DETERMINING HOW THE PHYSIOLOGICAL AND GENOMIC INTERACTION BETWEEN WAX AND STAYGREEN AFFECTS YIELD AND WATER USE IN *SORGHUM BICOLOR* UNDER DROUGHT AND HEAT STRESS

A Dissertation

by

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ABSTRACT

The balance between plant internal heat requirements and water use is achieved through a combination of physiological, biochemical and genetic processes. The use of physiological traits in crop modeling and breeding programs has been notoriously inaccurate. A contributing problem is the low understanding and low emphasis on the complexity of the subtle interactions between the physiological traits and their genetic regulation. The goal of this study was to highlight how the characteristics of the leaf surface can contribute to staygreen, water use and yield. This was investigated using the following objectives: 1) define the relationship between wax and transpiration in regulating TD among staygreen NILs and non staygreen inbreds and how this affects yield potential under heat, drought and a combination of both stresses, 2) determine if there is physiological and reproductive significance of the bloom (*Bm*, glaucous) phenotype compared to the bloomless (*bm*, non-glaucous) phenotype, and 3) determine allelic diversity and significant gene interaction controlling the bloomless trait.in sorghum. We used sorghum recombinant inbred lines (RILs) and staygreen near isogenic lines (Stg NILs) derived from Tx642 and Tx7000. We also used ethylmethanesulfonate (EMS) mutagenized Tx623 to investigate allelic diversity, inheritance and the photochemical effect of the bloomless mutation. Under objective 1, partitioning data into near high and low leaf wax load (WL) genotypes with uniform duration to flowering (DTF) was important. It revealed that the relationship between wax and canopy temperature depression/ cooling (TD) adopts a quadratic trend among the high wax genotypes under stress with a novel threshold beyond which further increase in wax load does not result in further cooler canopy. Genotypes that expressed the staygreen (Stg) trait and had high WL clearly outperformed all the other phenotypic classes in grain yield. We also noted that with the advancement in phenology, the fluctuations between whole plant evapotranspiration (ET) and WL were antagonistic and polynomial. Canopy temperature changes were better predicted by the interaction between ET and WL than by the individual traits separately. Under the second objective, we determined that the *bm*

mutation is associated with altered heat receptors important in signaling the regulation of stomatal aperture. These alterations may be linked to a modification in the C4 pathway that increases their overdependence on CO_2 influx and open stomata under heat and drought. In the third objective, the results pointed to a potentially large diversity in the alleles, including dominant mutant alleles that can influence the production of the bloomless sorghum. Our observations agreed with both the one-gene and the two-gene models. However, based on the observed complex interactions between some of the alleles, loci and gene products, we have proposed that more detailed studies and validation steps may be required to ascertain the different inheritance patterns.

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To my extended family of friends and relatives of goodwill, I wish we could do it all over again.

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

1.1 Executive summary

If high crop yields and nutrition are the prime targets for many breeding and crop-based sciences, plant stress management is obviously a necessary precursor to that goal. Drought and heat stress lead to major crop losses and are an expensive risk factor in farm commodity programs in the US, while in some parts of the world, it means famine. Floral fertility can be markedly reduced by abiotic stress. For instance, low water potential during male and female gamete development reduces the viability of both pollen and ovules. This might be accompanied by a loss of green leaf area (GLA) due to early onset of senescence. In high temperature-sensitive crop varieties, resulting low fertilization and poor seed set can lead to reduced yield despite vegetative growth being good. This can result in very low seed number on a fully formed panicle, incomplete emergence of panicle, or completely undeveloped flowers. By definition, 'staygreen' therefore includes the expression of stress resistance favorable for seed formation.

Because there is still an insufficient understanding of how plants ameliorate complex cues from water deficit and heat stress, testing the role of staygreen trait in preserving yield during stress is important. Resistance strategies include modified leaf morphology and cuticular wax or trichomes, stomatal aperture, adaptive root architecture, and ability to resist senescence (staygreen). These interact in complex and varied ways. However, determining reliable synergism could be exploited in targeted breeding. This study attempted to define the intersect between leaf cuticular characteristics and the staygreen trait, and the effects this relationship may have in plant physiological performance, water-use and yield.

The *central hypothesis* is that leaf-level stress reducing strategies, such as increased leaf wax, are intrinsically associated with reduced pre-anthesis and post-

anthesis senescence and hence improved floral viability and seed set, respectively. Cuticular wax act as a reflective barrier to excessive photosynthetic and heat generating irradiation to mediate a cooler plant canopy thus reducing temperatures, transpiration and their vulnerability to drought and heat stress. Both tissue water deficit and high ambient temperature and light lead to elevated internal leaf temperatures which can induce reproductive senescence and premature onset of vegetative senescence. Overall plant canopy temperature status was determined by temperature depression (TD – the air temperature minus leaf temperature; higher TD means cooler canopy). We *hypothesized* that the relationship between wax and TD is significant in defining a potential yield difference between staygreen and non-staygreen genotypes, and that leaf surface waxes contribute to the staygreen trait by constitutively lowering canopy temperature and in this manner reducing injury to the photosystems which might induce senescence-related cascades.

Transpiration through stomata is the main cooling mechanism employed by plants and accounts for about 90% of water lost from the above ground plant tissues, whereas cuticular water loss can be up to 10%. Cooling and water conservation are therefore two opposing but necessary processes under heat and drought stress conditions. *We investigated the hypothesis that* by acting as a cuticular physical barrier to cuticular water loss, leaf wax load is temporally antagonistic to transpiration rate in modulating canopy temperature and in this way, high leaf wax concomitantly reduces water demand from transpirational cooling, and thus increases water-use efficiency under water deficit and high temperature stress**.**

Leaf and stem wax morphology or structural architecture are reportedly different between glaucous (bloom - *Bm*) and the non-glaucous (bloomless - *bm*) plants. The carbon chain-length of the precursor fatty acids may also be implicated in the final visible differences. However, there is little research on how these dissimilarities contribute the overall plant response to abiotic stress. A knowledge gap also exists in how the bloom phenotype is regulated and its significance in stress coping strategies. Normal, wildtype sorghum is *Bm.* We investigated the hypothesis that glaucousness

(*Bm*) is simply inherited and that it offers physiological advantage over non-glaucousness (*bm*) in modulating resistance to water deficit and heat stresses.

The *goal of this study* was to improve our effectiveness of using staygreen and compositional surface waxes in breeding by classifying and understanding how these two traits interact in relation to transpiration, canopy temperature, and photochemical efficiency. This knowledge will be important in improving the efficacy of predictive modeling techniques for water-use and productivity of crops in dry and hot conditions.

The following objectives were used to test our hypotheses:

Objective 1: Define the relationship between wax and transpiration in regulating TD among staygreen NILs and non staygreen inbreds and how this affects yield potential under heat, drought and a combination of both stresses.

There were *two related hypotheses* for this objective. The *first* is that the relationship between wax and TD is significant in defining the yield potential and the difference between staygreen and non-staygreen genotypes, and that leaf surface waxes contribute to the staygreen phenotype by constitutively lowering canopy temperature and in this manner reduce injury to the photosystems which might induce senescence-related cascades. The *second* is that by acting as a cuticular physical barrier to cuticular water loss, leaf wax load is temporally antagonistic to transpiration rate in modulating canopy temperature and in this way, high leaf wax increases water-use efficiency under water deficit and high temperature stress**.**

Objective 2: Determine if there is physiological and reproductive significance of the bloom compared to bloomless phenotype. The *central hypothesis* of this objective is that bloom offers physiological advantage over bloomless in modulating resistance to water deficit and heat stresses.

Objective 3: Determine allelic diversity and significant gene interaction controlling the bloomless trait in *Sorghum*, in relation to wax production.

Under this objective it was *hypothesized* that the genes controlling bloomless trait are simply inherited.

1.2 Review of relevant literature

In cereals, rapid physiological changes occur as the plant prepares to undergo anthesis. The ability of plants to maintain floral viability despite abiotic stress is an essential objective in breeding for stress tolerance. In determinate crops such as *Sorghum*, this is critical in rescuing yield from the effects of transient heat and water stress that might hinder floral development, flowering and grain growth.

In some dry regions the proportion of water returned to the atmosphere by evapotranspiration may be 90% or more (Morison et al., 2008) . As such, increasing water-use efficiency would be a worthy target. That is, we want to produce more biomass and grain yield with a smaller amount of water. Waxes are long hydrocarbon skeletons on the outer surface of leaves with varied functional end groups. Cuticular waxes can be embedded with phenolic lipids that function in defense against bacteria and fungi (Sobrado, 2008), and may also mediate the leaf-air interface in a manner that lowers the potential of plant desiccation (Kirkham, 2005; Monneveux et al., 2007; Seibt et al., 2008). Thus, wax may reduce water demand during drought conditions by acting as a physical barrier. Our study aims to determine how sorghum uses water in relation to the changes in wax load and how this influences plant productivity.

