photochemistry, and $\Psi_o/(1 - \Psi_o)$ is an expression related to electron transport (Bacarin, *et al.*, 2011).

Statistical analysis were performed using JMP 11.1 (SAS Institute, Cary, NC) using an analysis of variance at an alpha level of 0.05. Significant differences between means were determined through Students t-test. Differences were considered statistically significant when $P<0.05$. For evaluating fluorescence induction transients, MPEA-Plus version 10 (a custom Windows® software package) was used.

RESULTS

The transient profile of chlorophyll fluorescence (ChlF) intensities with time after start of the measurement of four cotton genotypes at two different temperature regimes in two growth room studies are presented in Figure 1A&B. At 30°C control there were differences in ChlF intensity between genotypes indicating innate differences in photosynthetic efficiency. The 40°C heat stress resulted in a significant decline of the transient response of all four genotypes (Fig. 1A&B). These decreases in fluorescence intensities are associated with the restriction in the flow of electrons between the two photosystems (PSII and PSI) in photosynthesis as well as a decrease in the plants ability to reduce $NADP^+$ to NADPH (Oukaroum *et al.*, 2013). There was a significant interaction between genotype responses to HS in both studies (Table 2, Fig 1). In both studies DP393 had the least change in fluorescence intensity (17 and 5% decline compare to the 30°C control) showing that it was more tolerant to HS. The other three genotypes, Arkot

9704, VH260 and DP 210 B2RF showed higher changes in fluorescence intensity indicating larger responses to high temperature (Table 2).

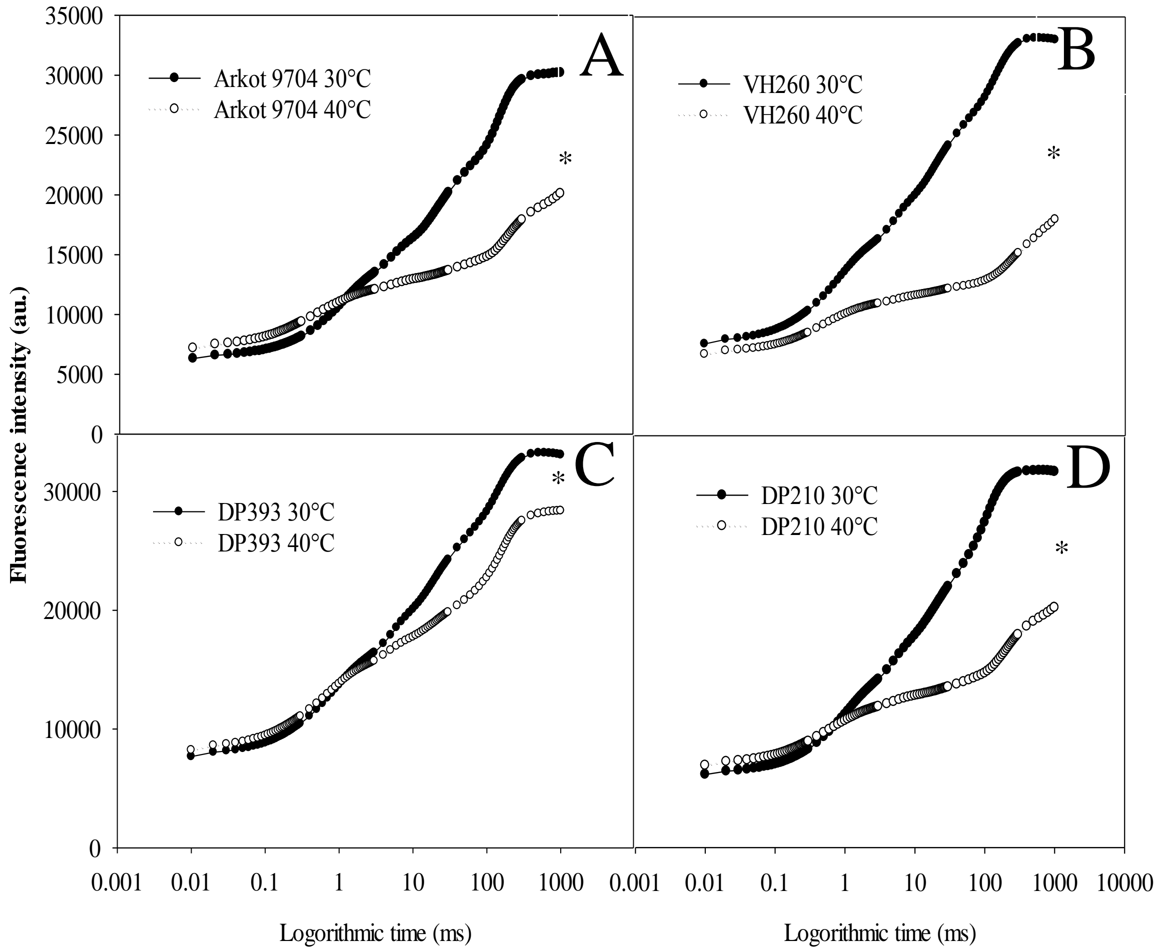

Figure 1A. Chlorophyll fluorescence intensity (arbitrary units) transient exhibited by intact leaves of four cotton genotypes during (A) Arkot 9704, (B) VH260, (C) DP393 and (C) DP 210 B2RF subjected to a 30°C treatment and a 40°C temperature regime. Study 1, Potchefstroom, South Africa. * = significant difference.

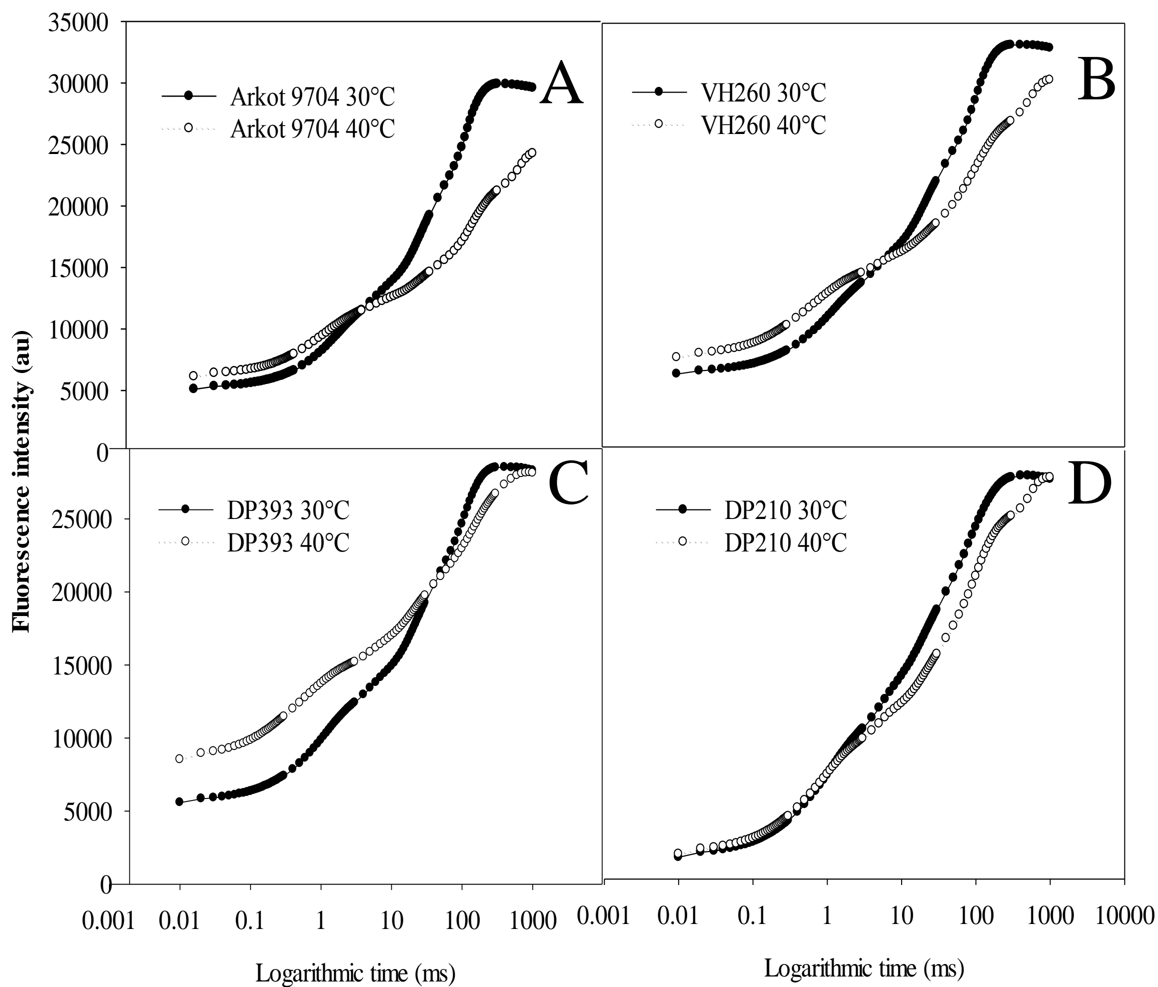

Figure 1B. Chlorophyll fluorescence intensity (arbitrary units) transient exhibited by intact leaves of four cotton genotypes during (A) Arkot 9704, (B) VH260, (C) DP393 and (C) DP 210 B2RF subjected to a 30°C treatment and a 40°C temperature regime. Study 2, Potchefstroom, South Africa.

Table 2. Chlorophyll fluorescence intensity (ChlF) at 0.3 ms of four cotton genotypes at two temperature regimes. Study 1 & 2, Potchefstroom, 2016 and 2017.

		Fluorescence Intensity (au)			
Study	Treatment	Arkot 9704	VH260	DP393	DP 210 B2RF
1	30°C	28,865b[1]	28,726b	33,208a	32,008a
	40°C	18,781c	14,252d	27,521b	18,147c
	% Change[2]	35	50	17	43
2	30°C	29,941a	28,464ab	27,938ab	29,596a
	40°C	20,997d	24,073c	26,482bc	26,531bc
	% Change	29	15	5	10

[1] The same letters in a row indicates no significant difference between genotypes ($P < 0.05$).
[2] Percentage change with the same letter for genotypes in a row do not differ significantly ($P < 0.05$).

Analysis of the differences in relative variable fluorescence (V*k*) of the two temperature regimes (Fig. 2) of Study 1 at 0.3 ms of the transient has been used to further interpret the fluorescence response to HS (Strasser, 2004). Measurements of relative variable fluorescence at 0.3 ms show clear peaks due to the fast fluorescence rise and the subsequent decrease of fluorescence intensity (Lazar *et al*., 1999), and is predominant under strong heat stress (Guissé *et al*., 1995; Strasser, 1997). Comparing V*k* between genotypes in Study 1 showed that DP393 had the least increase in relative variable fluorescence indicating greater tolerance to HS and Arkot 9704 had the largest response, indicating more sensitivity to heat stress (Fig 2). When ranking the genotypes according to heat tolerance using variable fluorescence, DP393 was the most heat tolerant, followed by VH260 and DP 210 B2RF, and Arkot 9704 was the most sensitive.

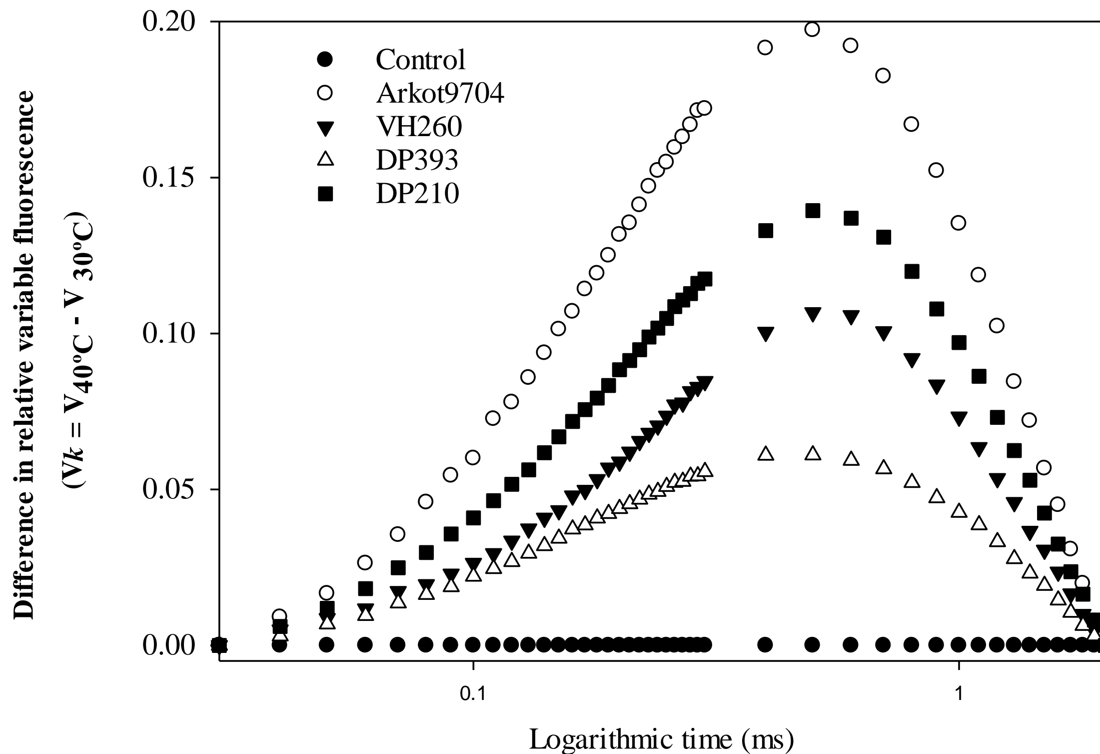

Figure 2. Difference in relative variable fluorescence (V*k*) measured at 0.3 ms after excitation in Study 1, exhibited by intact leaves of four cotton genotypes Arkot 9704 (open circle), VH260 (filled triangle) DP393 (open triangle) and DP 210 B2RF (filled square) at 40°C HS compared to a 30°C control temperature (filled circle). The control was a summary of the four cultivars at the 30°C control. Potchefstroom, South Africa.

Maximum efficiency of PSII (F_v/F_m) is the most widely used parameter in ChlF research (Kalaji *et al.*, 2016). Changes in maximum efficiency of PSII (F_v/F_m) of 4 genotypes and 2 temperature regimes are presented in Table 3. In both studies HS resulted in significant decreased F_v/F_m values for all four genotypes after the 40°C HS treatment and revealed differences in the response of the four different genotypes to HS. In study 1, DP393 was the least affected by HS (Table 3) compared to the other 3 genotypes, suggesting that DP393 is a heat tolerant genotype, and in Study 2, both DP 210 B2RF and DP393 were the least affected by the heat stress.

Table 3. Maximum efficiency of PSII (F_v/F_m) of four cotton genotypes at two temperature regimes. Study 1 & 2, Potchefstroom, 2016/2017.

Study	Treatment	Maximum fluorescence efficiency (F_v/F_m)			
		Arkot 9704	VH260	DP393	DP 210 B2RF
1	30°C	0.787a[1]	0.808 a	0.765a	0.803a
	40°C	0.606c	0.585c	0.696b	0.629c
	% Decrease[2]	23.0a	28.0a	9.0b	22.0a
2	30°C	0.825a	0.813ab	0.798b	0.796b
	40°C	0.737d	0.748cd	0.750cd	0.767c
	% Decrease	11.0b	8.0b	6.0c	4.0a

[1] The same letter in a row indicates no significant difference between genotypes ($P < 0.05$). [2] Percentage decrease with the same letter in a row do not differ significantly ($P < 0.05$).