1.2.1 The staygreen trait and drought tolerance

Staygreen (Stg) has been traditionally regarded as an integrative drought adaptation trait characterized by sustained greenness (of leaves and stem) especially at grain filling under water limiting conditions. In sorghum, extension of green leaf area (GLA) and higher photosynthetic rate have been cited as ways in which the *Stg* genomic loci when introgressed into single or different genetic backgrounds contribute to higher grain yield (Borrell et al., 2014a; Borrell et al., 2014b; Harris, 2007; Jordan et al., 2012; Kassahun, 2010; Vadez, 2011). This may be due to a reduced leaf size thereby conserving water for grain filling (Borrell et al., 2014a), or higher leaf nitrogen which might suggest a higher photosynthetic rate among some *Stg* genotypes (Harris, 2007). Below ground root architecture in the active phase of leaf metabolism (Borrell et al.,

2014a; Kassahun, 2010) has also been linked to staygreen. To date, no consistent yield penalty has been associated with the *Stg* genetic loci. Even though biomass accumulation is radiation-limiting under high-yielding (non-stress) conditions, some studies have not shown that reduced leaf area negatively impacts the radiation intercepted under sufficiently optimized yield (ALa et al., 2012; DeLacy et al., 2010; Dingkuhn et al., 2006; Kouressy et al., 2008; Lafarge and Hammer, 2002; Rosenow et al., 1983).

On the contrary, a reduction in canopy size or leaf size has been associated with increased grain yield under post-anthesis drought stress when biomass is a function of water availability (Borrell et al., 2014a; van Oosterom et al., 2011). This has been linked to *Stg* QTLs compared to alleles from the senescent recurrent parent, TX7000 (van Oosterom and Hammer, 2008). Such generalizations blur clarity as to which *Stg* QTL (or a combination of *Stg* QTL) has the greatest potential to achieve this under a combination of water deficit and heat stress. Since water-use regulation is a key factor for drought tolerance advantage among the *Stg* loci in sorghum, determining how the cuticular layer balances stomatal transpiration among four well known *Stg* sorghum NILs would be useful. By employing photosynthetic and water-use indicators, we investigated how leaf waxes and transpiration crosstalk to modulate leaf internal water and temperature required for productivity. The aim was to understand the cumulative physiological role and behavior of the genes resident at these *Stg* genomic regions.

1.2.2 Temperature regulation in optimal photosynthetic performance

Optimum gas exchange allows the leaf photosynthetic machinery to access carbon dioxide (CO_2) but high leaf temperature reduces the diffusion and solubility of CO_2 versus O_2 in the leaf palisade cells and air spaces (Chen et al., 1994; Fock et al., 1979). Investigations of *Yucca glauca* in the Colorado shortgrass prairie differentiated photosynthetically optimum temperatures from threshold temperatures (Newell, 1996). The former is associated with the natural morning conditions while the latter is linked to higher midafternoon time, and that both of these adjust accordingly as the growing season progresses. The study also established that it is the threshold temperature which

promotes stomatal closure in hot afternoon summer temperatures. The net photosynthesis and stomatal conductance were therefore depressed under hot conditions. By monitoring leaf temperature and leaf-to-air water vapor concentration differences (Δw) in simulated summer heat conditions using potted plants, the study found that decreases in photosynthetic rate appeared to be due primarily to high leaf temperatures. Decrease in stomatal conductance was attributed mainly to high Δw values as well as leaf temperatures exceeding a critical threshold value, even when Δw was artificially maintained at a constant level. Taken together, it is obvious that leaf temperature status is a key determinant of photosynthetic performance, stomatal conductance and potential plant productivity. Modulating leaf temperature is therefore an essential stress tolerance parameter. Determining the role of the interplay between leaf wax and transpiration in regulating optimal leaf temperature would offer valuable insights into heat and drought tolerance.

1.2.3 Senescence

Programmed cell death (PCD) is a necessary physiological and developmental process in both plants and animals. Reape and Greenberg (Greenberg, 1996; Reape, 2008) outlined three necessary criteria to qualify a tissue as undergoing PCD. First, tissue location and time of cell death need to be predictable. Second, cell death somehow benefits the plant at the developmental level. Third, that cell death is hereditary (fingerprinted in the heritable genetic material). This excludes necrotic cell death due to accidental damage, or injury as a result of exposure to a toxic environment and is distinct in some respect to developmental senescence (Gepstein, 2004b; Gepstein et al., 2003b; Pennell and Lamb, 1997). The leaf grows through a series of cell division and differentiation steps into a mature photosynthetic organ, and then enters the final stage of development which is *senescence.* The dramatic changes during senescence involve an orderly degradation of cellular structures beginning with the chloroplast, while the mitochondria and nucleus remain intact until the final stages of leaf senescence (Lim and Nam, 2005). Senescence (as opposed to PCD) is considerably

slowed due to remobilization of nutrients (Guiamét et al., 1999). Remobilization of degradation products becomes a necessary phase of (though not synonymous with) developmental aging, which is masked in staygreen.

Grain sorghum is a perennial grass managed as an annual crop. If these remobilized macromolecules are assimilated to plant parts such as developing seeds and fruits (Hinderhofer and Zentgraf, 2001; Hörtensteiner, 2009; Lim and Nam, 2005; Noodén et al., 1997), then we should expect that the less stress tolerant non-staygreen lines will fill grain post-anthesis to near normal size and weight, irrespective of GLA.

Senescence has also been classified into mitotic and post mitotic senescence (Gan and Amasino, 1997; Lim et al., 2003). Mitotic senescence occurs at active cell division and if this happens in the developing floral meristems, fertilization and seed formation could be hampered. This strengthens our speculation that *Stg* sorghum has the inherent propensity to resist the degradation of essential metabolic structures pre-anthesis, or through remobilization of these degradation molecules to new growth parts (in our case seed tissue structures) post-anthesis, irrespective of GLA. In this way, we should be able to delineate and redefine staygreen functionality, timing and expression under drought and heat stress.

1.2.4 Pre-anthesis senescence as a risk to optimal yield

The actual number of potential florets specified by floral meristems, even under optimal conditions, often exceeds the realized number of fruits or grains that are brought to maturity (Jordan, 1983). The previously vegetative apex initiates inflorescence as a signal of the floral meristem development. Environmental cues, both abiotic and biotic may signal pre-anthesis mitotic and meiotic senescence which may lead to impaired germinal cell differentiation and development (Dolferus, 2011; Ji et al., 2010). For determinate crops such as sorghum, the reproductive potential of the crop is fixed by the total number of floral meristems formed within a period of about 30 days (Jordan, 1983). The two factors determining the number of florets produced are the relative rate of their initiation and their development (Aspinall and Husain, 1970; Husain and Aspinall,

1970). Of these, the rate of floral meristem initiation is more sensitive to severe water deficit (Moss, 1971; Saini, 1997) and high tissue temperatures (Fischer, 1979), and is the main determinant of the number of florets formed for potential grain development (Fischer, 1979; Inuyama, 1976). Observed effects of floral failure include complete absence of floral parts, aborted but fully formed florets (Jordan, 1983), reduced or failure of fertilization at anthesis (Boyer and McLaughlin, 2007; Dolferus, 2011; Ji et al., 2010; Moss, 1971; Parish et al., 2012) or failure of the panicle to fully exert (Inuyama, 1976; Jordan et al., 2012). An association was also found between reduced kernel number in barley with failure of photosynthate production and allocation to spike during pre-anthesis periods when spike dry matter is accumulated (Fischer, 1979). Such sources of variation in actual number of grains developed may be drastic when this reproductive flexibility becomes a function of large genotype-by-environment interactions due to stress. In this context, we hypothesize that sorghum genotypes resistant to pre-anthesis senescence will intrinsically possess leaf-level water-conserving strategies such as increased wax that allow for effective photosynthesis and photosynthate allocation to support the floral development, fertilization and seed development.

1.2.5 Some molecular insights into the staygreen trait

At the molecular level, expression studies support the hypothesis that the onset of senescence involves genes whose products play a role in senescence-related biochemical and cellular changes (Gepstein, 2004a; Gepstein et al., 2003a). Examples of SAGs (senescence associated genes) in response to different senescence-inducing treatments have indicated the existence of a complex regulatory network in leaf senescence processes (He et al., 2002; Lim and Nam, 2005; Lim et al., 2003; Noh and Amasino, 1999; Quirino et al., 2000). Some senescence up-regulated genes belong to various transcription factor families and include SARK (Senescence-Associated Receptor Kinase), GTP-binding protein, WRKY (WRKY amino acid signature-containing DNA-binding domain) , EREBP (Ethylene Responsive Elements Binding Protein), NAC (No Apical Meristem), and MYB (Myeloblastoma) families (Quirino et al., 2000; Yoshida et

al., 2002). Of these, the genes for WRKY53, a MYB protein, and zinc finger protein may show transiently increased expression at a very early stage of leaf senescence (2001; Buchanan-Wollaston et al., 2003; Guterman et al., 2003; Hinderhofer and Zentgraf, 2001) with MYB having additional role in meristem differentiation. The possible importance of Ca^{++} in the regulation of senescence is indicated by elevation of genes for a calcium-binding protein and a Ca^{++}-dependent protein kinase such as CIPK (Batistic and Kudla, 2004; Guterman et al., 2003; Yu et al., 2007).

1.2.6 The dynamics of chlorophyll-related proteins during senescence and their relationship to stress tolerance

The thylakoid-bound proteins LHCP (light harvesting complex) exist as a pigment-protein complex with chlorophyll (Hidema et al., 1992; Hörtensteiner, 2009; Hörtensteiner and Feller, 2002; Park et al., 2007). The LHCP degradation during senescence requires simultaneous catabolism of chlorophyll (Hörtensteiner and Feller, 2002) since the disassembly of the pigment-protein complex causes the release of hazardous chlorophyll which induces photooxidative damage (Hidema et al., 1992; Hörtensteiner, 2009; Hörtensteiner and Feller, 2002; Lim et al., 2003). Chlorophyll degradation is followed by the dismantling of thylakoid proteins by proteases present in chloroplast. Remarkably, nitrogen present in chlorophyll may not immediately be exported from senescing leaves, but might remain in the form of linear tetrapyrrolic catabolites that accumulate in the vacuole after release from the ruptured chloroplast. In such situations where remobilization fails, chlorophyll degradation, though an energy-expensive process might not be carried out in order to mobilize the nutrients, but rather to remove the potentially toxic free chlorophyll (Lim and Nam, 2005; Noodén et al., 1997; Quirino et al., 2000). Vegetative storage proteins (VSPs) have been suggested to serve as a storage buffer for such nitrogen sources (Lim and Nam, 2005). It can therefore be speculated that staygreen leaves might either be maintained through dysfunctional chlorophyll degradation or have their free chlorophyll immobilized by chlorophyll binding proteins (CBP) so as to remain unavailable for the extraplastidal enzymes.

1.2.7 Identifying a reliable staygreen phenotype

How do we identify the wax-dependent staygreen phenotype? Chloroplast efficacy in productivity is of a universally acknowledged significance. Leaves with reduced chlorophyll (chl) count are considered to have a slower photosynthetic rate since the efficiency of this biochemical process has been associated with abundance of chlorophyll-dependent light harvesting complexes. On this basis, chlorophyll degradation during senescence is expected to automatically lead to reduced photosynthesis, while sustained greenness or delayed senescence (staygreen) is associated with high photosynthetic productivity. However, a direct relationship of chlorophyll content to sustained productivity has been debunked by a cosmetic staygreen as opposed to functional staygreen (Hörtensteiner, 2009; Palma et al., 1995; Thomas and Howarth, 2000). While reduced greenness might lower the overall photosynthetic output, the amount of greenness and longevity of greenness do not automatically correlate with sustained photosynthetic efficiency (Passioura, 2012). Martino (2007) using chlorophyll fluorescence, showed that it is possible to maintain reasonable photochemical efficiency even with degreening of bananas treated with 1MCP (1-methylcyclopropane) (Martino et al., 2007), as has been observed for similar stress responses in many other crops (Palma et al., 1995). Consequently, determining whether delayed senescence is cosmetic or not in breeding objectives may require that underlying genetic control be understood in light of the physiological mechanism. Photochemical efficiency seems to be an agreed output of functional chlorophyll machinery. Part of this study focused on factors regulating the functionality of staygreen by looking at leaf level conditions necessary for optimal quantum yield (QY) of photosystem II among genetically near-isogenic lines of staygreen sorghum.

1.2.8 The efficiency of photosystem II as a proxy to the photosynthetic efficacy

Lesions in carbohydrate metabolism are a hindrance to allocation of photosynthates to the panicle (Boyer and McLaughlin, 2007; Dolferus, 2011; Passioura, 2012). Here, we considered QY of electron transfer in photosystem II (ϕPSII) as

indicative of QY for carbon dioxide exchange (ϕCO_2). But the question has remained as to whether stomatal dynamics would affect both ϕPSII and ϕCO_2 in a colinear manner, for one variable to be used as a proxy in place of the other. Conditions limiting CO_2 fixation (e.g. stomatal closure) may lead to enhancement of alternative electron sinks such as photorespiration and a reduction of molecular O_2, reduced assimilation of NO_2^- to NH_4, or increased heat dissipation. Fluorescence emission is one pathway for chlorophyll de-excitation. Fluorescence is highest when photochemistry and heat dissipation is lowest. Therefore, changes in fluorescence reflect changes in photochemical efficiency and heat dissipation (Sinsawat, 2004). In a study of maize, ϕPSII and ϕCO_2 were found to be similar in different leaves (Fracheboud et al., 1999). The ϕPSII/ ϕCO_2 ratio decreases when more electrons are used in photorespiration. The ratio is near-1 with near optimal ϕCO_2.The ϕPSII measures the fraction of photons absorbed by PSII that is used in photochemistry.

1.2.9 Wax morphology and regulation in the bloom and the bloomless variants

Waxes include a very diverse collection of aliphatic compounds. Primer substrates for waxes are synthesized by fatty acid synthase (FAS) in plastids. FAS products are extended by fatty acid elongases (FAEs) and ketoacyl-CoA synthases (KCSs) to give skeletons with as many as 36 carbons (Kunst and Samuels, 2009). The enzymes in associated pathways localized in the endoplasmic reticulum convert the long carbon skeletons into a broad range of compounds. A handful of genes participating in wax biosynthesis and their translocation within the palisade mesophyll cells have been cloned and characterized (Samuels et al., 2008). But there are many missing links in the biosynthetic pathway, their regulation and transport. It is also not clear what intervening processes confer varying degrees of glaucousness under stress. Knowledge of this will help in exploiting cuticular wax in the management of stress in plants.

1.2.10 Known inheritance of bloomless trait in Sorghum

Jenks et al. (Jenks et al., 1992; Jenks et al., 1994) and Peters et al. (Peterson et al., 1982) reached the conclusion that bloomlessness is controlled by a single nuclear gene. They had used the M4 progeny of P954035, from Purdue Sorghum Improvement Center, which had been mutagenized by EMS or diethyl sulfate (at a single concentrations). Both studies agreed that the limited number of *bm* mutant alleles (24) reported so far could not have represented complete saturation of possible gene mutations for the bloomless trait. Single recessive nuclear gene inheritance pattern was also reported using F2 progeny of irradiation-induced bloomless parent and a bloom wild-type (Burow, 2008). But an earlier inheritance study of bloomless mutants in *Sorghum*, established that two gene loci could be conditioning the bloomless phenotype (Peterson et al., 1982). These include *bm1* and *bm*2 for bloomlessness, while sparse bloom (*h*) is conditioned by three or four genes. Some bloom X bloomless crosses failed to satisfy the two-gene (9:7) inheritance model, partly attributable to too many bloom in a cross with one parent and too many bloomless in a cross with another parent. The study suggested that this deviation could be due to a general preponderance of bloomless type apportioned to chance.

An interesting pattern of inheritance was observed in a sparse-bloom X bloomless (Peterson et al., 1982). Bloom phenotype resulted from one locus containing at least one *Bm* allele and the other at least one wild type H (*Bm_H_*). It also resulted from a sparse-bloom phenotype with at least *Bm* in one locus and a homozygous *hh* on the other locus (*BmBmhh* or *Bmbmhh*). Conversely, the bloomless phenotype resulted from one locus being homozygous for *bm* regardless of the alleles at the other locus (*bmbmHH*, *bmbmHh, or bmbmhh*). The study proposed further analysis to establish accurate segregation pattern. Clearly, there is plenty of uncertainty on the inheritance of the bloomless trait. We sought to verify the gene number and to elucidate some probable gene interactions that confound this seemingly simply inherited trait. Our study used Tx623 germplasm, chemically mutagenized at three different concentrations of ethylmethanesulfonate. This was expected to improve the saturation for most of the

possible mutations for the trait. The goal was to identify potential genetic loci regulating the bloom phenotype.

CHAPTER II
UNDERSTANDING THE LIMITS OF CANOPY TEMPERATURE BASED ON EPICUTICULAR WAX LOAD

2.1 Overview

The balance between plant internal heat requirements is achieved through a combination of physiological, biochemical and genetic processes. Plant surface wax has been implicated in canopy temperature modulation which is an important factor influencing yield. Generalizations and sometimes underestimation of the association between leaf wax and canopy temperature depression (TD) has made it difficult to select for wax as a proxy to improved yield-related parameters. In this regard, one trait that warrants precise monitoring is wax load (WL), especially in the absence of soil water and transpiration data. In this study, we demonstrated that partitioning germplasm into the number of days to flowering (DTF) and into WL levels provides a more informative behavior of the WL-TD relationship. The study materials were selected from a population of 100 recombinant inbred lines (RILs) segregating for WL. The RILs were clustered into DTF bins and also into high WL or low WL genotypes, above or below the WL of both parents respectively, across three selection environments. Random samples in both high wax and low wax genotypes were treated to water deficit and heat under greenhouse conditions in two seasons, and data collected beginning at 50% anthesis. Compared to non-partitioned data, the partitioning significantly improved the variance of TD explained by WL. Also, the WL-TD relationship was linear in normal temperature and well-watered conditions, but adopted a quadratic function with a defined threshold under heat and water deficit treatments. Further, the high WL genotypes experienced lower seed set failure, hence higher grain number than their low WL counterparts, under drought and heat stress. These results provide an improved understanding of plant canopy temperature modulation and the potential for improved adaptive trait breeding targeting heat and water deficit stress.

2.2 Introduction

Plant canopy temperature has been suggested as a good secondary predictor of grain yield under conditions where transpiration is limited due to severe water deficit (Amani, 1996; Balota, 2007; Fan T., 2005). The extent to which transpiration counterbalances a plant's canopy cooling cannot be understated. However, the relationship between increased epicuticular wax load and decreased canopy temperature has not been established in a manner predictable enough to warrant its selection as a stress adaptive trait. This study sought to verify the effects of partitioning germplasm into duration to flowering (DTF) and wax load (WL) clusters, based on the *hypothesis* that such binning would improve the use of WL as a functional predictor of other dependent integrative traits such as canopy temperature.