PI_{ABS} is a measurement of the accumulation of all PSII's responses to energy capture and use in chlorophylls (Oukarroum *et al*., 2007), and is used to quantify PSII behaviour in response to HS. In both studies, HS plants had lower values for all four genotypes compared to the control temperature (Table 4), thus indicating the negative effect of HS on PSII function. In Study 1 after HS, genotypes differed significantly with DP393 (3.1%) having the highest $PI_{ABS,}$ compared to Arkot 9704 (2.4%), VH260 (1.5%) and DP 210 B2RF (2.4%). Although genotypes did not differ significantly in Study 2, the same tendency was found, with DP393 and DP 210 B2RF, exhibiting higher PI_{ABS} values compared to Arkot 9704 (4.8) and VH260 (4.6). In Study 1 (Table 4) the lowest reduction in PI_{ABS} from HS was obtained by DP393 (46%), indicating heat tolerance. In study 2 (Table 4), both DP 210 B2RF (45%) and DP393 (48%) resulted in the lowest reductions of PI_{ABS} and therefore considered to be heat tolerant.

Table 4. Performance index on absorption basis of chlorophylls (PI_{ABS}) of four cotton genotypes at two temperature regimes. Study 1 & 2, Potchefstroom, 2016/2017.

Study	Heat	Performance index on absorption basis (PI_{ABS})			
		Arkot 9704	VH260	DP393	DP 210 B2RF
1	30°C	8.3b[1]	12.3a	5.7c	9.8b
	40°C	2.4a	1.5d	3.1d	2.4d
	% Change[2]	71a	88a	46b	76a
2	30°C	16.4a	13.0b	9.8c	9.2c
	40°C	4.8d	4.6d	5.0d	5.0d
	% Change	71a	64a	48b	45b

[1] The same letters for each genotype in a row indicates no significant difference between genotypes (P < 0.05). [2] Percentage change with the same letter in a row do not differ significantly (P < 0.05).

To further study and interpret genotype response to high temperature stress electron transport flux per leaf cross section (ET/CS) was used as it expresses photosynthetic activity (Strasser *et al*., 2004). All four genotypes in Study 1 showed significant decreases in ET/CS when HS was applied (Fig 3A). In Study 1, the lowest change was obtained from DP393 compared to Arkot 9704, VH260 and DP 210 B2RF. In Study 2, DP393 again had the lowest change in ET/CS which differed significantly from Arkot 9704, but not from VH260 and DP 210 B2RF (Fig 3B). In Study 1 (Fig 3A) after HS, ET/CS differed significantly for the interaction temperature x genotypes. DP393 had the smallest changes in ET/CS, indicating tolerance to the HS treatment. VH260 had the lowest ET/CS showing that it was the most sensitive to HS. In Study 2 (Fig 3B) again significant differences were present for the interaction temperature x genotypes. After HS, DP393 and DP 210 B2RF had the highest (ET/CS) compared to VH260 and Arkot 9704, meaning DP393 had the most efficient electron transport flux, and Arkot 9704 had the least efficient electron transport flux.

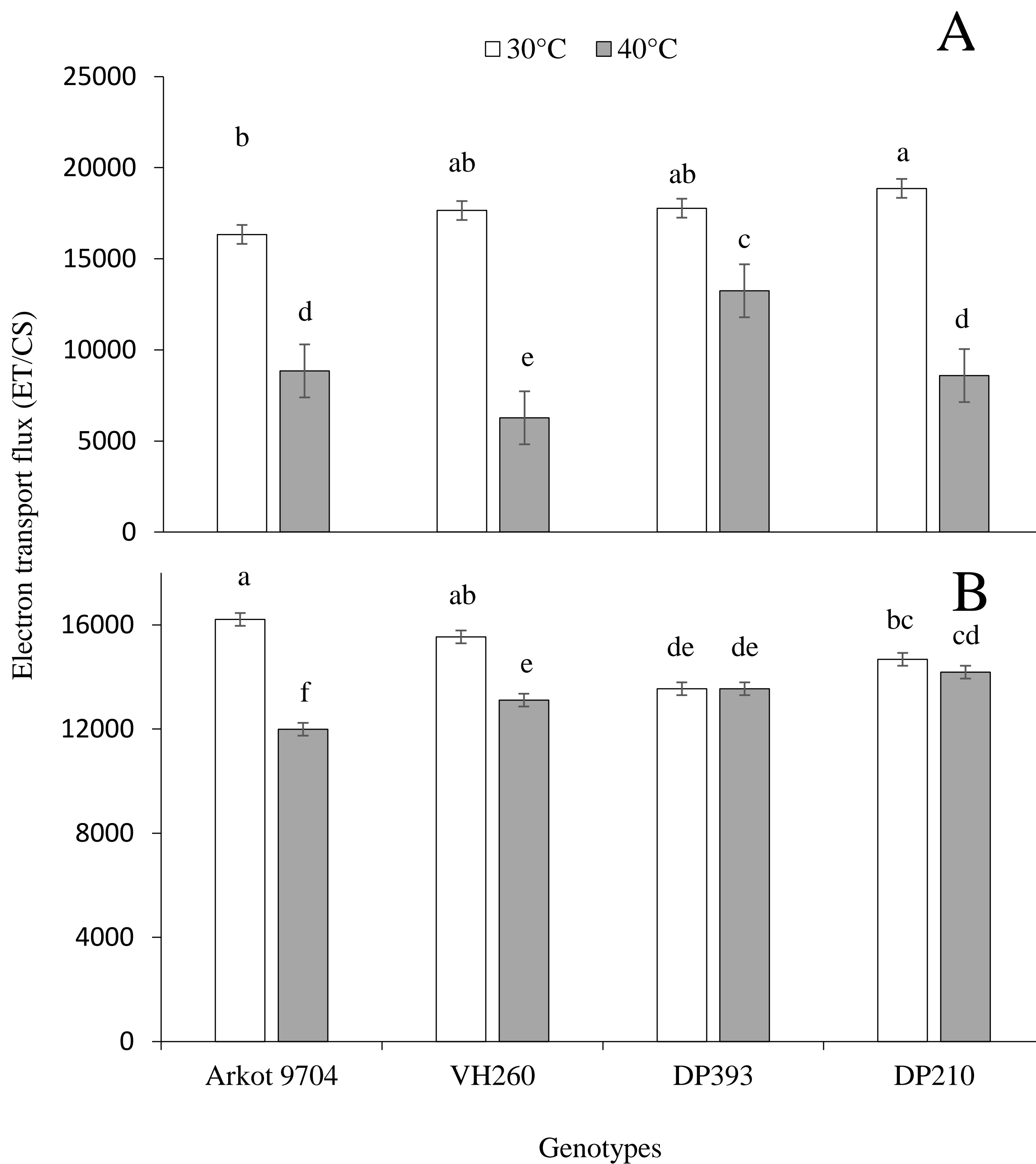

Figure 3. Electron transport flux (ET_0/CS) of the four genotypes for Study 1 (A) and Study 2 (B). Different letters between the 30°C and 40°C treatments for each set of columns indicate a significant difference ($P < 0.05$). Error bars indicate the standard error at $\alpha = 0.05$.

DISCUSSION

Elevated temperatures due to climate change are projected to cause substantial losses in cotton production (Bange *et al*., 2016). Cotton is an important multi-purpose crop grown in warm climates across the world, and it is therefore of vital importance to minimize the onset of HS by selecting higher yielding cotton genotypes under high temperature stress. Several authors have tried various techniques to measure and document genotypic tolerance in cotton, including ML (Bibi *et al*., 2008: FitzSimons, 2016) and chlorophyll fluorescence (Bibi *et al*., 2008; Cottee *et al*., 2010; Pilon *et al*., 2016; Wu *et al*., 2014), but with varying success. Chlorophyll fluorescence is considered to be the most indicative and reliable method for detecting plant stress (Yan *et al*., 2013; Kalaji *et al*., 2016).

Chlorophyll fluorescence is an indication of the fate of excitation energy in the photosynthetic apparatus (Yamada *et al*., 1996) and evaluations of chlorophyll fluorescence have been used to describe and detect the effect of multiple environmental stresses in plants of diverse habitats (Larcher, 1995). Photosystem II and specifically the oxygen evolving complex in PSII is the most sensitive plant process to heat stress ((Havaux *et al*., 1993, 2004) Murata *et al*., 2007). Measurement of losses in energy fluxes and transportation of electrons in PSII can be a strong indicator of the adverse effect of high temperature damage to plants (Strasser *et al*. 2000, 2004). In cotton, above optimum temperatures leads to disruptions in the structure and functioning of the PS11 system in photosynthesis (Cottee *et al.*, 2014; Law *et al*., 2001; Snider *et al*., 2010). The chlorophyll fluorescence transient is sensitive to environmental stressors (Krüger *et al*., 1997; Tsimilli-Michael *et al*., 1998, 1999, and analysis of the transient polyphasic rise in

fluorescence (Srivastava *et al*., 1997; Strasser *et al*., 2000) provides a mean to quantify photosynthetic performance of plants and PSII function (Strasser *et al*., 2004; Tsimilli-Michael, 2013). Numerous parameters can be derived from the ChlF transient curve, and in the current study we used five of those parameters namely; fluorescence intensities, V*k*, F_v/F_m, PI_{ABS} and ET/CS to identify HS in cotton genotypes.

In the current study, decreases in fluorescence intensities under elevated temperature shows that the functioning of Photosystem II had been adversely affected. Fluorescence intensities were decreased by HS for all four genotypes in both studies (Table 2). Similar results of decreased fluorescence intensities with HS have been reported by Wu *et al*. (2014) for cotton (*Gossypium hirsutum* L) and by Srivastava *et al*. (1997) for pea (*Pisum sativum*), showing that PSII function are negatively affected by HS. There were clear genotypic differences with DP393 exhibiting the least change in ChlF intensity from HS in Study 1 (17%) and in Study 2 (5%), indicating greater tolerance to HS (Table 2).

To further investigate and confirm the effects of HS on fluorescence an analysis of the relative variable fluorescence (V*k*) was conducted for Study 1. This analysis uses the ChlF transient response curve at 0.3 ms after the start of HS measurement to differentiate genotype responses to the high temperature. Increases in variable fluorescence in 0.3 ms heat-stressed samples was attributed to a decrease of electron transport between the OEC and the reaction centers of PSII (Srivastava and Strasser, 1997). This has been shown as the most heat susceptible site in PSII in wheat leaves (Brestic *et al*., 2012). Relative variable fluorescence (V*k*) of the four cotton genotypes in Study 1 (Fig.2) showed that DP393 had the least increase in V*k* indicating greater

tolerance to the heat stress. Arkot 9704 had the largest increase in V*k*, indicating more damage in PSII and more sensitivity to heat stress. When ranking the genotypes for heat tolerance according to the V*k*, DP393 was the most heat tolerant, followed by VH260 and DP 210 B2RF with intermediate tolerance, and Arkot 9704 was the most heat sensitive. These results are in agreement with research done by Yan *et al*. (2013) who found with sweet sorghum (*Sorghum bicolor*) at severe HS of 48°C that an increase in V*k* was a specific indicator for the heat-induced damage to the oxygen evolving complex (OEC) in PSII. Martinazzo *et al*. (2012) also reported for *Prunus persica* that higher V*k* occurred at high temperatures > 40°C. The variable fluorescence response analysis supported the ChlF intensity and maximum efficiency of PSII measurements that DP393 exhibited the most tolerance to HS of the four genotypes studied.

The maximum efficiency of PSII (F_v/F_m) is the most widely used parameter in chlorophyll fluorescence research to document stress (Kalaji *et al*., 2016; Strasser *et al*., 2005). In my study, F_v/F_m was decreased after HS for all four genotypes in both studies (Table 3). Strasser *et al*. (2004) defined the boundary level for a fully functional PSII system to be 0.750 F_v/F_m, and concluded that higher values indicated a higher ability to use and move electrons into the electron transport chain. In my study, HS decreased F_v/F_m values of all four genotypes below the 0.750 boundary level for fully functional PSII system. In Study 1, DP393 had the lowest decrease of 9 % in F_v/F_m from the HS compared to the 30°C control, indicating the most heat tolerance of the genotypes (Table 3). In Study 2, DP 210 B2RF and DP393 had the lowest decreases of 4.0 and 6.0 %, respectively, showing heat tolerance (Table 3). When ranking the genotypes according to heat tolerance using F_v/F_m, DP393 was the most heat tolerant, followed by DP 210 B2RF and VH260, with Arkot 9704 being the most sensitive. Decreased ratios of

F_v/F_m in stressed plants was likely due to damage to the PSII system (Maxwell and Johnson, 2000) and indicates photo inhibitory damage in HS plants as shown in research done on soybean and cotton by Inamullah and Isoda (2005). Li *et al*. (2012) found decreased F_v/F_m values for cotton under drought stress as did Wu *et al*. (2014) for cotton under HS. Živčák *et al*. (2008) and Oukarraum *et al*. (2007) however found F_v/F_m to be an insensitive measurement to early changes of plant photosynthesis in drought stress studies. Photosynthetic efficiency (F_v/F_m) is one of the most employed parameters, as it provides evidence about the amount of light absorbed by chlorophyll in PSII for photochemical processes (Genty *et al.*, 1989), but this parameter only utilizes extreme values of minimal variable fluorescence (F_o) and maximal variable fluorescence (F_m) of chlorophyll fluorescence.

The advancement of the ChlF technique by Strasser *et al*. (2000) led to the introduction of a multi-parametric expression called performance index (PI_{ABS}). PI_{ABS} takes into account all main photochemical processes of the PSII reaction center complex, such as light energy absorption, trapping of excitation energy, electron transport further than primary plastoquinone (Q_A) and dissipation of excess excitation energy. Olsen *et al.* (2016) found PI_{ABS} to be a more sensitive and better reflection of water stress in sugarcane than the F_v/F_m ratio. PI_{ABS} is considered as a very good indicator of the changes in photosynthetic activity as it is sensitive to environmental stressors that damage the photosynthetic apparatus in plants (Krüger *et al*., 1997; Stirbet and Govindjee., 2011). We recommend the use of PI_{ABS} in conjunction with F_v/F_m to identify genotypes for heat tolerance. PI_{ABS} is a measurement of the accumulation of all PSII's responses to energy capture and use (Oukarroum *et al*., 2007) and was considered by Tsimilli-Michael and Strasser, 2013 as the most sensitive parameter of ChlF to stress and an efficient tool to quantify

stress in plants. Photosystem II were negatively affected by HS, as significant decreases in PI_{ABS} were noted after HS in both studies (Table 4). PI_{ABS} values in Study 1 (Table 4) was the highest for DP393 after HS indicating heat tolerance. In Study 2, PI_{ABS} (Table 4) for genotypes DP 210 B2RF and DP393 was the highest after HS, indicating that both genotypes had tolerance towards HS. The lower PI_{ABS} values may have been caused by absorption of energy by inactive reaction centers (Martinazzo *et al*., 2012).

Electron transport flux per leaf cross section (ET/CS) provides a quantification of photosynthetic activity (Strasser *et al*., 1999). In the current studies, ET/CS in Study 1 (Fig 3A) showed the highest values for genotype DP393, and in study 2 the highest ET/CS (Fig 3B) values were obtained from DP 210 B2RF, DP393 and VH260 in decreasing order. In both studies the lowest ET/CS was obtained with VH260, indicating heat sensitivity. Change in ET/CS in both studies showed that DP393 had the lowest change, indicating that DP393 had a more efficient electron transport flux under HS. These results confirm above mentioned results for measurements of fluorescence intensities, V_k, F_v/F_m, and PI_{ABS}.

The difference in measuring F_v/F_m compared to PI_{ABS}, is that F_v/F_m is calculated from the two endpoints of the ChlF transient, whereas PI_{ABS} is a composite of the kinetics parameters of electron absorption (ABS/RC), trapping (TR/RC) and electron transport from PSII to PSI (ET/RC) (Olsen *et al*., 2016). Brestic and Zivcak (2013) reported that some studies have shown that the parameters PI_{ABS}, Vk and ET/CS show a greater sensitivity to heat than the conventional parameters such as F_v/F_m and that it is caused by the fact that F_v/F_m represents an average value

of the efficiency for all the PSII units in the measured excited cross-section but also the units with inactivated reaction centers.