Some studies have suggested that to be an effective selection parameter, canopy temperature depression (TD, increased TD means reduced canopy temperature and vice versa) must be determined for individual environments (Balota, 2007; Blum, 2005a). However, some investigators have overgeneralized TD to assume that similar basic responses to drought-related stress have been reported in all crops (Blum, 2005a). Still, those that have delved into the relationship between wax and TD (Ebercon, 1977; Mondal et al., 2014; Sánchez et al., 2001) have only showed general wax-TD relationships which make it difficult to use wax as a proxy predictor for TD.

In this study, we determined how the pattern of canopy temperature depression is affected by different wax load clusters in sorghum under simulated stress conditions.

2.3 Materials and methods

2.3.1 Germplasm

The 100 F12 RILs used in this study were derived from a cross between Tx7000 and Tx642. Tx642, formerly referred to as B35, has been a useful source staygreen for research and development of drought resistant commercial hybrids (Harris, 2007; Tao, 2000). It is an inbred BC_1 derivative from IS12555 durra sorghum from Ethiopia. It is

susceptible to pre-flowering drought stress but highly resistant to post-flowering drought stress (staygreen trait)(Rosenow et al., 1983) and has relatively high yield potential (Sanchez, 2002; Van Oosterom, 1996; Walulu R. S., 1994; Xu, 2000). The Tx7000 is an elite line released and distributed in the 1940s as Caprock, an open pollinated, grain sorghum variety, by Texas Agricultural Experiment Station at Lubbock. It was derived from a 'Kafir' x 'Milo' cross, and was later used as a male parent in the late 1950s (Xu, 2000). It is currently a public line in the United States (Subudhi, 2000). It is a pre-flowering drought resistant line, but drought susceptible during post-flowering periods (Rosenow et al., 1983; Xu, 2000).

2.3.2 Partitioning wax load into clusters

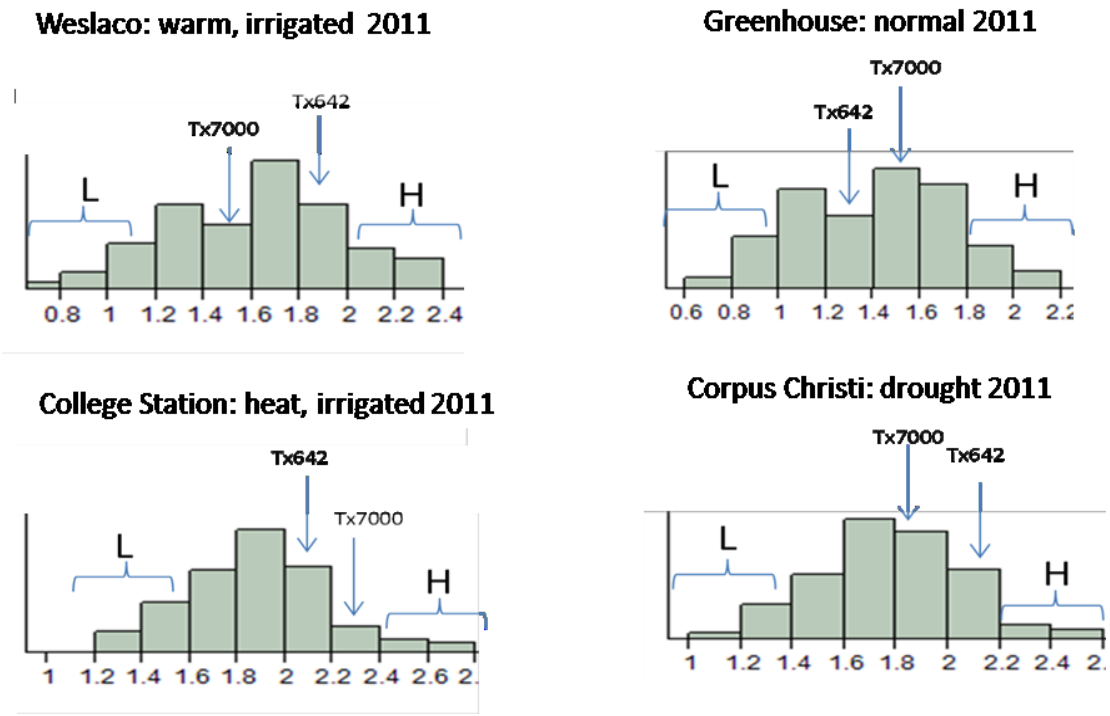

Figure 1: The distribution and two-tailed selection of mean wax load for 100 RILs from Tx642 X Tx7000, across three field locations and the greenhouse. The individual selected were then checked for consistency across the environments for high wax load (H) and low wax load (L). The threshold of at least 1.5mg/dm^2 wax load below (L) the lower wax load parent and above (H) the higher wax load parent. The wax values are log transformed. The mean location and genotypic variation of wax load values were computed from 4 plants per genotype in two reps per environment and treatment, at 50 percent flowering. Wax was extracted from 10 leaf disks per plant. The consistency of these genotypes was reevaluated in the greenhouse under elevated

Using data previously obtained from three field experimental locations at the Texas A&M AgriLife field stations in College Station (high temperature, irrigated, 2011), Weslaco (normal temperature, irrigated, 2011) and Corpus Christi (water deficit, 2011), we identified genotypes which consistently showed higher wax load and lower wax load than the values of both parents (Figure 1).

These were binned in groups falling within 5d of duration to flowering (DTF), using a dendrogram hierarchical clustering based on WARD's data standardization algorithm (Ward, 1963).

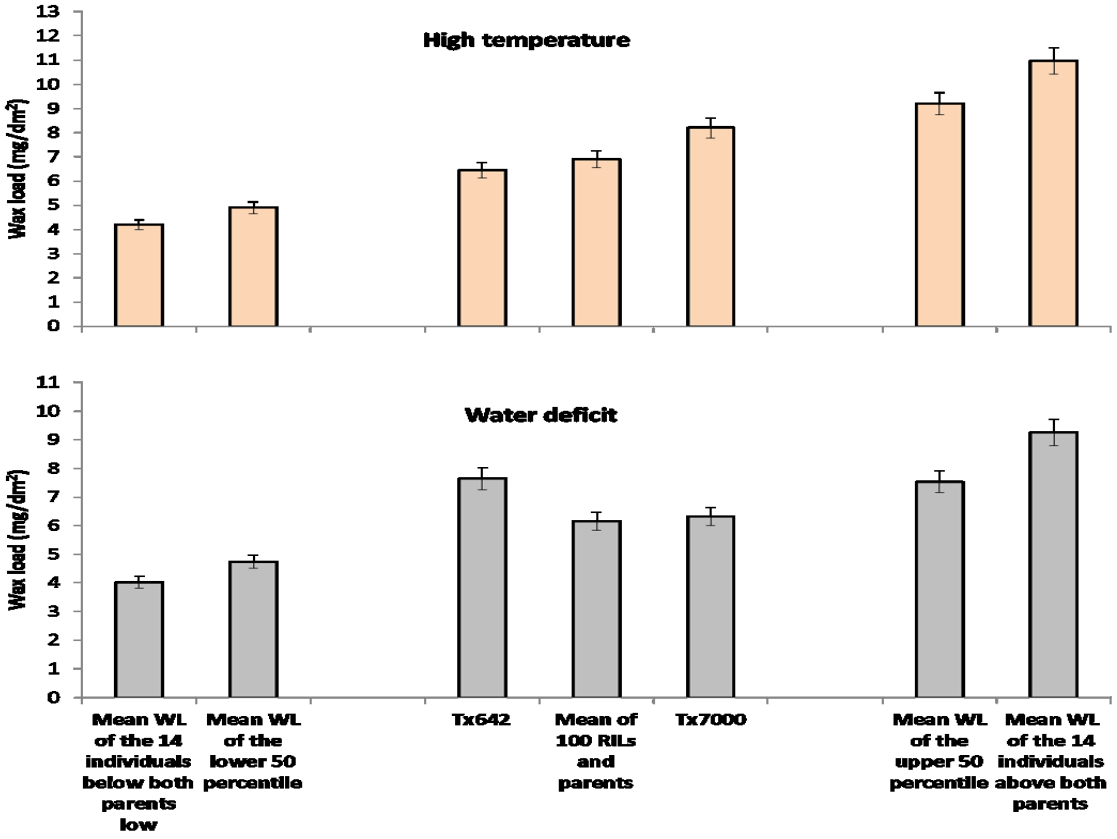

Figure 2: Partitioning based on mean WL of the RILs selected from a two-tailed normalized distribution, under water deficit and high temperature stresses. The means of the 14 low-WL and 14 high-WL lines are on the far left and far right, respectively. These were had similar flowering time and showed stability in WL between stress treatments and between field locations. The means of the lower 50 percentile and the upper 50 percentile and the mean of the total 100 RILs are also shown alongside the two parental lines Tx642 and Tx7000.

Wax load and DTF bins were confirmed in in the greenhouse (Figure 2) using the same 100 RILs treated to water deficit in one greenhouse and another set to elevated

temperatures in another greenhouse, from two weeks to flowering until physiological maturity. Plants in the two sets of treatments were sown in February 2012.

From a normalized distribution of the wax load values in each treatment, a two-tailed grouping was selected from high wax genotypes with values greater than both parents, and low wax genotypes with values less than both parents. These clusters contained the high and low wax genotypes previously identified in the field.

To allow for enough reps, only 14 genotypes consistently showing the highest WL and 14 with the lowest WL were analyzed separately under drought (water deficit), heat (high temperature) and normal greenhouse conditions in summer 2012 and 2013 (drought and heat shown, Figure 2). A fourth set of 14 high-WL and 14 low-WL was also treated to a combined heat and water deficit, but wax values were not taken from this set due to the relatively poor condition of the leaves of most genotypes.

2.3.3 Evaluation conditions and treatments

One hundred-and-forty plants (14 RILs X 5 reps X 2 plants per rep) from the low and high WL clusters were used in each and treatment. Thinning was done to 2 plants per 12l pots filled with forest potting soil (MetroMix 366, Schulenburg, Texas) and watered normally up to the time of stress imposition. The treatments were: elevated temperature & well-watered (heat, H); water deficit & normal temperature (WD); well-watered & normal temperature (WW), and H & WD. The greenhouse conditions were as follows: from two weeks to flowering, 2L of reverse osmosis-distilled water was supplied to the WW and H every 2d, 0.5L every 2d for WD and H & WD; temperatures were set at 31^0C d, 25^0C n for WW and WD; 43^0C, 27^0C for the H and for the H and WD. The heat treatment and the combined drought were set up in a single greenhouse and the two treatments separated by at least 5 feet horizontal space. The WW treatment and the WD were also set up in a different greenhouse with at least 10 feet separating the two treatments. Humidity and light were similarly regulated for all the treatments.

2.3.4 Phenotypic evaluation

Ten fresh leaf disks (7 mm diameter) were obtained from the flag leaf of each plant at 50% anthesis and analyzed for WL using the standard colorimetric method (Ebercon, 1977) and the Beer-Lambert conversion formula (Paynter, 1981). The temperature of flag leaves was taken between 12pm and 2pm when the correlation of TD at anthesis and yield is highest (Balota, 2007) and plant water stress is expected to be high (Fan T., 2005). The leaf temperatures ($\mathbf{T_l}$) for all the reps were averaged using a hand-held IRT (model OAKTon Pro, Class 2(ii) Laser Product, Output Wavelength 630 – 670nm, with external probe). The incident laser point was beamed at 45^0 tilt to the horizontal and incident 5 inches perpendicular to at least 6 different points on the subject contact flag leaf. The external probe was simultaneously used to record the ambient air temperature ($\mathbf{T_e}$). The difference ($\mathbf{T_e}$-$\mathbf{T_l}$) was taken as the temperature depression (TD).

The yield parameters measured included: seed weight per panicle (head weight), 100 seed weight, grain number per panicle and dry biomass.

2.3.5 Data analysis

Data was analyzed using Excel and JMP software. Log-transformed cuticular wax load and raw data for flowering time were used in clustering using WARD algorithm in SAS-JMP. We first determined the main parameters and their interactions that had significant effects on wax load and canopy temperature across the two growing years. To do this, we used combined, full factorial stepwise regression in a forward direction with unbound variance components, to partition the effects of genotype (RILs), wax load status (classification of high or low), treatments (heat, drought and control), years (2012 and 2013). Next, the significant parameters were used as bins within which to determine the effects of wax load on canopy temperature using either linear or polynomial fit depending on the best fit. The LSMeans of yield factors were graphed and means contrasted using the Studentized t-test. Relevant statistical parameters and assumptions (Ott, 2010) were considered in arriving at the inferences.

2.4 Results

As seen in in the greenhouse screening for high-wax and low-wax among the 100 RILs and their parents (Figure 1), the high-wax genotypes remained higher in the mean wax load compared to the low-wax group, the population mean and the means of each parent. The low-wax group also remained consistent.

2.4.1 The effects of wax load-based clustering on the correlation between wax and temperature depression (TD)

Twenty-eight genotypes showing uniform duration to flowering (DTF) were selected. Among these were 14 high wax load genotypes (above higher-WL parent; >8 mg/dm^2), and 14 low wax load genotypes (below lower-WL parent; <6 mg/dm^2). A combined analysis (Table 1) shows that only wax load status (classification into high wax group or low wax group), and treatment had significant effects on the changes of both wax load and canopy temperature. Genotype and year, and the terms containing their interactions were not significant.

Table 1. Combined analysis of the effects of wax load classification on canopy temperature across treatments, genotypes and years. All experiments were separated by greenhouses, in both 2012 and 2013.

	Canopy Temperature		Wax Load	
Model R^2	0.905934		0.861945	
Model Std Error	0.739939		1.237305	
Effect Tests	**F Ratio**	**Prob > F**	**F Ratio**	**Prob > F**
Genotype	1.6633	0.2004	2.9265	0.0905
Treatment	251.8737	**<.0001**	30.014	**<.0001**
Year	1.7773	0.6003	2.04532	0.1094
WL Status	27.0956	**<.0001**	456.0497	**<.0001**
Genotype*Treatment	0.8189	0.4867	0.3573	0.7839
Genotype*Year	1.58109	0.8299	1.42801	0.1902
Genot*WL Status	1.3218	0.2533	3.1313	0.0802
Treatment*Year	1.8775	0.1672	2.882	0.0907
Treatment*WL Status	33.611	**<.0001**	4.0108	**0.0099**
Year*WL Status	0.9339	0.3244	0.8381	0.4821
Genotype*Treatment*Year*WL Status	2.6879	0.0911	0.7705	0.5135

WL Status: wax load, either in the high or low classification. Bolded numbers are significant at $\alpha = 0.5$

That is, the relative change in the mean wax load and the mean canopy temperature between the individual plants remained rather stable (high remained high and low remained low relative to each other within a treatment, and between the two years).

We then checked if relationship between wax load (WL) within each wax load cluster and canopy temperature (TD – temperature depression determined as the air ambient temperature minus leaf internal temperature) remained similar between the treatments. The results show that the WL-TD relationship was a derivative of a quadratic function among the high wax genotypes under heat and under drought stress but was linear under the well-watered, normal temperature conditions (Figure 3).

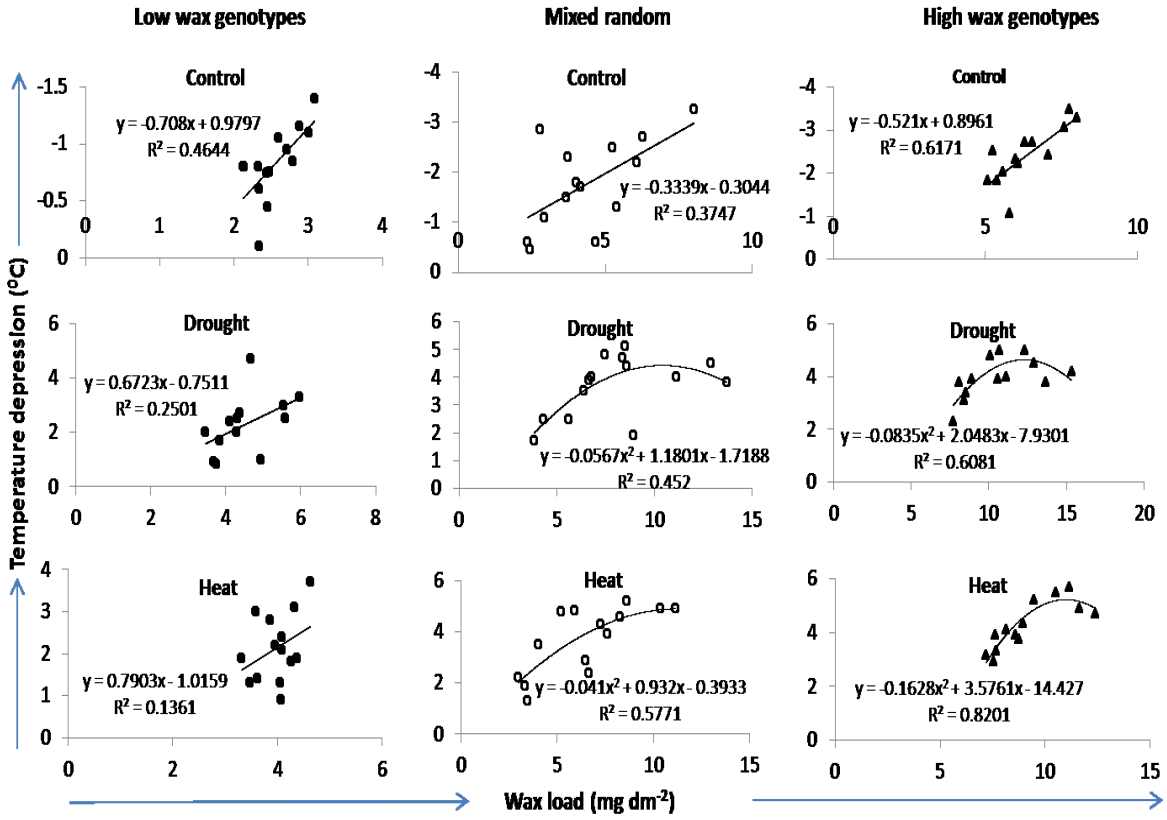

Figure 3. The effects of WL partitioning on WL-TD relationship. Left and right: genotypes showing consistency in high or low wax load at anthesis. Center: genotypes picked at random (the same genotypes in all the treatments). Each data point represents 10 plants X 2 seasons. The limited number is due to the mostly uncommon consistency of WL across the different selection treatments. The WL – TD relationship was stronger and non-linear for high wax genotypes; wax load threshold was established under stress. Note the differences in scale.