Measurements of ChlF appear to offer the most accurate and practical method of quantifying temperature tolerance in cotton genotypes (Kalaji *et al*., 2016). Xu *et al*, (2014), compared three methods to identify heat tolerance in grapevine (*Vitus vinifera*) and found ChlF more practical and sensitive than ML and gas exchange for investigating heat injury. Lepedus *et al*. (2012) also confirmed this finding with ChlF research on maize (*Zea mays* L). Development of high-temperature resistant cotton genotypes can ameliorate yield losses in response to elevated temperature (Zahid *et al*, 2016). Early identification of genotypes for HS is an objective that many plant breeders prioritize, and in this study on cotton, it was shown that ChlF measurements can detect HS differences between diverse genotypes in growth chambers, but both F_v/F_m and PI_{ABS} should be measured before recommendations are made. Measuring ChlF has become an attractive means of obtaining rapid information on photosynthesis and effects of stress and is being used by an increasing number of researchers both in the laboratory and field. My results show that ChlF provides a quantitative measure of genotypic differences in response to high temperature stress. However, use of this method to differentiate genotypic differences may be more appropriate in field conditions with larger genotype entries and larger replication.

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CHAPTER III

Evaluation of Screening Methods to Detect Heat Stress in Cotton Genotypes in Field Studies.

ABSTRACT

The growth and yield of cotton (*Gossypium hirsutum* L.) are decreased by high temperature during reproductive development, but information on genotypic variation to heat stress is lacking. Above optimal temperature affect physiological functions and decrease yield. The impact of heat stress (HS) on cotton genotypes was evaluated with different screening methods in field trials at Rustenburg (South Africa) from 2013 to 2017 and in Marianna, Arkansas (USA) during 2015. Four diverse cotton (*Gossypium hirsutum L.*) genotypes were tested namely Arkot 9704, VH260, DP393 and DP 210 B2RF BRF. Measurements were made of membrane leakage (ML), chlorophyll fluorescence (ChlF), glutathione reductase (GR) and carbohydrate content of leaves at early flowering during a high temperature period and compared to measurements in a lower temperature period. High temperatures increased ML in all genotypes in all years and locations, but there were no clear difference in genotypic response to high temperature. Chlorophyll fluorescence was increased at temperatures higher than 30°C and was generally increased at all locations, but with no significant genotypic differences to high temperature. Glutathione reductase was increased, starch was decreased, and sucrose and total carbohydrate concentrations were increased by high temperature, with no genotypic differences. Although ML and ChlF techniques were practical, fast and gave reliable results of heat stress, they were

not able to detect genotypic differences in the genotypes studied. The genotypes used in this study did not show significant or consistent tolerance to heat stress which was related to modern genotypes having less tolerance to heat stress than older obsolete cultivars and wildtype cotton. Measuring indicators of heat stress in the field on cool days compared to hot days was not a suitable method to detect genotypic tolerance, and it was shown than measuring these indicators in cool early morning compared to hot midday temperatures may provide a better indication of genotypic difference to HS.

Abbreviations. HS = heat stress; ML = membrane leakage; ChlF = chlorophyll fluorescence; GR = glutathione reductase.

INTRODUCTION

Global temperature has increased by approximately 0.6°C since the late 19th century and is projected to increase by another 1.4 to 5.8°C by the end of the current century (Houghton *et al*., 2001). Above optimum temperatures during critical stages of plant development will become a major factor limiting crop production (Hall, 1992). Climate change effects on crop yields suggest losses of productivity due to projected surface temperature increases by the end of the 21st century (Reddy *et al*., 2002; Peng *et al*., 2004). Almeselmani (2006) summarized plant physiological processes that are significantly injured by HS as photosynthesis, dark respiration, membrane stability and mitochondrial respiration. High temperatures during the reproductive development of cotton in Arkansas reduced yield and there was a strong negative correlation between temperature and yield, where high temperatures during the flowering period resulted in

lower yields (Oosterhuis, 2002). High temperatures (>35 °C) throughout the growing season affect growth, yield and fiber quality of cotton negatively (Hearn and Constable, 1984).

Cotton is produced worldwide under a wide range of temperatures, but the ideal range for cotton is from 20 to 30°C (Reddy *et al.*, 1991). High temperatures of above 35°C during the growing season are commonplace in cotton production areas worldwide and exceed the thermal kinetic window for which metabolic activity is most efficient in cotton plants, thereby limiting plant function, growth and yield (Hodges *et al*., 1993; Burke *et al*., 1988; Burke and Wanjura, 2010). Because typical daily high temperatures in Arkansas are often in excess of the optimum range during the reproductive stage, high temperature represents a major limitation to crop development and productivity (Snider, 2010).

Cotton leaf temperature can be substantially below air temperature due to evaporative cooling, and leaf cooling is significantly correlated with fruiting prolificacy and yield during the hottest period of the year (Radin *et al*., 1994). These authors also reported that selection for improved heat resistance (fruit set during heat stress) of irrigated Pima (*Gossypium hirsutum L.*) cotton has been accompanied by increasing stomatal conductance and decreasing leaf temperature, especially during the afternoon. Lu *et al*. (1997) reported that lower leaf and canopy temperatures at critical developmental stages associated with flowering and fruiting during July for Pima cotton in Arizona appear to favour higher yields.

There is a need to understand cotton plant response to high temperature and determine the best method of detecting and quantifying plant responses to heat stress. The development of a rapid

and reliable screening tool for genotype specific thermotolerance can potentially improve the efficiency of breeding programs and the development of high-yield genotypes for hot growing regions (Constable *et al*., 2001). Wise *et al*. (2004) stated that growth chamber experiments have shown that measurement of processes such as electron flow through the photosystem may be used to quantify heat stress in plants. Other measurements that have been used to quantify heat stress include photosynthesis (Salvucci and Crafts-Brander, 2004), respiratory enzyme viability (de Ronde *et al*., 2000), cell membrane disruption (Sullivan, 1971; Blum and Ebercon, 1981), and chlorophyll fluorescence (Bibi *et al*., 2008). Membrane disruption in plant cells alter water, ion and organic solute movement, photosynthesis and respiration (Cristiansen, 1978). Possible methods to alleviate the detrimental effects of heat stress include the planting of genotypes that are heat tolerant, earlier planting to avoid heat stress during flowering, plus managing irrigation to cool the crop during heat stress, and the application of plant growth regulators.

The objectives of these studies were to study physiological effects of high temperature stress on the growth and yield of cotton genotypes in the field, and to use physiological measurements to quantify the effect of high temperature stress of cotton genotypes for screening for temperature tolerance. It was hypothesized that high temperatures cause physiological responses in cotton leaves that effect growth and yield, and that these responses can be used to screen for temperature tolerant genotypes.

MATERIALS AND METHODS

Field trials and genotypes

Field trials were conducted to evaluate the effect of high temperatures on physiological processes of field-grown cotton and evaluate genotypic heat tolerance using four diverse cotton (*Gossypium hirsutum* L.) genotypes namely: Arkot 9704, VH260, DP393 and cultivar DP 210 B2RF (Table 1). Genotypes were selected based on earlier screening done by Bibi *et al*. (2008) and Bourland and Jones (2009). Arkot 9704 was chosed because of its performance in the national cotton variety trials (http//rbtn.cottoninc.com/files – 2006 results). VH260 was chosen as it was identified as heat tolerant by Zhang (2013). DP393 gave good yields in Dr Bourlands trials, and DP210 B2RF had unknown tolerance to heat, and is planted as a commercial cultivar in South Africa.

Table 1. Pedigree information for the genotypes used in field studies in South Africa and the USA during 2012 to 2016.

Genotypes	Area of origin	Parent lines
VH260	Pakistan genotype grown at temperatures of 45°C (Zhang, 2013)	S12 x H1692 VH55 XLRA5166
Arkot 9704	Arkansas Agricultural Experiment Station (Bourland and Jones, 2009)	Ark 9108-04 + 8 M331RKN
DP393	USA, Deltapine & Pineland & Co.	PVP 200400266
DP 210 B2RF	South Africa, Monsanto	DP560BGIIx2[B1][B2] /COKER312[R2].

Localities and seasons were; Rustenburg (South Africa) 2013 to 2017 and Marianna, (Arkansas, USA) 2015. During the 2017 season, fluorescence was measured on one day, namely 12 February, in the morning at 6.00 AM and at noon, 12.00 PM to evaluate diurnal response of plants to HS. Locations latitudes and longitudes and soil types are summarized in Table 2. The cotton was grown under adequate nitrogen supply (150 N kg ha^{-1}) applied in two side dressings, 4 and 8 weeks after planting. Trials were designed as completely randomized block designs with 6 replications. Each plot was 20 m^2 (5 m x 4 rows) with a 1 m inter-row spacing and 0.20 m intra-row spacing. Two to three seeds were planted by hand at each planting station and the seedlings were thinned to a single plant per station when they were approximately 0.15 m tall, resulting in a plant population of 70 000 plants ha^{-1}, the recommended plant population for cotton grown under irrigation. Plants of the middle 2 rows per plot were sampled during early flowering at 12.00 AM each day and the leaves used for measurements of membrane leakage (ML), chlorophyll fluorescence (ChlF), glutathione reductase activity (GR), and carbohydrate

content. Seed cotton yield was determined by handpicking the cotton. Weather data were collected from the national weather stations closest to the trial sites for information on minimum and maximum temperatures and rainfall (Appendix 4, Fig. 1). During the 2017 season, fluorescence was measured on a single day, namely 12 February, in the morning at 6.00 AM and at noon, 12.00 PM in order to measure ChlF at a cool and hot temperature in the same day.

Table 2. Location, season, latitude, longitude and soil types of the heat tolerant field trials in South Africa and the USA during 2013 to 2016.

Locality	**Season**	**Latitude**	**Longitude**	**Soil type**
Rustenburg	2014	25.66°S	27.2500 °E	Hutton (Arcadia)
Rustenburg	2015	25.66 °S	27.2500 °E	Hutton (Ventersdorp)
Rustenburg	2016	25.66 °S	27.2500 °E	Hutton (Arcadia)
Rustenburg	2017	25.66 °S	27.2500 °E	Hutton (Arcadia)
Marianna	2015	34.77 °N	90.7650 °W	Calloway silt loam

Measurements

Measurements of ML, ChlF, GR and carbohydrates were made in the five field trials (Table 2) during a hot and a cooler day each growing season at 12.00 AM on the day of measurement. Membrane leakage (ML) was measured using the method of Sullivan (1971) and FitzSimons

(2016). Membrane leakage was determined by sampling three 10 mm discs per plant at first flower with a cork borer. Ten plants per replicate were sampled at 11.00 pm, in the morning. The samples were taken from the youngest fully expanded main-stem leaf of a plant and veins were avoided. Leaf discs were placed in separate test tubes with 10 mL de-ionized water and rinsed three times to remove excess electrolytes. The samples were placed in the dark for 24 hours, after which electrical conductivity (EC) was measured with an EC meter (Primo 5, HANNA Instruments, USA) and recorded as the initial ionic leakage. Tubes were capped and autoclaved for 20 minutes to dissociate all cellular cytosols into solution. After cooling to room temperature, the EC was again measured as total ionic leakage. Calculations were performed as an injury index percentage (eq. 1) at 100 °C, and the final EC measurements were taken after cooling down to room temperature.

$$1 - \left(\frac{Final - Initial}{Initial}\right) * 100 \qquad \text{(eq. 1)}$$

Where final and initial are the EL measurements at that time.

Chlorophyll fluorescence of attached leaves at Marianna (USA) was measured with a modulated chlorophyll fluorometer OSI-FL (Opti-Sciences, Tyngsboro, MA). With this instrument, chlorophyll is excited by a 660 nm solid-state white source with filters blocking radiation longer than 690 nm. The average intensity of the modulated white was adjusted to 1 µE. Detection was in the 700-750 nm range using a PIN silicon photodiode. To measure ChlF response to increasing temperature the leafTech method (Snider, 2010) was used at Rustenburg in 2012/2013 on leaves harvested at dawn and transported to the laboratory and stored in the dark in the laboratory. Discs were then measured at 5 minute intervals at temperatures of 20, 25, 30, 35 and 40 °C with the new Leaftech instrument (Plate 1). Chlorophyll fluorescence of attached leaves at

Rustenburg, South Africa, was measured with a Plant Efficiency Analyser (PEA, Hanzatech Instruments LTD., Norfolk, UK). The actinic light was 1500 $\mu mol.m^{-2}.s^{-1}$ provided by an array of six high intensity light-emitting-diodes (the peak wavelength at 650 nm) with the duration of 5 s. Measurements were conducted at noon. All measurements were replicated with five different leaves.

Measurements of the fourth main-stem leaf to determine non-structural carbohydrates were done according a modification of the Hendrix, (1993) protocol with modifications by Zhao (2010) and, modified further by FitzSimons & Loka, 2013). Three leaves per plot were sampled and oven dried for three days at 50 °C before analyses. Forty mg of ground leave tissue were extracted 3 times with 80 °C aqueous ethanol (800 ml ethanol/L) and the samples were centrifuged after each extraction at 5000 rpm and the fraction were pooled. Active charcoal was then added to the pooled fractions in order to remove substances that could interfere with the carbohydrate measurements and the samples were centrifuged again at 3500 rpm. The supernatant was immediately stored at – 80°C for determination of sucrose and hexose with a Multiscan Ascent Microplate Reader (Thermo Fisher Scientific Inc., Waltham, MA). The glucose (HK) assay kit from Sigma (Sigma Chemical Company, St Louis, MO) was used. A 20 µl aliquot of each extract was pipetted into a well of a micro titration plate and the plate was incubated at 50 °C for 40 minutes to evaporate ethanol. 10 µl of water were then added to each well along with 100 µl of glucose assay reagent and the plate was incubated again for 15 min at 30 °C. The absorbance was measured three times at 340 nm using a microplate reader. 0.25 EU of phosphoglucose isomerase was added to the extracts in each well of the plate and the absorbance was again measured at 340 nm. Eighty three units of invertase were added to the

extracts and the micro titration plate was incubated at 30 °C for 60 min. Absorbance was measured three times at 340 nm and the results were expressed in mg carbohydrate/mg dry weight with the help of a standard curve made of known glucose concentrations.

Glutathione reductase activity (GR) was measured using the method of Anderson *et al*. (1992) (App. 3). Three leaves per plot were sampled in liquid Nitrogen and transported to a -80 °C freezer. Leaf tissue was homogenized using a mortar and pestle in an ice-cold extraction solution comprised of 50 mM Pipes (1,4-Piperazine diethanesulfonic acid) buffer (pH 6.8), 6mM cysteine hydrochloride, 10mMd-isoascorbate, 1mM ethylenediaminetetraacetic acid, 0.3% Triton X-100 and 1 % (w/v) soluble Polyvinylpyrrolidone (PVP). Solutions were further blended for 1 minute in a tube containing 0.25 g insoluble PVP and 1 drop of antifoam using a homogenizer (Model Polytron; Brinkman Instruments Inc., Palo Alto, CA). Samples were centrifuged at 21 000 g for 20 minutes (4 °C) and the supernatants were stored at -80 °C for determination of glutathione reductase content according to Shaedle and Bassham (1977), with modification. To each well of a 96-well micro titration plate, a 15.7 µl aliquot of enzyme extract from each ample was added to a 300 µl reaction solution containing 50 mM Tris-HCL buffer (pH=7.5), 0.15 mM reduced nicotinamide adenine dinucleotide phosphate (NADPH), 0.5 mM oxidized glutathione, and 3 mM MgCL2. Oxidation of NADPH was determined as the decrease in absorbance at 340 nm during a 1 min reaction time using an Ascent Multiscan microplate reader (Molecular Devices Corporation, Sunnyvale, CA), and glutathione reductase activity was expressed as GR units/g fresh weight.

Comparison of ML, ChlF, GR, carbohydrates and seedcotton yield between temperature regime and genotype were made using a two-way ANOVA and the student's t test at ($\alpha < 0.05$). Comparison analysis was performed using JMP 11.1 software (SAS Institute, Cary, NC).

RESULTS

Weather Data and Temperature Regimes Measured

Mean maximum temperatures over the trial environments on the day of measurement ranged from 23.0°C to 35.0°C, and minimum temperatures ranged from 13.0°C to 24.0°C (Table 3, Appendix 1). At Rustenburg in 2014 a high temperature regime of 35°C and a low temperature regime of 31°C were measured on two different dates. At Rustenburg in 2015 a high temperature regime of 32°C and a low temperature regime of 27°C were recorded. In 2016 Rustenburg experienced hot weather (record highs in 50 years) and a high temperature regime of 35°C and a low temperature regime of 32°C were recorded. In 2017 in Rustenburg, a low of 22.7°C and a high temperature of 29.3°C were measured on the measuring date (12 February 2017). At Marianna in 2015, the high temperature was 34°C and the low temperature was 32°C (Table 3). The daily changes in maximum and minimum temperatures and precipitation for the four localities are given in Appendix 2, Fig 1.

Table 3. Minimum and maximum temperatures on measuring day at the weather station on the Institutes at Rustenburg and Marianna (2013-2016).

Year	Temperature Regime	Minimum and maximum temperatures (°C)			
		Rustenburg		Marianna	
		Max °C	Min °C	Max °C	Min °C
2014	Low	31	14	–[1]	–
	High	35	17	–	–
2015	Low	27	13	32	21
	High	32	19	34	24
2016	Low	32	15	–	–
	High	35	19	–	–
2017	Low	23	15	–	–
	High	29	18	–	–

–[1] No trial was planted during this season.

Membrane Leakage (ML)

Membrane leakage was increased significantly by the high temperature regime compared to the low temperature in all locations and years (Fig. 1). In the four experiments in South Africa at Rustenburg, the high temperature was 3 to 5°C higher than the low temperature treatment (Table 3), and above the 30°C optimal temperature for cotton (Reddy *et al*., 1991). At Rustenburg in 2014, ML differed significantly between the two temperature regimes (low 31°C and high 35°C). The high temperature of 35°C had the highest ML of 88.8 % compared to the low temperature regime of 31°C with an ML of 74.4 %, an increase of 14.4 % in ML. During 2015 in Rustenburg, ML in the two temperature regimes (low 27°C and high 32°C) differed significantly (Fig. 1). The high temperature regime 32°C resulted in the highest ML of 88.8 % compared to 72.3 % in the low temperature regime of 27°C, a 16.5 % reduction in ML. At

Rustenburg in 2016, ML again differed significantly between the two temperature regimes (Fig 1). The high temperature regime 35°C gave the highest percentage ML 77.4 % compared to the 59.6 % for the low temperature regime (32°C). During 2017 in Rustenburg, again ML of the two temperature regimes differed significantly. The low temperature regime (23°C) had the lowest ML (40.4 %), compared to the 52.9 % of the high temperature regime (29°C) (Fig. 1). At Marianna, the high and low temperature regimes were not too different (32.0 and 34.0°C) and above the 30 °C optimum, but a significant increase in ML (30.4 %) at the higher temperature regime was still recorded, compared to the low temperature regime (32.0°C) with a ML of 27.1 %.

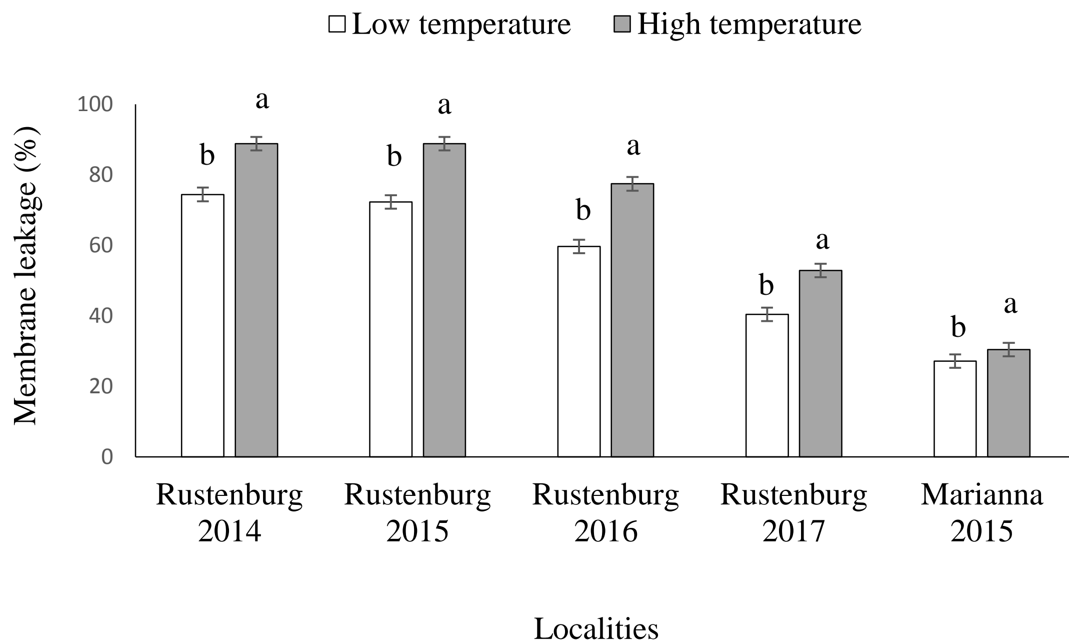

Figure 1. Membrane leakage of two temperature regimes meaned over four genotypes measured at Rustenburg 2014 (31.0°C) low temperature, (35°C) high temperature; Rustenburg 2015, low temperature (27°C), high temperature (32°C); Rustenburg 2016, control (32°C), HS 35°C; Rustenburg 2017 was sampled on 12 February at 6.00 am, low temperature (22.7°C) and at 12.00 pm, high temperature (29.3°C); and Marianna 2015, low temperature (32°C) and high temperature (34°C). Pairs of columns with the same letters are not significantly different ($P<0.05$). Error bars indicate the standard error at $\alpha = 0.05$.

At the low temperature regime there were inconsistent differences in ML between genotypes in the four studies. During 2014 at Rustenburg, significant differences were not found between genotypes at the low temperature regime (data not shown). In 2015 at Rustenburg in the low temperature regime, DP 210 B2RF (64.6 %) gave significant lower ML percentages than VH260 (76.2 %) and DP393 (75.2 %) but not than Arkot 9704 (73.2 %) (Fig. 2A). In 2016 in Rustenburg (Fig 2. B) Arkot 9704 (50.8 %) gave significantly lower ML in the low temperature regime than VH260 (69.4 %) but not DP393 (56.5 %) and DP 210 B2RF (61.6 %). At Rustenburg in 2017, significant genotypic differences were not present (Fig. 2C). At Marianna in 2015, in the low temperature regime, the lowest ML of 22.6 % was obtained from DP393 and Arkot 9704 (24.2 %) which differed significantly from VH260 (28.2 %) and DP 210 B2RF (33.6 %) (Fig 2. D).

In the high temperature regimes variable results in ML were obtained for genotypes. During the 2014 season at Rustenburg, significant differences were not found between genotypes at the high temperature regime (data not shown). At Rustenburg in 2015 (Fig. 2A) significant differences were not present between genotypes in the high temperature regime. At Rustenburg in 2016 Arkot 9704 (62.4 %) had significantly lower ML than VH260 (85.4 %), DP393 (80.0 %) and DP 210 B2RF (81.8%). 2B). At Rustenburg in 2017 significant differences were present, with the lowest leakage with Arkot 9704 (39.3%), DP393 (49.2 %) and DP 210 B2RF (51.7 %) compared to the highest ML of Arkot (71.3 %) (Fig. 2C). At Marianna in 2015, in the high temperature regime, DP393 gave the lowest ML of 27.9 %. This differed significantly from DP 210 B2RF (33.7 %) but not Arkot 9704 (29.4 %) and VH260 (30.6 %) (Fig. 2 D).

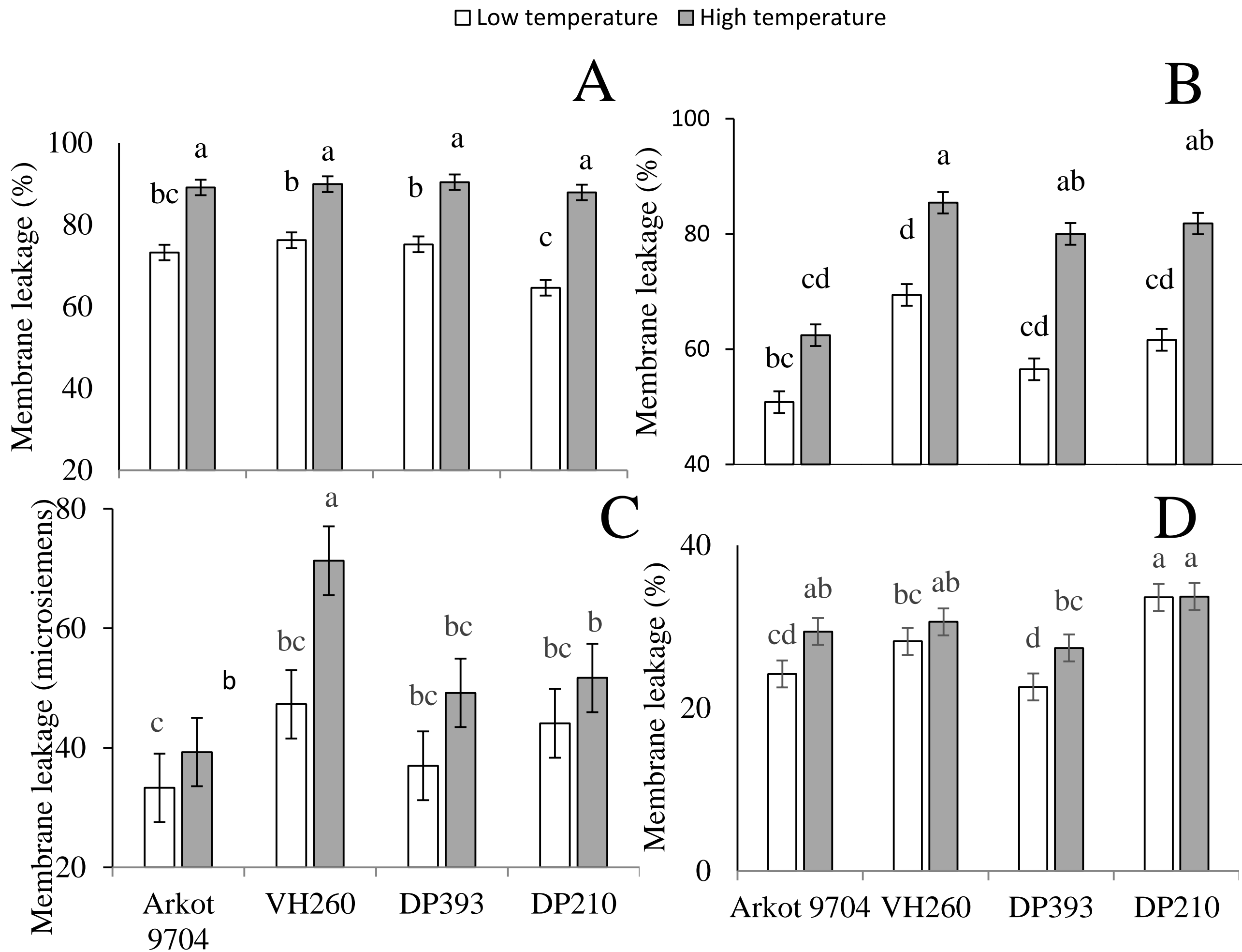

Figure 2. Membrane leakage of four genotypes at two temperature regimes, high and low at (A) Rustenburg in 2015, (B) Rustenburg in 2016, (C) Rustenburg in 2017 (micro Siemens), and (D) Marianna in 2015. Columns with the same lowercase letters are not significantly different (P<0.05). Error bars indicate the standard error at α = 0.05.

Comparison of the percentage change between the low and high temperature regime showed consistently that higher temperatures led to higher ML, but inconsistent results were found between the genotypes. During the 2014 season at Rustenburg percentage change in ML between the low and high temperature regime were non-significant, but the lowest numerical

value was obtained by VH260 (11.7 %) compared to 14.9 % for DP 210 B2RF, 15.2 % for DP393 and 15.9 % for Arkot 9704 (Table 4). At Rustenburg in 2015, VH260 (17.0 %), DP393 (20.0 %) and Arkot 9704 (21.0 %) had significantly lower % change in ML than DP 210 B2RF (36.0 5) between the low and the high temperature regime (Table 4). At Rustenburg in 2016 percentage change in ML for Arkot 9704 (22.0 %) and VH260 (23.0 %) was significantly lower than DP 210 B2RF (32.0) and DP393 (41.0 %). At Rustenburg in 2017 percentage change in ML for DP 210 B2RF (12.1 %) and Arkot 9704 (18.0 %) was lower than for VH260 (50.7 %) and DP393 (33.0 %). At Marianna in 2015, DP 210 B2RF (0.3 %) significantly gave the lowest percentage change in ML, which differed from Arkot 9704 (21.0 %), DP393 (23.4 %) and VH260 (8.5 %) (Table 4).

Table 4. Percentage increase in membrane leakage from the low temperature regime to the high temperature regime at four localities.

	Percentage increase in ML				
	Rustenburg				Marianna
Genotypes	2014	2015	2016	2017	2015
Arkot 9704	15.9a[1]	21.0b	22.0c	18.0c	21.0a
VH260	11.7a	17.0b	23.0c	50.7a	8.5b
DP393	15.2a	20.0b	41.0a	33.0b	23.4a
DP 210 B2RF	14.9a	36.0a	32.0b	12.1c	0.3c

[1] Columns with the same letter are not significantly different at P<0.05.

Chlorophyll fluorescence (F_v/F_m)

Chlorophyll fluorescence was measured during early flowering in Rustenburg 2013 with the Leaftech instrument described in Snider (2010) (Appendix 1) at 5 minute intervals at temperatures of 20, 25, 30, 35 and 40 °C (Fig. 3). Chlorophyll fluorescence was high at 25°C, but decreased significantly from 30 to 40°C (Fig. 3). There was a similar trend for fluorescence with temperature recorded in the growth chamber studies reported in Chapter 1 of this dissertation. A chlorophyll threshold value for temperature stress was reported to be 35°C (Bibi *et al*., 2008).

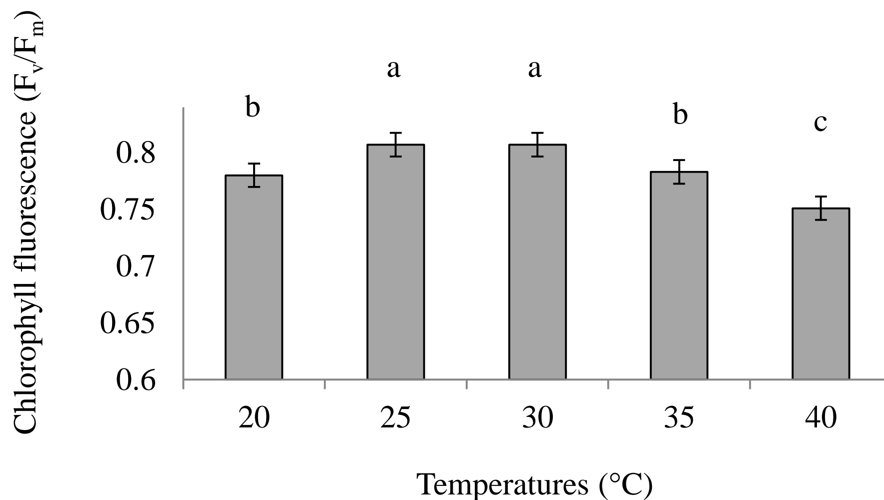

Figure 3. Chlorophyll fluorescence (F_v/F_m) measured with the Leaftech of five different temperatures on fluorescence in a field study in Rustenburg, South Africa in 2013. Columns with the same lowercase letters are not significantly different ($P<0.05$). Error bars indicate the standard error at $\alpha = 0.05$.