All the low wax genotypes had significant linear WL – TD relationships (and lower TD, <3.5^{0}C, compared to the up to ~6^{0}C among the high-WL) in all treatments, suggesting that the nature of WL – TD relationship is both condition and WL-dependent.

The R^2 among the high wax RILs were stronger than among the low wax RILs and the randomly selected 14 RILs. For the high-WL genotypes under stress, the TD plateaued at a WL value of 10.5 and 12.5 mg/dm^2 for the greenhouse high temperature (H, heat) and water deficit (WW, drought) treatments, respectively. Earlier field data produced a close 11 and 13 mg/dm^2 under H and WD, respectively.

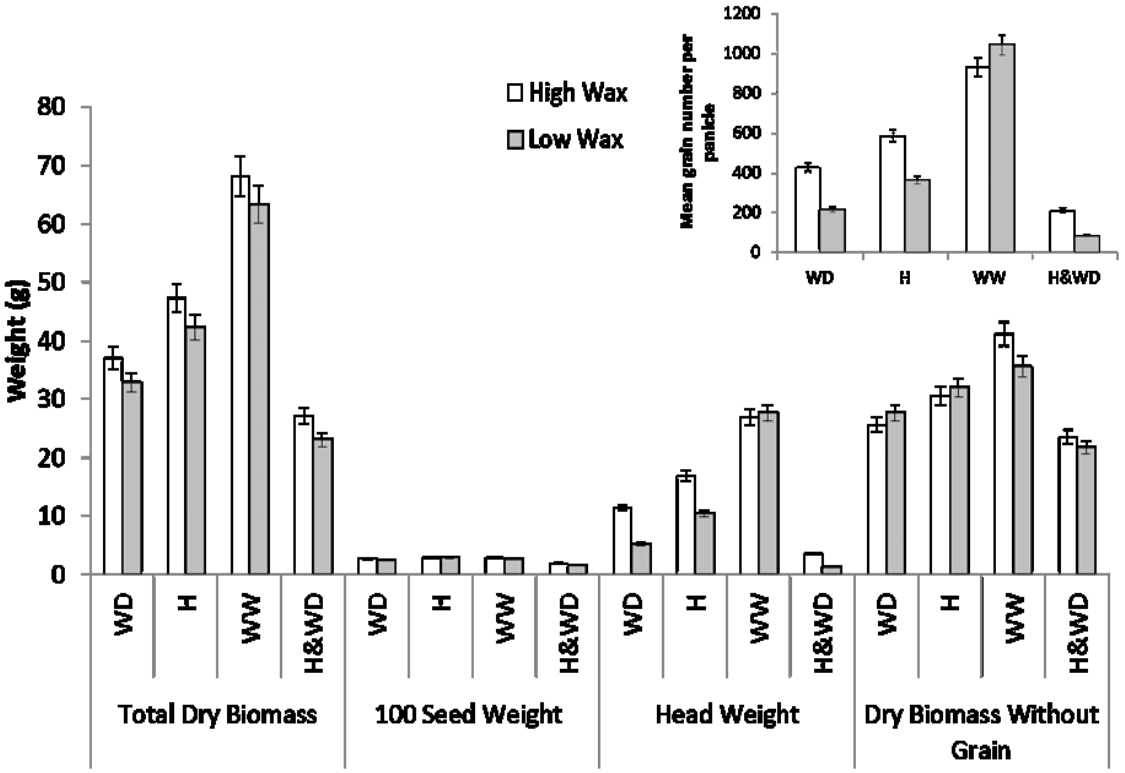

Figure 4. Dry biomass and grain yield for 14 RILs selected for high WL and 14 RILs selected for low WL at reproductive stage. Each bar represents LS Means for 112 plants (14 RILs X 2 reps X 2 plants per rep X 2 seasons). Thinning was done to 2 plants per 3-galon filled with forest potting soil and watered normally up to 40 days after germination (DAG). Treatments were: elevated temperature & well-watered (heat, H); water deficit & normal temperature (drought, WD); well-watered & normal temperature (control, WW), and H &WD. Greenhouse conditions were as follows: from 40 DAG, 2L of reverse osmosis-distilled water every two days for the WW and H, 0.5L every 2d for the WD and H&WD; temperatures at 31^{0}C d, 25^{0}C n for WW and WD; 43^{0}C d, 27^{0}C n for the H and H&WD. The head weight represents grain weight per panicle; the total dry biomass represents above ground biomass (including grain); biomass without grain is dry biomass minus grain. 100 seed weight used due to the very high rate of seed set failure under H&WD.

These observations support the hypothesis that positive wax-TD relationship is constrained within a threshold under given condition. These observations also vindicate the essence of data partitioning as an aid in decision-making.

2.4.2 Determining the relationship between wax load and yield

The genotypes with higher total compositional wax load (WL) on their leaves showed slightly significant higher grain number (GN) per panicle, corresponding to higher MSHW (Figure 4a & b) than the low wax genotypes under stress. There was no significant difference in both GN and MSHW under the well-watered conditions. A similar observation was made for total dry biomass weight (TDBMW). These observations suggest that wax load is one of the factors important in stress modulation during reproductive processes.

2.5 Discussions

The objective was to determine how high wax load and low wax load affects canopy temperature under different growth conditions.

2.5.1 Canopy temperature depression estimation improves by increase in wax load and uniformity in the length of time to flowering

The improved association between WL and TD with increased WL (Figures 3) regardless of treatment shows the importance of wax load-dependent partitioning. Compared to the lower-WL RILs, the higher-WL RILs were a better estimator of leaf cooling potential in the absence of transpiration data. This is a novel finding with no direct reference in the literature. Higher WLs may increase the stability of the associated transpirational cooling.

Because drought and heat stress slow down transpirational cooling due to stomatal closure (Jagadish et al., 2011; Kemanian, 2005; Lilley and Fukai, 1994; Mittler, 2006), plants need efficient reflection of incident heat-generating irradiation to alleviate the heat stress (Sánchez et al., 2001). We speculate that as a desiccation avoidance

strategy, sorghum plants constitutively lay down more wax in anticipation of such extended cooling requirements during early reproductive development. Within similar duration to flowering RIL bins,, partitioning data according to genotype-dependent WL allowed for a better understanding of the effects of wax on the plant's energy balance. This might be important in refining drought screening protocols and redefining criteria for TD-based selection.

2.5.2 Leaves have internal temperature threshold which affects WL-TD relationship

Increasing wax was associated with increasing negative TD (T_e-T_l = negative) in the cooler control temperatures (~31^0C), while the relationship remained all-positive under the stress treatments. This shows that for the same genotype, incremental wax accumulation can be achieved both under reduced temperature and elevated temperature below or above (respectively) what is optimal for a plant's internal biochemical processes. More studies on the effect of extreme low temperatures on wax load might provide a better insight into this area. However, our observation indicate that ambient air temperature can trigger interior leaf temperature responses that redefine the role of leaf surface wax into either heat preservation (insulation) within the leaf, or as incident heat barrier to keep plant cooler. The two possibilities also justify the treatment of TD as absolute values.

2.5.3 The change of canopy temperature depression with change in wax load may be linear or polynomial

A plot of TD against WL produced a linear gradient under normal growth conditions, but drought and heat-treated samples showed quadratic curves, with TD peaking at WL ~12.5mg/dm^2 and 11mg/dm^2 for drought and heat respectively (Figure 3). All the low-WL genotypes in all the treatments and the high-WL genotypes in well-watered treatment showed linear relationships. Their highest WLs were also less than those under either heat or drought treatments. A similar observation was made in previous field data obtained under different field stress conditions.

These observations lend new insights into the physiological association between leaf surface wax and canopy temperature depression. The few, scattered previous studies on wax-TD relationship, all report either linear relationship or no relationship (Ayeneh, 2002; Mason et al., 2010; Mondal et al., 2014; Sánchez et al., 2001). Such reports did not take into consideration the profound effects of partitioning genotypes into high wax and low wax or the differences in duration to flowering under differing stress conditions. Our study also highlights the discovery of a possible threshold WL ($\mathbf{WX_t}$) between 10mg/dm^3 and 13mg/dm^3 under heat (air temperature between 43 - 45^{0}C) and drought (air temperatures between 28 – 32^{0}C) at which sorghum plants experience the most cooling (TD). Higher wax load above this $\mathbf{WX_t}$, did not concomitantly lead to increased plant cooling; instead, genotypes showed a slight decline in canopy cooling, probably due to the negative interaction between wax and residual transpiration. Genotypes able to accumulate up to the wax threshold value did show positive wax-TD correlation under stress. These results may be important in improving the interpretations of datasets obtained from diverse population panels.

2.5.4 Higher cuticular WL may ameliorate the constraints on grain yield due to low seed set

The head weights were significantly higher in the higher-WL RILs than those in the lower-WL category. The reduction in mean grain number per panicle was more severe with concomitant reduced mean grain weight per panicle in the lower wax genotype than in the higher wax genotypes, under water deficit, elevated temperatures and in a combination of both stresses. There was not a significant difference under the well-watered, normal temperature (WW) treatment. We propose that under heat, high wax may reduce cuticular transpiration and improve the reflective capacity of the leaf against heat generating irradiation. This may allow for a reduced stomatal transpirational cooling while maintaining photoassimilate partitioning to floral structures under heat. But this functionality might be limited when tissue water becomes constraining due to severe soil water deficit. With continued open stomata, more severe desiccation occurs

under water deficit, while, if compounded by elevated temperatures, stomata may close resulting in increased tissue photochemical malfunction. There was thus a slightly higher mean grain number under heat than under water deficit stress among both the high WL and the low WL genotypes.

2.6 Conclusions

Generally, leaf surface wax and absolute temperature depression show a positive correlation. However, we have demonstrated that accounting for the duration to flowering and the inherent relative wax load improves the estimation accuracy of the WL to TD relationship. As a result, we have also shown that the wax to TD relationship is not always as linear as some studies have previously reported, but can be either a linear or a polynomial function depending on the amounts of wax load or the stress imposed. This also points to a possible threshold beyond which a further increase in wax load does not correspond to lowered canopy temperature. In any case, by inference, high WL phenotype on average contributed to lower seed set failure compared to the low WL phenotype, under heat, drought and a combination of both stresses.

CHAPTER III

DETERMINING THE EFFECTS OF THE INTERACTION BETWEEN LEAF WAX AND STAYGREEN ON SORGHUM GRAIN YIELD UNDER WATER DEFICIT AND ELEVATED TEMPERATURE STRESS

3.1 Overview

The genetic association between the staygreen trait and accumulation of cuticular waxes is fairly a new concept, and little empirical evidence associating its importance to crop stress exists. This study sought to establish such a connection using staygreen near-isogenic lines segregating for wax load (WL) and recombinant inbred lines (RILs) developed from the staygreen Tx642 and the non-staygreen Tx7000 parents. The *objective* was to determine if WL and the staygreen trait interact to affect yield performance under stress. A hundred RILs were screened for the staygreen trait and wax load and grouped into four phenotypic classes. These four classes were staygreen high wax (SHI), staygreen low wax (SLO), non-staygreen high wax (RHI) and non-staygreen low wax (SLO). Evaluations were done in the greenhouse under elevated temperatures, water deficit and a combination of the two stresses in 2012 and 2013. The yield determinants included grain number (GN), 100 seed weight, mean single head weight (MSHW), dry biomass and yield penalty under stress (ratio of the difference under stress and that under control). The results showed that the SHI clearly outperformed all the other phenotypic classes in mean grain number and MSHW under stress but not under control. SHI also suffered the least penalty in grain number and MSGW under water deficit and under heat. The staygreen alleles expressing high WL also showed a corresponding reduction in overall grain yield penalty under stress, though the timing of stress also had significant effects on both the RILs and NILs. These results suggest that a plant wax can improve staygreen-related stress tolerance under certain stress conditions. This study is one initial step highlighting the potential empirical agronomic significance of the crosstalk between staygreen and cuticular waxes in defining yield under water deficit and heat.

3.2 Introduction

Environmental cues, both abiotic and biotic may signal pre-anthesis senescence which leads to a faulty germinal cell differentiation and development (Dolferus, 2011; Ji et al., 2010); (Jordan, 1983). Water stress accelerates the senescence of lower leaves in sorghum (Jordan, 1983; Jordan et al., 1984), while delayed senescence (staygreen) can improve prolonged dry matter portioning to the grain (Borrell et al., 2014b; Harris, 2007; Jordan et al., 2012; Kassahun, 2010; Tuinstra et al., 1998; Vadez, 2011; Xu et al., 2000). Genotypes varying in the onset and rate of leaf senescence can also be show differences in specific leaf nitrogen (N) and the N uptake during grain filling (Harris, 2007; Hörtensteiner, 2009; Hörtensteiner and Feller, 2002). At the canopy level, leaf waxes can improve reflectance of heat-generating irradiation (Mijitaba, 2004) thus helping prevent premature desiccation of the leaves and potentially securing completion of inflorescence and seed set. However, little is known of a composite effect of the association between cuticular waxes with staygreen in stress tolerance.

Thus it can be hypothesized that by improving reflectance, waxes can reduce premature desiccation and thus help maintain the plant nitrogen intake and retention and hence staygreenness. This possibility merits the consideration of staygreen and wax traits in contributing to enhanced seed set during embryo and pollen maturation and development during stress.

The timing of phenotyping for staygreen has traditionally been done during post-flowering phases, especially from late grain filling stages based on visual scores (Gous, 2015; Guterman et al., 2003; Thomas and Howarth, 2000; Van Oosterom, 1996; Xu et al., 2000). Hence staygreen has been regarded solely as a post-flowering trait in most of these studies including some recent ones (Gous, 2015). Pre-flowering stress-related studies targeting the staygreen trait have largely been ignored. Only a few recent studies show that the genotypes expressing post-flowering staygreen phenotype can have constitutive pre-flowering staygreen-specific expressions. Stress-related gene transcripts expressed including elevated proline levels in water-stressed staygreen sorghum seedlings but not in non-staygreen genotypes (Johnson et al., 2015; Pasini et al., 2014),

in Arabidopsis seedlings (Grassl et al., 2012) and from booting onwards in sorghum (Burke et al., 2010). Proline could therefore one of the important AA in pre-anthesis staygreen.

Proline, an amino acid, for instance plays a highly beneficial role in plants exposed to various stress conditions. Besides acting as an excellent osmolyte, and also act as a metal chelator, an antioxidative defense molecule and a signaling molecule (Hayat et al., 2012). A stressful environment thus results in an overproduction of proline in plants which in turn imparts stress tolerance by maintaining cell turgor or osmotic balance; stabilizing membranes thereby preventing electrolyte leakage; and normalizing the concentrations of reactive oxygen species (ROS. Thus its association with staygreen sorghum under stress as early as the seedling stage may well suggest that at the molecular level, staygreen expression may not only be associated with the post-anthesis phenotypic expression. Staygreen QTLs have also been found to co-localize with QTL for leaf wax when phenotyped at flowering (Awika, 2011). The potential of this association in stress alleviation has not been addressed. In addition, no consistent yield penalty has been associated with the Stg QTLs (Borrell et al., 2014b; Jordan et al., 2012; Vadez, 2011). However, this conclusion has not been tested for genotypes expressing varying degrees of wax load under prolonged stress. In this study, we imposed stress at 40 DAE and 14 DAE in 2012 and 2013, respectively.

3.3 Materials and methods

3.3.1 Plant materials

A population of F_{12} recombinant inbred lines (RILs) and four staygreen near isogenic lines (NILs) developed from a cross of Tx642 and Tx7000 (Subudhi, 2000; Tao, 2000) were used. The parental line Tx642, formerly referred to as B35, has been a useful source staygreen for research and development of drought resistant commercial hybrids (Harris, 2007; Tao, 2000). It is an inbred BC_1 derivative from IS12555 durra sorghum from Ethiopia. It is considered susceptible to pre-flowering drought stress but highly resistant to post-flowering drought stress (staygreen trait)(Rosenow et al., 1983)

and has relatively high yield potential (Sanchez, 2002; Van Oosterom, 1996; Walulu R. S., 1994; Xu, 2000). The Tx7000 is an elite line released and distributed in the 1940s as Caprock, an open pollinated, grain sorghum variety, by Texas A&M AgriLife Research Center in Lubbock. It was derived from a 'Kafir' x 'Milo' cross, and was later used as a male parent in the late 1950s (Xu, 2000). It is currently a public line in the United States (Subudhi, 2000) and is a pre-flowering drought resistant line, but drought susceptible during post-flowering periods (Crasta, 1999; Rosenow et al., 1983; Xu, 2000).

The staygreen NILS (Stg1, Stg2, Stg3 and Stg4) are allelic variants developed from the BTx642/Tx7000 through a series of backcrosses into Tx7000 as the recurrent parent (Crasta, 1999; Tao, 2000; Xu, 2000)**.** We used these staygreen NILs to determine if the interaction between the individual staygreen alleles and cuticular wax is associated with the differential drought and heat tolerance.

3.3.2 Selecting for staygreen-expressing and non-staygreen-expressing RILs

In order to determine the effects of a combined staygreen and wax among individuals segregating for both traits, a population of 100 RILs derived from Tx642 / Tx7000 was exposed to a terminal dry-down from 5 DAP to physiological maturity. These were screened for visual expression of staygreen as previously reported (Borrell et al., 2000; Haussmann, 2002; Subudhi, 2000; Xu et al., 2000). The RILs and the two parents were planted in three reps (pots) with 3 plants per rep, in two separate greenhouses.