Chlorophyll fluorescence (F_v/F_m) was measured in the field at Rustenburg in 2015, 2016 and 2017, and in Marianna in 2015 on days of high and low temperatures. Chlorophyll fluorescence (F_v/F_m) generally showed decreased F_v/F_m at the high temperature regimes compared to the lower temperature regimes (Fig. 4). At Rustenburg in 2015, F_v/F_m between the two temperature regimes differed significantly (Fig. 4). The low temperature regime of 30°C gave significantly higher F_v/F_m values (0.813) compared to the high temperature regime of 34°C (0.680). At Rustenburg in 2016, F_v/F_m differed significantly at temperature regimes. The low temperature regime of 32 °C gave higher F_v/F_m (0.698) than the high temperature regime of 35°C (0.665). At Rustenburg (2017) although not significant the low temperature regime of 23°C gave higher F_v/F_m (0.787) than the high temperature regime of 29°C (0.778). Chlorophyll fluorescence at Marianna in 2015 differed significantly between the two temperature regimes, 32°C and 34°C, with the low temperature regime having higher F_v/F_m (0.517) than the high temperature regime (0.357). However, at Rustenburg in 2017, F_v/F_m did not differ significantly at temperature regimes. This was related to the lower day temperatures (both low and high) than the other years which were within the optimum range for cotton of 20-30°C (Reddy *et al*., 1999).

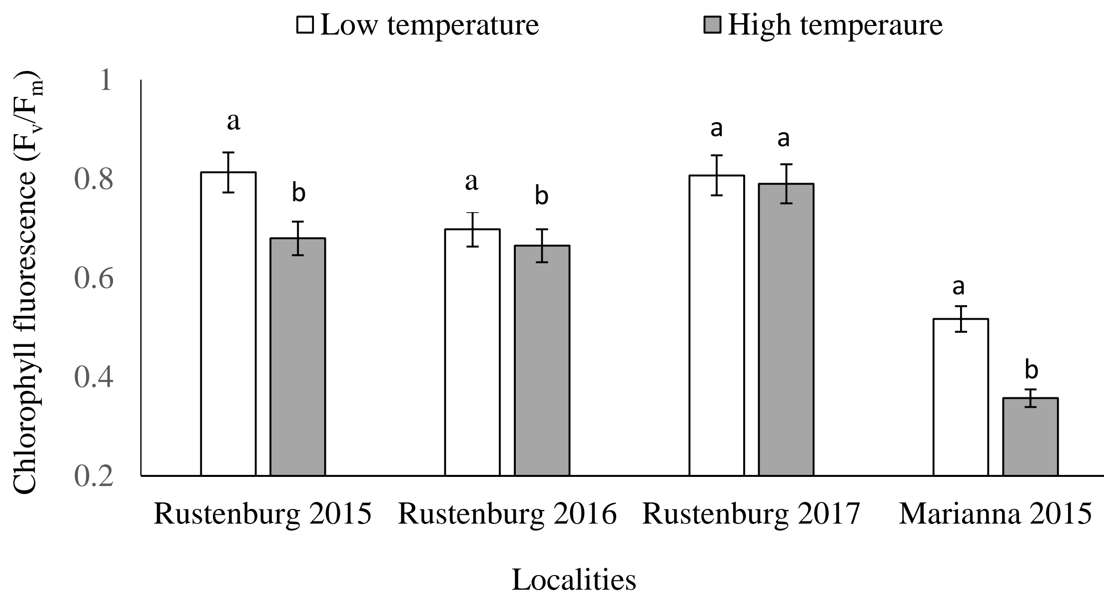

Figure 4. Chlorophyll fluorescence (F_v/F_m) of two temperature regimes meaned over genotypes measured at (A) Rustenburg (2015), 30°C and at 34°C; (B) Rustenburg (2016) 32°C and 35°C; and at (C) Rustenburg (2017), 23°C and at 29°C, and (D) Marianna (2015) 32°C and 34°C. Pairs of columns with the same lowercase letters are not significantly different ($P<0.05$). Error bars indicate the standard error at $\alpha = 0.05$.

The objective of these studies was to determine if we could find differential heat tolerance of genotypes by recording if fluorescence was either maintained or reduced on hot days compared to fluorescence recorded on cool days. Fluorescence values (F_v/F_m) recorded in the field on low and high temperature days for four genotypes at four locations are presented in Figure 5. Fluorescence (F_v/F_m) was generally decreased in the high temperature measurements compared to the low temperature regimes, but genotypic responses to the heat treatment were variable and inconsistent (Fig. 5 A-D). In Rustenburg in 2015, F_v/F_m for DP 210 B2RF (0.664) differed significantly (Fig. 5A) from Arkot 9704 (0.688) andVH260 (0.692) but not from DP393 (0.676). In Rustenburg in 2016 (Fig. 6 B) and Marianna in 2015 (Fig. 5D) genotypes did not differ significantly for the high temperature regime. In Rustenburg in 2017 (Fig. 5C), DP 210 B2RF

(0.773) gave lower F_v/F_m than VH260 (0.779), DP393 (0.780) and Arkot 9704 (0.791). The temperatures on the days of these measurements at the four locations/years varied: Rustenburg 2015 was 32°C, Rustenburg 2016 was 35°C, Rustenburg 2017 was 29°C, and Marianna 2015 was 34°C.

The threshold level of fluorescence F_v/F_m for a fully functional PS11 system was defined by Strasser *et al*. (2004) as 0.750 F_v/F_m. In my study in three years (Rustenburg 2015-2017 and Marianna 2015) F_v/F_m was significantly below the 0.750 the threshold, and the maximum temperature during the measurement were above 32°C (Fig. 5). Whereas in Rustenburg in 2017 there were no significant effects of temperature as the high temperature of 29°C was well within the optimum range for cotton (Reddy *et al*., 1999), and the F_v/F_m values were above the threshold F_v/F_m (Fig. 5).

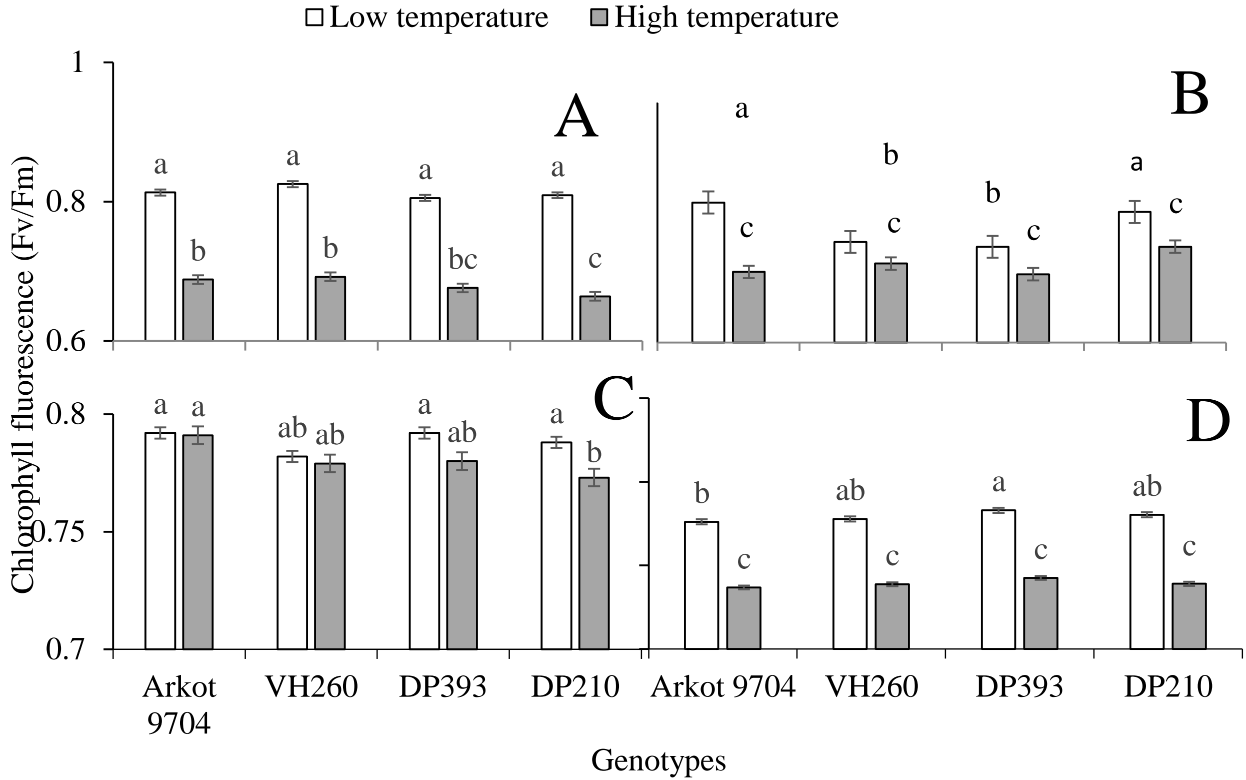

Figure 5. Chlorophyll fluorescence (F_v/F_m) of four genotypes, Arkot 9704, VH260, DP393 and DP 210 B2RF at a low and high temperature at (A) Rustenburg in 2015, (B) Rustenburg in 2016, and (C) Rustenburg 2017, and (D) Marianna in 2015. Columns with the same lowercase letters are not significantly different ($P<0.05$). Error bars indicate the standard error at $\alpha = 0.05$.

When the fluorescence values (F_v/F_m) were compared with the temperatures at which the field measurements were made (Fig. 6), a pattern was observed with a significant >15% decrease in (F_v/F_m) at 30°C, and a sharp fall thereafter to 35°C. A decrease of 15% in fluorescence from the normal or control value has been defined as a significant effect of heat stress (Maxwell and Johnson, 2000; Snider *et al.*, 2010). An upper limit for optimum cotton growth has been reported to be 30°C (Reddy *et al.*, 1999) and a decrease in fluorescence efficiency above this temperature would be expected.

There was little difference between the four genotypes in the pattern of F_v/F_m changes with increasing temperature (Fig. 6). They all showed a similar plateau of ChlF as temperature increased up to 27°C, after which a significant decline of 15% at 30°C and a sharp fall thereafter to 35°C. These results show that there was little difference between the genotypes in response of fluorescence (F_v/F_m) to heat stress. When the fluoresence response at the highest temperatures 33-35°C was analysed, the genotype DP393 showed a slightly improved (higher F_v/F_m) response at the higher temperature 34-35°C (data not shown).

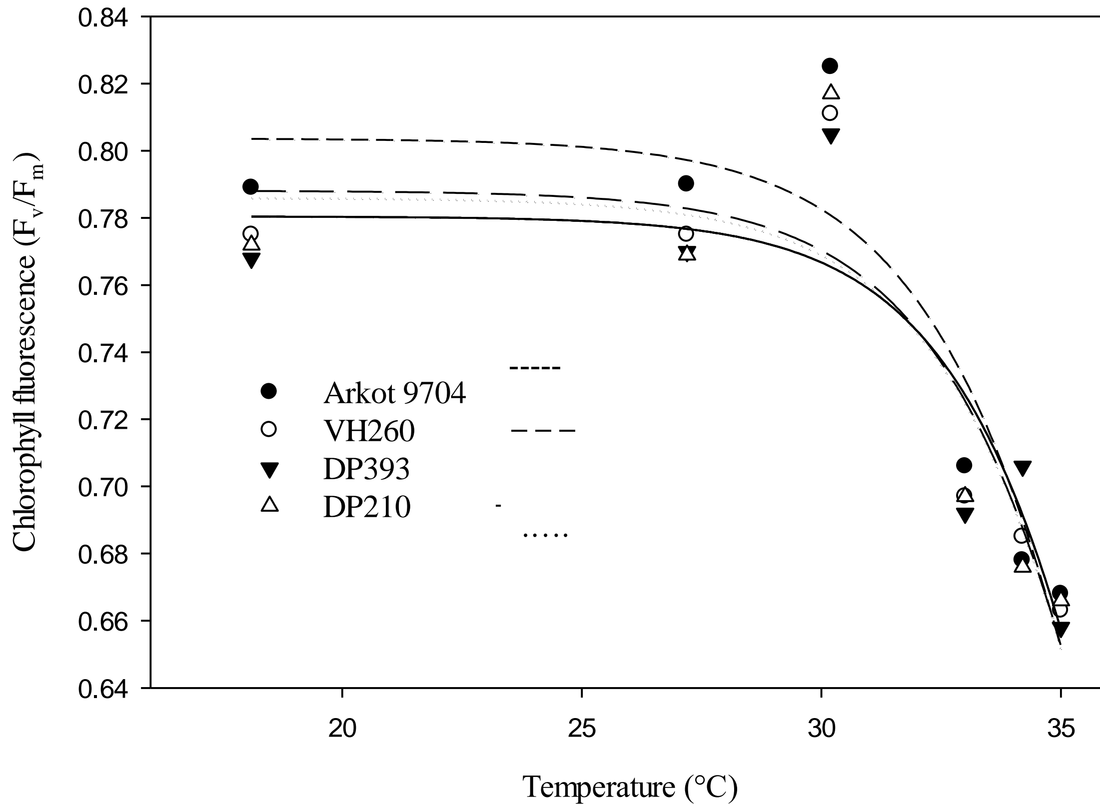

Figure 6. Chlorophyll fluorescence (F_v/F_m) of four genotypes, Arkot 9704, VH260, DP393 and DP 210 B2RF over the temperatures at which they were recorded in the field.

The percentage decrease in F_v/F_m between low and high temperature treatments in each year was calculated to see if there were genotypic differences in ability to tolerate the higher temperatures (Table 5). There were no significant decreases between genotypes in three of the four years, indicating no real genotypic differences in tolerance of the higher temperatures. This may be due to the inability of the technique to determine the small differences between the genotypes.

Table 5. Percentage decrease in F_v/F_m for four genotypes from the low temperature regime to the high temperature regime at four locations.

	Percentage decrease in F_v/F_m			
	Rustenburg	Rustenburg	Rustenburg	Marianna
Genotypes	2015	2016	2017	2015
Arkot 9704	12.5a	5.8a	0.9a	15.8a
VH260	13.3a[1]	1.9b	1.5a	14.3a
DP393	12.9a	2.3ab	1.2a	16.1a
DP 210 B2RF	14.5a	2.9ab	2.2a	16.5a

[1] Columns with the same letter are not significantly different at $P<0.05$

During the final season at Rustenburg (2017), membrane leakage and F_v/F_m were measured at 6.00 AM and 12.00 PM on the same day to provide a low and high temperature in order to determine the effect of the increased temperature on ML and F_v/F_m. The measurements were taken on 15 December 2016 and 12 January 2017 (Fig. 7). On 15 December at 6.00 AM temperature was 19°C and at 12.00 PM 24°C. On 12 January, temperature at 6.00 AM was 21°C and at 12.00 PM 26°C. Membrane leakages at each measuring date resulted in higher ML at 12.00 PM (Fig. 7A&B). However, increases in F_v/F_m were experienced on 15 December 2016 (Fig. 7C) and decreased F_v/F_m was experienced on 12 January 2017 (Fig. 7D). This was related to the clear skies on 12 January (909 MJ/m^2) and cloudy weather on 15 December (535 MJ/m^2).

It was suggested that measurement of chlorophyll fluorescence should only be measured on days without clouds and when temperatures are high enough to cause damage to PSII efficiency.

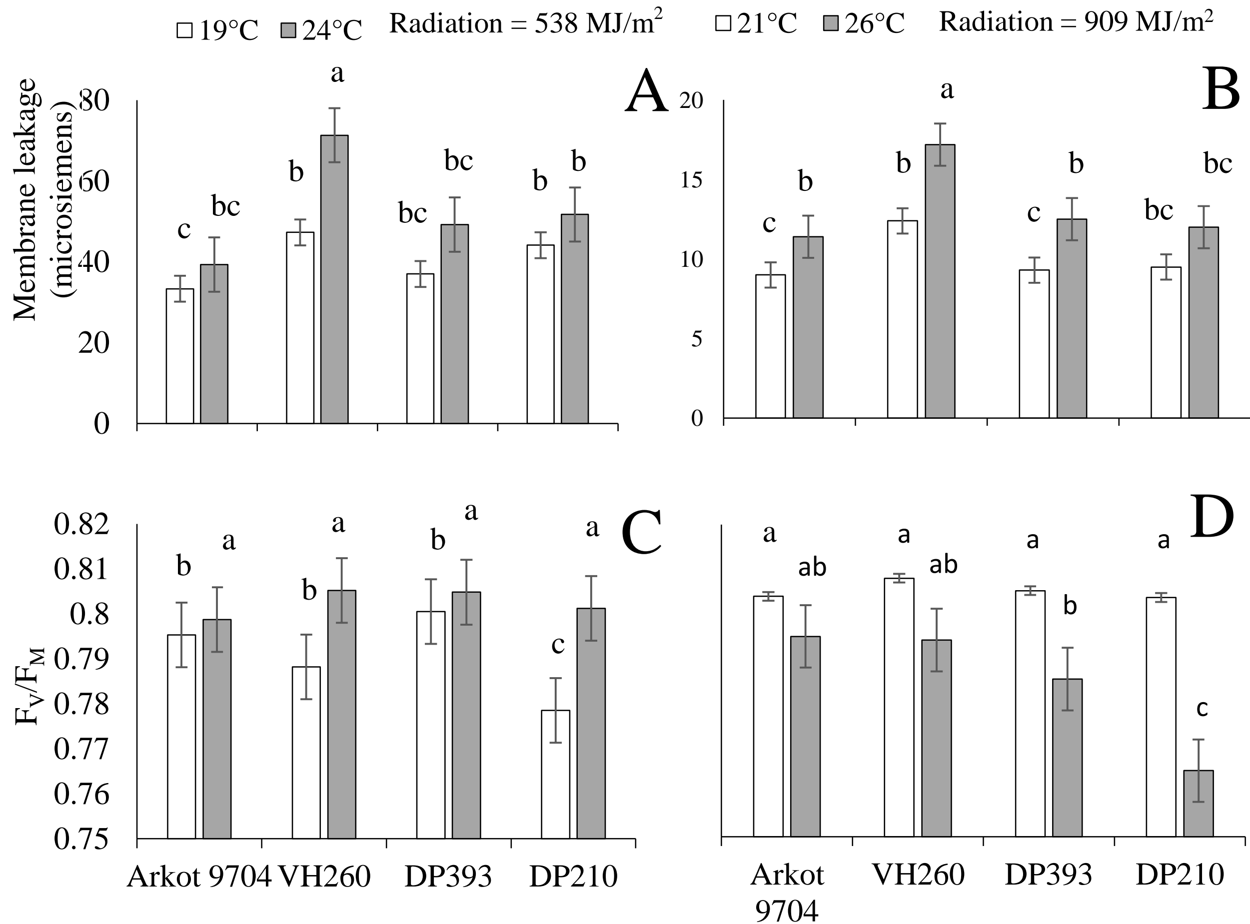

Figure 7. Membrane leakages and chlorophyll fluorescence as measured on 15 December 2016 and 12 January 2017 at Rustenburg at 2 temperatures, morning (6.00 AM) and midday (12.00AM). (A) – ML at 6.00 AM, (B) – ML at 12.00 AM, (C) F_v/F_m at 6.00 AM, F_v/F_m at 12.00 PM. The temperature regimes and radiation for each day of measurement are shown.

Glutathione reductase (GR)

Glutathione reductase activity of leaves was significantly increased by high temperatures (35°C). At Rustenburg in 2014, GR differed significantly between temperature regimes (Fig. 8). The

high temperature regime 35°C gave the highest GR of 131.7 g dry weight^{-1} compared to the 105.9 g dry weight^{-1} for the low temperature regime (31°C) (Fig. 8). There were no significant genotype differences in GR response to higher temperatures (Fig. 9) but DP 210 B2RF had a lower GR content of 137.3 g dry weight^{-1} than Arkot 9704 (146.3 g dry weight^{-1}), VH260 (142.9 g dry weight^{-1}) and DP393 (160.0 g dry weight^{-1}).

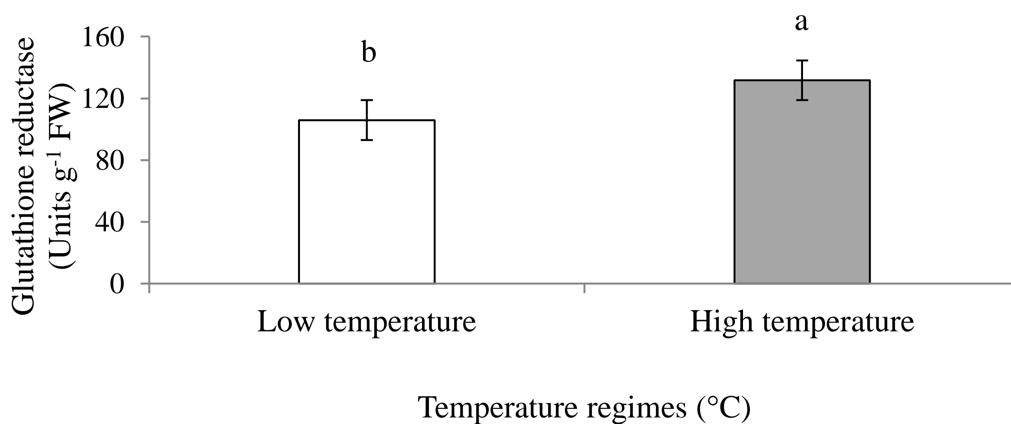

Figure 8. Glutathione reductase content (units g^{-1} FW) of leaves at two temperature regimes low (31°C) and high (35°C) meaned over genotypes measured at early flowering at Rustenburg in 2014. Columns with the same lowercase letters are not significantly different ($P<0.05$). Error bars indicate the standard error at $\alpha = 0.05$.

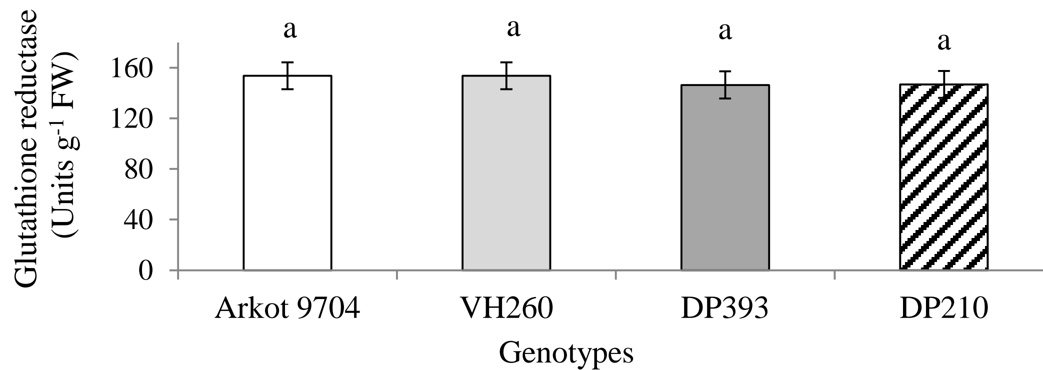

Figure 9. Glutathione reductase content (Units g^{-1} FW) meaned over temperature regimes of four cotton genotypes at Rustenburg in 2014. Columns with the same lowercase letters are not significantly different (P<0.05). Error bars indicate the standard error at $\alpha = 0.05$.

Carbohydrates

There was a significant effect of the higher temperature on leaf starch, sucrose and total carbohydrate content (Fig. 10). At Rustenburg during 2014, temperature regimes differed significantly regarding starch contents in leaves (Fig. 10A). At the low temperature regime significant differences did not exist for starch content between the four genotypes, but at the high temperature regime starch content was higher at VH260 (0.016 mg/g^{-1} DW), Arkot 9704 (0.014 mg/g^{-1} DW) and DP 210 B2RF (0.014 mg/g^{-1} DW) than at DP393 (0.012 mg/g^{-1} DW).

Temperature and genotypes differed significantly for both sucrose and total carbohydrates with increased concentrations at the high temperature regime (Fig. 10B&C). In the low temperature regime, the highest sucrose contents were present in VH260 (0.073 mg/g^{-1} DW) and Arkot 9704 (0.071 mg/g^{-1} DW), and this differed significantly from DP393 with 0.058 mg/g^{-1} DW, but not

from DP 210 B2RF (0.064 mg/g^{-1} DW). When heat stressed, sucrose contents of leaves increased and showed no significant differences between genotypes in the high temperature regime.

For total carbohydrate contents, there were significant differences between genotypes at the low temperature regime (31°C) with decreasing concentrations at the high temperature regime (35°C), but again genotypes differences were not present at the high temperature regime. At the low temperature regime (31°C), the highest total carbohydrate contents were at VH260 (0.073 mg/g^{-1} DW) and this only differed significantly from DP393 with a total carbohydrate content of 0.068 mg/g^{-1} DW, but not from Arkot 9704 (0.079 mg/g^{-1} DW), or DP 210 B2RF BRF (0.076 mg/g^{-1} DW).

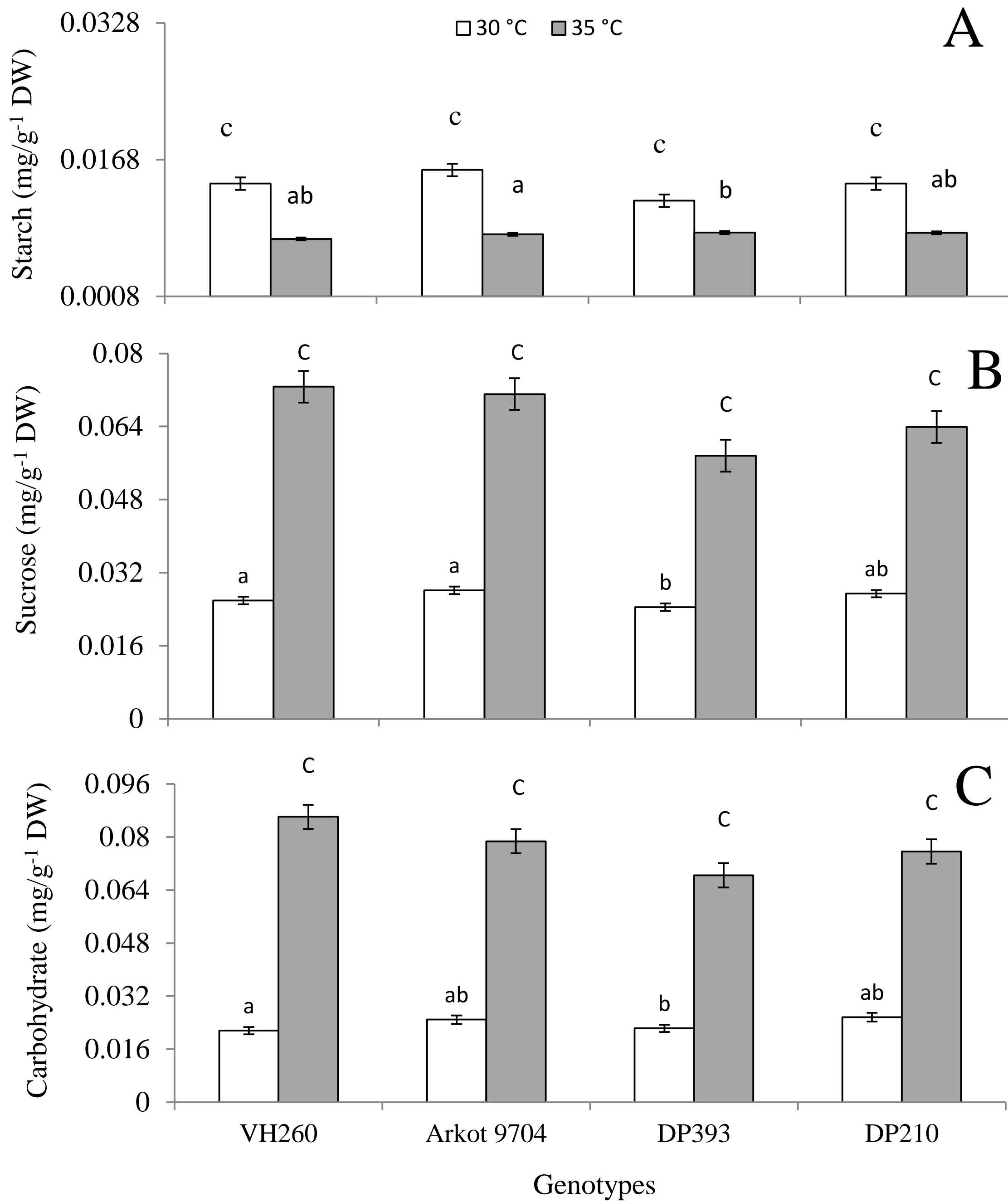

Figure 10. Starch (A), sucrose (B) and total carbohydrate content (C) of leaves measured at 30°C and at 35°C at Rustenburg in 2014. Columns with the same lowercase letters are not significantly different ($P<0.05$). Error bars indicate the standard error at $\alpha = 0.05$.

Lint yield

Genotypes differed significantly in fiber yield at Rustenburg in 2014, 2015 and 2016 as well as in Marianna in 2015 (Fig. 11). Different genotypes yielded the best at different localities and seasons. During the 2014 season at Rustenburg, VH260 gave the highest fiber yield of 1849 $kgha^{-1}$, and this differed significantly from Arkot 9704 (1528 kg ha^{-1}), DP393 (1332 kg ha^{-1}) and DP 210 B2RF (1397 kg ha^{-1}) (Fig. 11A). At Rustenburg in 2015, Arkot 9704 gave the highest fiber yield (1063 kg ha^{-1}), but was not significantly higher than DP393 with 1007 $kgha^{-1}$, and only differed from VH260 (899 kg ha^{-1}) and DP 210 B2RF (642 kg ha^{-1}) (Fig. 11B). At Rustenburg in 2016, VH260 (2281kg ha^{-1}) and DP393 (2127 kg ha^{-1}) gave the highest yield compared to Arkot 9704 (1332 kg ha^{-1}) and DP 210 B2RF (1764 kg ha^{-1}) (Fig. 11C). At Marianna in 2015, DP393 outperformed the other genotypes with the highest yield of 2451 kg ha^{-1}. Arkot 9704 yielded 2171 kg ha^{-1}, VH260 yielded 2076 kg ha^{-1} and DP 210 B2RF yielded 1982 kg ha^{-1} (Fig. 11D).

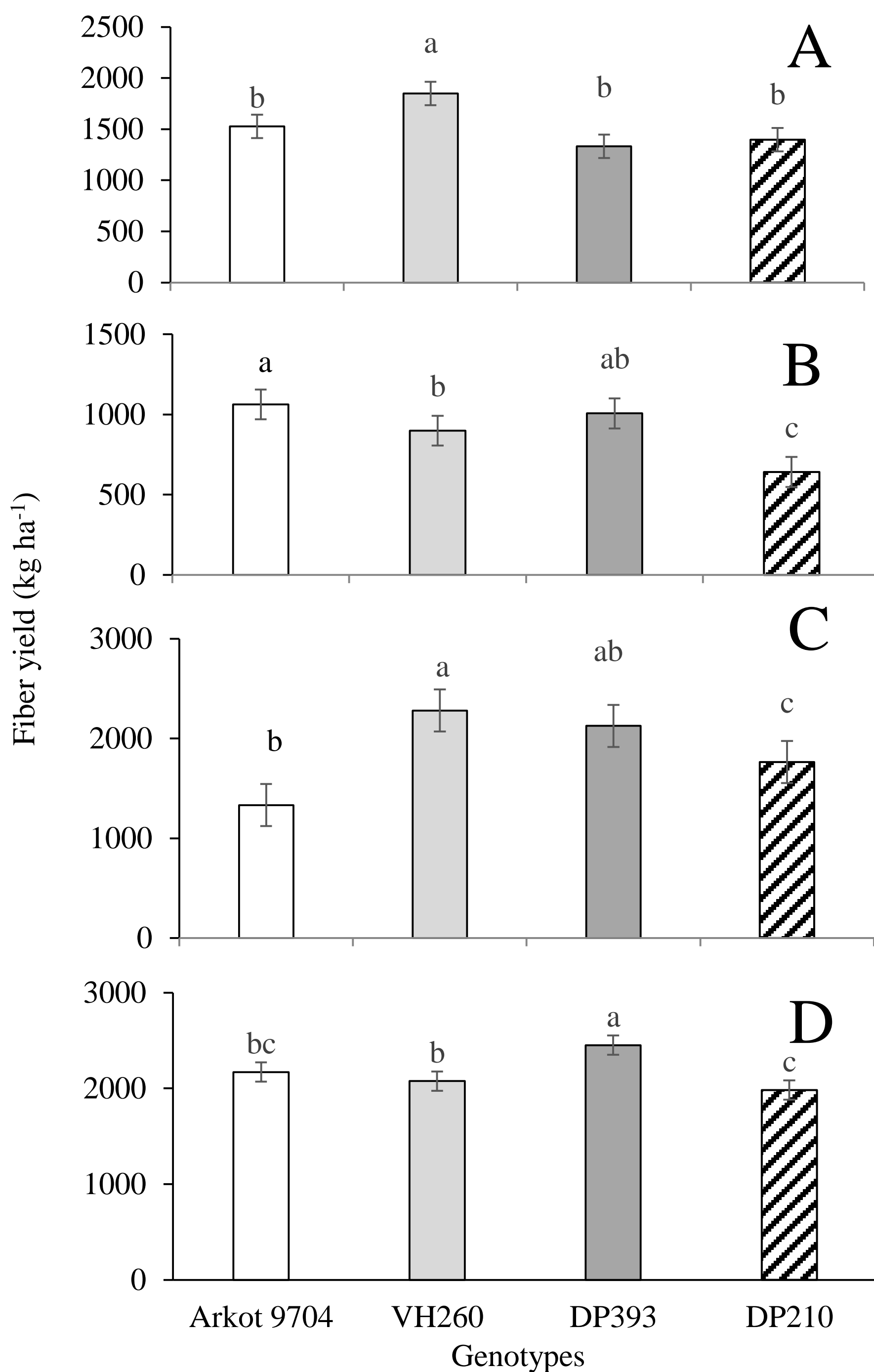

Figure 11. Lint yield (kg ha^{-1}) of four cotton genotypes at (A) Rustenburg in 2014, (B) Rustenburg in 2015 and (C) Rustenburg 2016 and (D) Marianna in 2015. Columns with the same lowercase letters are not significantly different ($P<0.05$). Error bars indicate the standard error at $\alpha = 0.05$.

DISCUSSION

High temperature stress is a major environmental factor that changes from season to season and undergoes daily fluctuations with actively growing plants highly sensitive to heat stress (Zrobek-Sokolnik, 2012). Limitations to normal growth and development in cotton under heat stress result from numerous adverse effects on the physiology of the plant. Some of these effects on physiological processes have been used to quantify the effects of heat stress on plant growth. A screening method is effective if it can show distinct differences in injury to a tissue or plant process (Srinivasan, 1996) and give consistent responses. Many of these studies on screening for temperature tolerance (Zhang, 2013; Sharma *et al.,* 2012) were conducted under growth chamber or greenhouse conditions and don't necessary reflect plant responses in natural field conditions, whereas the current studies were done in field environments to determine if the selected physiological responses would still show heat stress effects in the more unpredictable and variable outdoor field environments. This would be essential if the techniques were to be used in breeding selection of a large range of genotypes for temperature tolerance.

Membrane dysfunction is a physiological process disturbed most by heat stress (Levitt, 1980; Quinn, 1989). The increased permeability and leakage of electrolytes due to stress, reduces photosynthesis and mitochondrial activity as well as the ability of the plasma lemma to retain solutes and water (Lin *et al*., 1985). In the studies reported here ML generally increased with higher temperatures (>30 °C) which agrees with published research on cotton (Bibi *et al*., 2008, Cottee, 2012, and Zhang, 2013), as well as with Sullivan (1971) with grain sorghum, and Blum and Ebercon (1981) with wheat. My results consistently showed increases in ML at higher

temperatures at Rustenburg in all three years as well as in Marianna in 2015 (Fig. 1). At low temperature there were inherent differences in ML values between the genotypes as would be expected due to their different pedigrees (Table 1). The high temperatures increased ML for all four genotypes at all locations (Fig. 2), but variable genotypic responses were obtained

Field measurements of chlorophyll fluorescence (F_v/F_m) with the Leaftech technique showed that F_v/F_m was significantly increased at temperatures of 30°C and above (Fig. 3). A similar result with cotton was shown in growth chamber studies (Chapter 1). An upper limit for optimum cotton growth has been reported to be 30°C (Reddy *et al*., 1999) and a chlorophyll threshold value for temperature stress in cotton was reported to be 32°C by Bibi *et al*. (2008). Thus a decrease in fluorescence efficiency above this temperature would be expected. My studies showed a significant decrease in F_v/F_m above 30°C (Fig. 3) which is in agreement published results (Bibi *et al*., 2008, Snider *et al*., 2010).

The threshold level of fluorescence F_v/F_m for a fully functional PS11 system was defined by Strasser *et al*. (2004) as 0.750 F_v/F_m. In my study in three years (Rustenburg 2015-2017 and Marianna 2015) F_v/F_m in the high temperature measurement was significantly below the 0.750 the threshold, and the maximum temperature during the measurement were above 32°C (Fig. 5). Whereas in Rustenburg in 2017 there were no significant effects of temperature as the high temperature of 29°C was well within the optimum temperature range for cotton (Reddy *et al*., 1999), and the F_v/F_m values were above the threshold F_v/F_m (Strasser *et al*., 2004) (Fig. 5). When the fluorescence values (F_v/F_m) were compared with the temperatures at which the field measurements were made (Fig. 6), a pattern was observed with a significant >15% decrease in

F_v/F_m at 30°C, and a sharp fall thereafter to 35°C. An upper limit for optimum cotton growth has been reported to be 30°C (Reddy *et al*., 1999) and a decrease in fluorescence efficiency above this temperature would be expected.

Chlorophyll fluorescence (F_v/F_m) was consistently decreased with the higher temperatures in 2015 and 2016 field trials at Rustenburg and in 2015 at Marianna (Fig. 5A-D). This is in agreement with Law and Crafts-Bradner (1999), Srinivasan *et al*. (1996); Zhang (2013); Papageorgiou and Govindjee (2004); Shaw *et al*. (2014) and Song *et al*. (2016) who reported declines in F_v/F_m ratios with increased leaf temperatures. Baker and Rosenqvist, (2004) reported that measurements of F_v/F_m gave quantitative assessment of inhibition or damage to electron transfer and provided a sensitive probe of the physiological status of leaves, which could provide rapid assessment of plant performance in a wide range of situations. Genty *et al,* (1989) demonstrated that F_v/F_m measurements could be used to estimate, rapidly and non-invasively, the operating quantum efficiency of electron transport through PSII in leaves. The decrease in F_v/F_m after heat stress is related to the malfunctioning of primary photochemical reactions, primarily involving inhibition of PSII (Berry and Bjorkman, 1980). Overall here results showed similar trends, that when heat stress occurs a decrease in F_v/F_m ensued. There was a decrease in F_v/F_m with the high temperature measurements at three of the four locations (Fig. 5). The exception was in Rustenburg in 2017 which experienced a much cooler season with lower temperatures, i.e., 29°C and 23°C maximum temperatures (Table 3). Clear genotypic differences in response to the higher temperatures were not apparent (Fig. 5) with all genotypes exhibiting similar F_v/F_m values at the higher temperature in each location. When the percentage decreases from the low to the high temperatures in F_v/F_m between the genotypes was considered, the results were

variable and inconsistent at the five locations (Table 5). It was concluded that with the measurement technique used in the field there were no appreciable and consistent differences in the genotypes to the elevated temperatures.

In a separate study in 2017 in Rustenburg, membrane leakage and F_v/F_m were measured at 6.00 AM and 12.00 PM on the same day and repeated on 15 December 2016 and 12 January 2017 to provide a low and high temperature in order to determine the effect of increased temperature (in a single day) on ML and F_v/F_m (Fig. 7). The weather on the two measuring days () was different with clear skies and warmer temperatures compared to slightly overcast conditions with lower temperatures. Membrane leakage was increased from the early morning measurement to the midday measurement regardless of the radiation and showed the effect of the difference in the two temperatures. However, fluorescence was unaffected by the elevated temperature when the weather was overcast (radiation 535 MJ/m^2), but showed differences on a clear day (radiation 909 MJ/m^2). These results indicate that it may be possible to determine the effects of elevated temperatures in the field without a temperature control as used in growth chamber studies, but by using the early morning temperature compared to a higher midday temperature, provided the fluorescence measurement is recorded on days without clouds or overcast conditions in order to illicit radiation damage to PSII efficiency.

The antioxidant glutathione reductase (GR) was increased in activity in response to high temperature stress (Fig. 8) has also been reported for cotton (Bibi *et al*., 2005; Snider *et al*., 2010, and Kawakami *et al*., 2013). Under stress, plants accumulate reactive oxygen species which are capable of damaging nearly every organic component of a living cell (Iba, 2002). As a

result, plants exposed to temperature stress respond with increased antioxidant enzyme activity (Gong *et al*., 1998). In the present study, GR of leaves was shown to increase in response to a high temperature regime, i.e., at Rustenburg in 2014 GR activity increased by 15.8 % at a temperature of 35°C compared to 31°C (Fig. 8). Although others have reported an increase in GR with heat stress, no significant differences between genotypes in the GR were recorded in my study (Fig. 9). Snider (2010) hypothesized that innate thermotolerance would be dependent upon prestress capacity for antioxidant defence in *G. hirsutum* leaves, but we did not record any genotypic differences in GR. The technique for measuring glutathione reductase is laborious and complicated requiring storage in a -80 °C freezer and detailed and expensive laboratory analysis. In my study, the lack of clear genotypic differences in GR activity and the difficult time consuming measurement required suggests that GR was not a suitable screening technique for heat tolerance in cotton.

In my study, heat stress caused a decrease in starch contents and an increase in sucrose and in total carbohydrates (Fig. 10). Increased sucrose contents was reported by FitzSimons (2016) for cotton under high night temperatures. The response in carbohydrates to high temperature by cotton leaves was consistent for all four genotypes (Fig. 10 B&C). Both high temperature and genotype had an effect on carbohydrates. For starch, there were no significant differences between genotypes, and the lowest decrease between control and HS plants was for DP393. For sucrose and total carbohydrates, DP393 showed the smallest percentage change with the elevated temperatures. It was concluded that measurement of carbohydrates was not a reliable screening method to detect stress, as no significant differences were found among the genotypes with heat

stress. Furthermore, the procedure is very laborious and time consuming and a laboratory and analytical instruments are needed.

Genotype differences for fiber yield existed (Fig. 11) but the results were variable between years, as would be expected due to the different seasonal conditions and locations (Appendix 2, Fig. 1-4). VH260 yielded the highest fiber yield in two out of the four trials. Arkot 9704 and DP393 were highest in only one of the four trials, and DP 210 B2RF was generally intermediate in yield ranking. These variable results of yield for the genotypes at the three locations do not show any consistent or useful trend for selecting for heat tolerance. Although my research was conducted at two locations over three years, the findings and trends in plant physiological responses to high temperature stress were consistent. In these studies, ML, ChlF, carbohydrates and antioxidants were measured and evaluated in field conditions as screening techniques to screen genotypes for high temperature tolerance. Membrane leakage was increased in all trials by higher temperatures, but no clear genotypic differences were found. Chlorophyll fluorescence was consistently decreased with the higher temperatures, but clear genotypic difference in response to the higher temperatures was not found. Glutathione reductase activity of leaves was significantly increased by the high temperature, but not between genotypes. Starch was decreased by heat stress, whereas sucrose and total carbohydrates were increased by heat stress, but there were no significant differences between genotypes in response to the high temperature. The genotypes used in this study did not appear to show much difference in thermotolerance. This may be related to the narrower germplasm pool in the current commercial cultivars compared to older obsolete cultivars and wildtype cotton. Modern commercial cultivars have been shown to have less tolerance compared to older obsolete (< 30 years old) cultivars (Brown and Oosterhuis,

2010) and also compared to wildtype cotton (Bibi *et al*., 2004). Furthermore, modern cultivars have shown increased year-to-year variability in yield with higher temperatures, especially when the heat stress occurs during reproductive development (Oosterhuis, 1999).

Overall, higher temperatures caused definite differences in membrane leakage, chlorophyll fluorescence, glutathione reductase and carbohydrate contents in cotton in field trials from 2013 to 2016 in South Africa and in 2015 in the USA. However, significant and consistent differences in the four genotypes studied were not evident. This may have been related to the narrower germplasm pool in the current commercial cultivars compared to older obsolete cultivars and wildtype cotton. The recommendation from the current research would be to use membrane leakage and fluorescence measurements for screening genotypes for temperature tolerance, but with a wider germplasm pool of genotypes, larger sample sizes, and on days with temperatures higher than 30°C. Preliminary research here indicated that it may be possible to determine the effects of elevated temperatures in the field without a temperature control as used in growth chamber studies, by using the early morning temperature compared to a higher midday temperature, provided the fluorescence measurement is recorded on days without clouds or overcast conditions in order to illicit radiation damage to PSII efficiency. The importance of genotype screening for high temperature tolerance for use in future breeding programs, and the adapted management practices in warmer climates is an important endeavour.

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APPENDICES

Appendix 1. Maximum and minimum temperatures, and rainfall data.

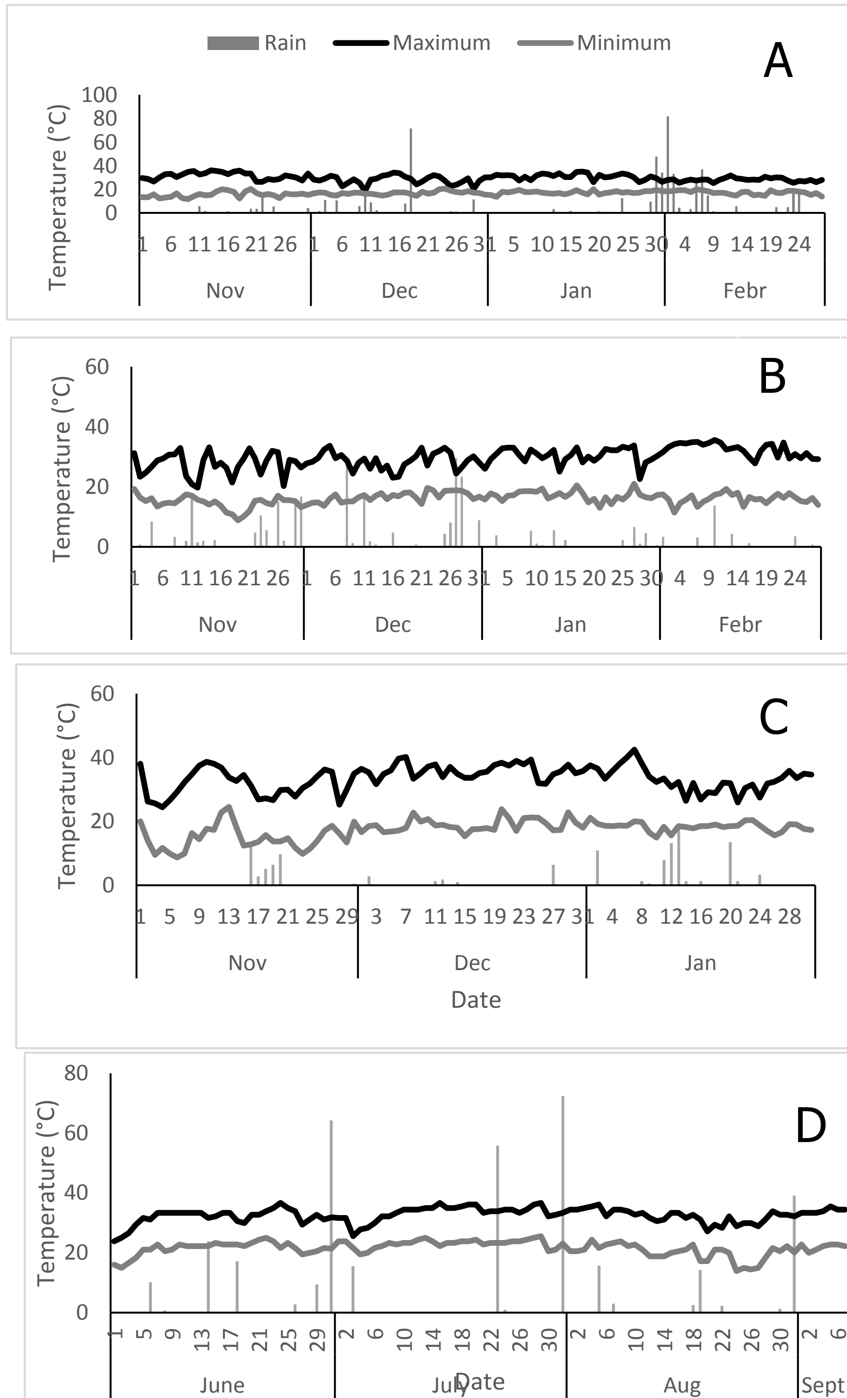

Figure 1. Maximum and minimum temperatures, and rainfall data, of the field studies in Rustenburg, South Africa in (A) 2013/2014; (B) 2014/2015 and (C) 2015/2016.

Appendix 2.

Table 1. Chlorophyll fluorescence of five different temperature regimes and four contrasting cultivars as an indication of the effect of heat stress on fluorescence in a field study in Rustenburg, South Africa in 2012/2013. Leaf temperatures were increased in 5°C increments from 20 °C up to 40°C, and ΦPSII determined with the Leaftech instrument after 5 min of incubation at each temperature.

Treatment	VH260	Arkot 9704	DP393	DP210 BRF
20 °C	0.787	0.779	0.784	0.774
25 °C	0.792	0.789	0.797	0.783
30 °C	0.787	0.785	0.775	0.771
35 °C	0.761	0.765	0.771	0.756
40 °C	0.740	0.728	0.748	0.756
T_{max}	**0.792**	**0.789**	**0.797**	**0.783**
T_{15}	**0.674**	**0.672**	**0.700**	**0.666**

Table 1. Membrane Leakage over seasons and locations of four cultivars.

Location	**Year**	**Cultivar**	**Temperature regime**	
			Low	High
Rustenburg	2014	VH260	76.2	87.9
		Arkot 9704	73.2	89.1
		DP393	75.2	90.4
		DP210	73.0	87.9
Rustenburg	2015	VH260	69.4	85.4
		Arkot 9704	50.8	62.4
		DP393	56.5	80.0
		DP210	61.6	81.8
Rustenburg	2016	VH260	63.6	86.5
		Arkot 9704	30.6	89.5
		DP393	59.7	90.7
		DP210	44.1	90.9
Marianna	2015	VH260	28.2	30.6
		Arkot 9704	24.2	29.4
		DP393	22.6	27.9
		DP210	33.7	33.6

Table 2. Chlorophyll Fluorescence over seasons and locations of four cultivars.

Location	**Year**	**Cultivar**	**Temperature regime**	
			Low	High
Rustenburg	2015	VH260	0.825	0.692
		Arkot 9704	0.813	0.688
		DP393	0.805	0.676
		DP210	0.809	0.664
Rustenburg	2016	VH260	0.684	0.666
		Arkot 9704	0.717	0.659
		DP393	0.680	0.657
		DP210	0.709	0.680
Marianna	2015	VH260	0.511	0.355
		Arkot 9704	0.504	0.347
		DP393	0.532	0.370
		DP210	0.521	0.356

Table 3. Percentage change from the low temperature regime to the high temperature regime in membrane leakages at four localities.

		Rustenburg		Marianna
Cultivars	2013/14	2014/15	2015/16	2015
VH260	11.7	16.0	22.9	2.4
Arkot 9704	15.9	11.6	58.9	5.2
DP393	15.2	23.5	31.0	5.3
DP210	14.9	20.2	46.8	0.1

Table 4. Percentage change from the low temperature regime to the high temperature regime in chlorophyll fluorescence at four localities.

		Rustenburg		Marianna
Cultivars	2013/14	2014/15	2015/16	2015
VH260	3.1	13.3	1.8	15.6
Arkot 9704	2.4	12.5	5.8	15.7
DP393	2.6	12.9	2.3	16.2
DP210	2.7	14.5	2.9	16.5

CHAPTER I

Membrane leakage Study 1

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Cultivar	3	3	216.32767	6.3924	0.0004*
Heat treat	1	1	16.72132	1.4823	0.2254
Cultivar*Heat treat	3	3	161.84846	4.7826	0.0033*
Measuring time	2	2	152.64754	6.7660	0.0016*
Cultivar*Measuring time	6	6	52.51059	0.7758	0.5901
Heat treat*Measuring time	2	2	16.77507	0.7435	0.4772
Cultivar*Heat treat*Measuring time	6	6	24.50512	0.3621	0.9018

Membrane leakage Study 2

Source	Nparm	DF	DFDen	F Ratio	Prob > F
Cult	3	3	138	6.4538	0.0004*
Time	2	2	138	6.8310	0.0015*
cult*time	6	6	138	0.7833	0.5844
heat trt	1	1	138	1.4966	0.2233
cult*heat trt	3	3	138	4.8285	0.0032*
time*heat trt	2	2	138	0.7507	0.4740
cult*time*heat trt	6	6	138	0.3655	0.8997

Membrane leakage Study 3

Source	Nparm	DF	DFDen	F Ratio	Prob > F
Cult	3	3	115	10.7129	<.0001*
heat trt	1	1	115	3.3472	0.0699
Cult*heat trt	3	3	115	0.5302	0.6625
Time	2	2	115	32.7675	<.0001*
Cult*time	6	6	115	0.4364	0.8532
heat trt*time	2	2	115	17.4360	<.0001*
Cult*heat trt*time	6	6	115	1.1773	0.3233

Fluorescence (Fv/Fm) Study 1

Source	Nparm	DF	DFDen	F Ratio	Prob > F
Meas time	2	2	334	244.5682	<.0001*
Cult	3	3	334	21.7738	<.0001*
Meas time*Cult	6	6	334	1.7953	0.0993
Heat trt	1	1	334	4.9007	0.0275*
Meas time*Heat trt	2	2	334	21.6312	<.0001*
Cult*Heat trt	3	3	334	0.1428	0.9342
Meas time*Cult*Heat trt	6	6	334	0.6394	0.6987

Fluorescence (Fv/Fm) Study 2

Source	Nparm	DF	DFDen	F Ratio	Prob > F
Cult	3	3	115	6.0965	0.0007*
heat trt	1	1	115	0.1300	0.7191
cult*heat trt	3	3	115	0.1235	0.9461
meas time	2	2	115	7.4271	0.0009*
cult*meas time	6	6	115	0.3840	0.8880
heat trt*meas time	2	2	115	1.2010	0.3047
cult*heat trt*meas time	6	6	115	0.9760	0.4448

Fluorescence (Fv/Fm) Study 3

Source	Nparm	DF	DFDen	F Ratio	Prob > F
Cult	3	3	115	0.3988	0.7541
Heat trt	1	1	115	0.4078	0.5244
Cult*Heat trt	3	3	115	0.4353	0.7281
Time	2	2	115	4.2597	0.0164*
Cult*Time	6	6	115	1.3744	0.2308
Heat trt*Time	2	2	115	0.9608	0.3856
Cult*Heat trt*Time	6	6	115	1.1508	0.3378

GR Study 1.

Source	Nparm	DF	DFDen	F Ratio	Prob > F
Meas time	3	3	188	9.1533	<.0001*
Heat trt	1	1	188	0.1775	0.6740
Meas time*Heat trt	3	3	188	10.7766	<.0001*
cult	5	5	188	5.4081	0.0001*
Meas time*cult	15	15	188	3.4792	<.0001*
Heat trt*cult	5	5	188	1.4074	0.2234
Meas time*Heat trt*cult	15	15	188	7.7552	<.0001*

Starch study 1

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Cult	3	3	0.00001730	0.3056	0.8211
Heat treatment	1	1	0.00000459	0.2433	0.6263
Cult*Heat trt	3	3	0.00002041	0.3606	0.7820

Total glucose, fructose and sucrose – Study 1

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Cult	3	3	0.00103204	2.7344	0.0659
Heat treatment	1	1	0.00000035	0.0028	0.9582
Cult*Heat treatment	3	3	0.00018023	0.4775	0.7009

CHAPTER II

Table 1: Fluorescence intensities Study 1

Source	Nparm	DF	DFDen	F Ratio	Prob >F
Genotype	3	3	77	29.9207	<.0001*
Temperature	1	1	77	257.6391	<.0001*
Genotype*Temperature	3	3	77	8.7096	<.0001*

Table 2: Fluorescence intensities Study 2

Source	Nparm	DF	DFDen	F Ratio	Prob > F
Genotype	3	3	42	2.8152	0.0507
Temperature	1	1	42	44.1732	<.0001*
Genotype*Temperature	3	3	42	5.7397	<.0001*

Table 3: V_k Study 1

Source	Nparm	DF	DFDen	F Ratio	Prob > F
Genotype	3	3	94.2	1.3899	0.2507
Temperature	1	1	94.19	167.3633	<.0001*
Genotype*Temperature	3	3	92.11	7.4121	0.0002*

Table 4: V_k Study 2

Source	Nparm	DF	DFDen	F Ratio	Prob > F
Genotype	3	3	94.2	1.3899	0.2507
Temperature	1	1	94.19	167.3633	<.0001*
Genotype*Temperature	3	3	92.11	7.4121	0.0002*

Table 5: Fluorescence (F_v/F_m) Study 1

Source	Nparm	DF	DFDen	F Ratio	Prob > F
Genotype	3	3	94.2	1.3899	0.2507
Temperature	1	1	94.19	167.3633	<.0001*
Genotype*Temperature	3	3	92.11	7.4121	0.0002*

Table 6: Fluorescence (F_v/F_m) Study 2

Source	Nparm	DF	DFDen	F Ratio	Prob > F
Genotype	3	3	56	5.9573	0.0013*
Temperature	1	1	56	62.2082	<.0001*
Genotype*Temperature	3	3	56	7.1952	0.0004*

Table 7: PI_{ABS} Study 1

Source	Nparm	DF	DFDen	F Ratio	Prob > F
Genotype	3	3	88	9.1425	<.0001*
Temperature	1	1	88.21	270.3973	<.0001*
Genotype*Temperature	3	3	84.78	21.5295	<.0001*

Table 8: PI_{ABS} Study 2

Source	Nparm	DF	DFDen	F Ratio	Prob > F
Genotype	3	3	56	7.3259	0.0003*
Temperature	1	1	56	0.4767	0.4928
Genotype*Temperature	3	3	56	5.7013	0.0018

Table 9: ET/CSm Study 1

Source	Nparm	DF	DFDen	F Ratio	Prob > F
Genotype	3	3	56	13.4520	<.0001*
Temperature	1	1	56	31.8014	<.0001*
Genotype*Temperature	3	3	56	0.0997	<.0001*

Table 10: ET/CSm Study 2

Source	Nparm	DF	DFDen	F Ratio	Prob > F
Genotype	3	3	56	6.1979	0.0010*
Temperature	1	1	56	99.8696	0.0001*
Genotype*Temperature	3	3	56	7.1996	0.0004*

CHAPTER III

Table 1: Field study 2 Rustenburg 2015 ML

Source	Nparm	DF	L-R ChiSquare	Prob>ChiSq
Cult	31	31	279.763403	<.0001*
Temp	31	31	0.00041548	1.0000
Cult*Temp	31	31	217.42864	<.0001*

Table 2: Rustenburg 2016 ML

Source	Nparm	DF	DFDen	F Ratio	Prob > F
Cult	3	3	85	6.3426	0.0006*
Temp	1	1	85	26.1256	<.0001*
Cult*Temp	3	3	85	0.5533	0.6473

Table 3: Rustenburg 2017 ML

Source	Nparm	DF	DFDen	F Ratio	Prob > F
Cult	3	3	35	5.5033	0.0033*
Temp	1	1	35	9.1391	0.0047*
Cult*Temp	3	3	35	0.9653	0.4200

Table 4: Marianna ML

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Cult	3	3	4855.4042	9.7588	<.0001*
Temp	1	1	1240.9901	7.4827	0.0065*
Cult*Temp	3	3	607.5721	1.2211	0.3014

Table 5: Fluorescence field study Rustenburg 2015

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Cultivar	3	3	0.00292132	3.4876	0.0269*
Planting	1	1	0.17687670	633.4927	<.0001*
Cultivar*Planting	3	3	0.00059744	0.7133	0.5513

Table 6: Fluorescence field study Rustenburg 2017

Source	Nparm	DF	DFDen	F Ratio	Prob > F
Cult	3	3	35	3.6035	0.0228*
Meas time	1	1	35	26.0716	<.0001*
Cult*Meas time	3	3	35	2.7069	0.0601

Table 7: Fluorescence field study Marianna 2015

Source	Nparm	DF	DFDen	F Ratio	Prob > F
Cult	3	3	344	3.1413	0.0254*
Time	2	2	344	233.1713	<.0001*
Cult*Time	6	6	344	3.1857	0.0047*

Table 8: Rustenburg Lint yield 2014

Source	Nparm	DF	F Ratio	Prob > F
Cultivar	3	3	5.4527	0.0031*
Heat trt	1	1	37.1318	<.0001*
Cultivar*Heat trt	3	3	0.7394	0.5348

Table 9: Rustenburg Lint yield 2015

Source	Nparm	DF	DFDen	F Ratio	Prob > F
Cult	3	3	35	9.9376	<.0001*
Heat trt	1	1	35	67.6739	<.0001*
Cult*Heat trt	3	3	35	1.3476	0.2748

Table 10: Rustenburg Lint yield 2016

Source	Nparm	DF	DFDen	F Ratio	Prob > F
Cult	3	3	28	12.7013	<.0001*
Heat trt	1	1	28	49.1482	<.0001*
Cult*Heat trt	3	3	28	2.7394	0.0621