All plants were well watered until 5 DAP at which point stress conditions were imposed. The pots were in completely randomized design, with pot positions being shifted every 1 week to reduce any position bias. In one greenhouse were heat (H) treatment and the combined drought (D) and heat under the following conditions: temperature was adjusted from 33^0C d and 25^0C n to 44^0C d, 28^0 n; water 2L every 2d for H and 0.5L every 2d for H and D, up to physiological maturity. In the second greenhouse were the well-watered control (C) and the conditions were: 33^0C d and 25^0C n during the experiment; water 2L every 2d until physiological maturity for C. Water

was adjusted 0.5L every 2d for D, up physiological maturity. In each greenhouse set-up the two treatments were separated by at least 5 feet horizontal space. Humidity and light were similarly regulated for all the treatments.

Two (6 plants) of the three reps were tagged for visual staygreen scoring at 10, 20, 25 DAP and at physiological maturity (sampling stages). The percent green leaf area (%GLA) was estimated visually at each sampling stage based on a scale of 0 to 10, with 0 being less than 10% GLA, 1 being 10% and 10 being 100% GLA. A weighted average GLA based on leaf size as reported by a related study (Kassahun, 2010), was calculated first per tagged plant, then per pot (rep) and then on a genotype basis, for each of the four sampling stages. The average dry-down GLAs (for all the sampling stages) were compared between each of the RILs and the two parents. The senescent parent Tx7000 had 20% average GLA while the staygreen source parent Tx642 had 80% average GLA. Of the 100 RILs, 41 had at least 20% GLA above the senescent parent Tx7000 and were classified as 'staygreen-expressing' RILs. The 48 RILs with equal or lower %GLA than Tx7000 were classified as 'non-straygreen-expressing' RILs. Eleven RILs (between the staygreen-expressing RILs and the senescent parent) accounting for about 11% margin of error of misclassification were left out.

3.3.3 Screening for differential expression of wax load among the staygreen-expressing and non-staygreen-expressing RILs

Wax load (WL) was evaluated for the 41 staygreen-expressing RILs and the 48 non-staygreen-expressing RILs at 50% anthesis, marking the stage at which the overlap of QTLs for staygreen and QTL for wax was previously determined (Awika, 2011). Wax was quantified as the mean of twenty 0.7mm leaf discs collected from the flag leaves of 4 plants per genotype (5 discs per plant) using colorimetric procedures (Ebercon et al., 1977). The flag leaf was used because it is considered the photo-assimilate leaf for the developing florets (Mijitaba, 2004).

The plants sampled for wax were those not tagged for the staygreen. Nevertheless, with the excised disc area adjusted for, we did not observe any significant

reduction in overall percent green leaf area due to leaf disc removal among the wax-sampled plants and those screened for staygreen.

The RILs with mean wax values higher than the higher parental wax load (WL) value (HPW) were classified as 'high wax' RILs. Those showing mean wax load lower than the lower parental WL value (LPW) were classified as 'low wax' genotypes. To qualify as 'high wax' RIL, a genotype had to have at least 1.5mg/dm^2 WL above the HPW; the 'low wax' RILs had to have at least 1.5mg/dm^2 WL lower than the LPW. Of the 41 staygreen-expressing RILs, there were 13 high wax RILs and 17 low wax RILs; 11 did not meet the 1.5mg/dm^2 threshold. Fifteen of the 48 non-staygreen RILs were high wax RILs, while 14 were low wax RILs. The relative consistency of these RILs and the NILs were also confirmed under heat.

3.3.4 Design and evaluation conditions for the effects of staygreen and wax load on yield

For further evaluations, we used 13 genotypes (RILs) each from the subsets expressing staygreen and high wax (SHI), those expressing staygreen and low wax (SLO), the non-staygreen and high wax (RHI) and the non-staygreen and low wax (RLO). Four treatments and two replications (pots) of each phenotypic subset SHI, SLO, RHI, RLO, the *Stg* NILs and the parents Tx642 and Tx7000 were set up in the spring of 2012 and spring of 2013. The treatments included elevated temperatures (heat), water deficit but normal temperatures (drought), a combined heat and drought, and normal temperature and well-watered (control). Each treatment was blocked within four benches, and the pots completely randomized with the reps nested within each block. The pots were randomly shifted within each treatment every 1 week to reduce position bias.

In 2012, stress was imposed 40 days after emergence (DAE), about two to three weeks before the onset of anthesis, until physiological maturity. In 2013, stress was imposed earlier from 14 DAE until physiological maturity. Through physiological and molecular methods, it has been found that staygreen genotypes can be distinguished

from the non-staygreen types earlier than the traditional post-anthesis periods in sorghum, such as from booting (Burke et al., 2010), at flowering (Awika, 2011; Kassahun, 2010) and more recently at seedling stage in Arabidopsis (Grassl et al., 2012) and sorghum (Johnson et al., 2015; Pasini et al., 2014). The different stress timings in 2012 and 2013 experiments were used to determine the effects it may have on staygreen and wax interaction.

The plants were thinned 7 days after emergence (DAE) to 2 plants per three-gallon pot filled with garden soil. In 2012, all pots were first watered to soil capacity before and after sowing, and thereafter water was added at 1kg kilogram (kg) every 2d until 1 week after thinning. Thereafter, water was adjusted to 2kg every 2d until 40 DAE to accommodate for the increased demand for water with increase in plant biomass. Between 40 DAE to 5 DAP, the plants were given 2.5kg every 2d under normal and heat treatments, but reduced to 0.5kg every 2d for drought treatment until physiological maturity of seeds. The temperatures were held at average 31^0C and 27^0C d/n throughout the growing cycle for the drought and control treatments. At 40 DAE, plants for the heat treatment were transferred to a 42^0C and 27^0C d/n greenhouse until physiological maturity.

The 2013 experiment had similar treatments as in 2012 except that drought and heat treatments were imposed from 7d after thinning (14 DAE), with watering under drought reduced to 1kg every 2d after thinning until 40 DAE, 1kg/2d between 40 DAE to 5 DAP, then 0.5kg/2d thereafter until physiological maturity. The combined heat and drought treatment had both the conditions described for heat treatment and for drought treatment.

3.3.5 Evaluation of reproductive response of stress

The reproductive response (how many seeded florets formed) was calculated as a mean grain number (GN) per panicle of all the plants (in each treatment) represented by a phenotypic group RHI, SHI, RLO, SLO, Stg1, Stg2, Stg3, Stg4, Tx642, or Tx7000. The other yield parameters measured included: mean single head weight (MSHW), 100

seed weight, dry biomass, and grain to biomass ratio (mean head weight divided by total dry biomass). To compare the relative change in the yield components, we calculated the percent reduction due to stress (the difference between performance under normal conditions and performance under stress, divided by the performance under normal conditions); the higher the percent reduction, the worse the performance under the stress treatment, and vice versa).

3.3.6 Data analysis

We used log-transformed cuticular wax load as the random variable, while the Stg status, treatment and year were treated as fixed estimators in a mixed, full factorial model. Yield parameters were the dependent variables. The effects of the estimators on the yield factors were determined by stepwise regression in a backward direction. This simplified the analysis by systematic identification and elimination of the higher order interaction terms that were not significant and to improve model fit. The LSMeans of yield factors were graphed and means contrasted using the Tukey HSD. Relevant statistical parameters and assumptions (Ott, 2010) were considered in arriving at the inferences. Data was analyzed using JMP software.

3.4 Results

3.4.1 Determining the effects of high and low WL on yield among the staygreen and non-staygreen RILs

Table 2 shows that treatment significantly affected all the yield components, while WL was significant in mean grain number (GN), and mean single grain weight (MSGW). Staygreen, as expected contributed significantly to 100 seed weight. On a mean basis, this was however, more dramatic under a combined heat and drought stress (Figure 5). The main effects, wax load and staygreen and their interaction significantly contributed to the MSHW, while the interaction between WL and treatment (Tr) was significant in GN. (Table 2). Staygreen was not.

Table 2: Combined analysis of effects of wax load and staygreen on yield parameters among RILs expressing staygreen and those expressing non-staygreen. All experiments where conducted in the greenhouse under well-watered treatment as control, water deficit as drought and a combined elevated temperature as temperatures and water deficit as heat and drought.

	Plant Biomass		Grain Number		100 Seed Weight		Mean Single Head Weight	
Model R^2	0.637215		0.813882		0.696739		0.852043	
RMSE	13.97519		16.74875		0.3379		5.07873	
Effect Tests	**F Ratio**	**Prob > F**	**F Ratio**	**Prob > F**	**F Ratio**	**Prob > F**	**F Ratio**	**Prob > F**
Wax load	1.1893	0.2815	25.807	**<.0001**	10.14	**0.0029**	8.6084	**0.0053**
Stg status	2.9355	0.0939	3.2124	0.0801	4.0283	**0.0491**	6.9637	**0.0115**
Treatment	21.6064	**<.0001**	45.833	**<.0001**	19.187	**<.0001**	69.1097	**<.0001**
Year	0.4096	0.8941	3.3133	0.0526	1.0045	0.7983	3.145	0.0537
Wax load*Stg status	0.9097	0.3455	0.9567	0.3335	0.1373	0.7131	10.4778	**0.0023**
Wax load*Treatment	0.0046	0.9996	3.1999	**0.0326**	0.7472	0.5309	2.5857	0.0654
Wax load*Year	0.1734	0.8825	2.1782	0.1109	3.0016	0.0772	2.0491	0.1202
Stg status*Treatment	0.2929	0.8303	0.8832	0.4574	0.2698	0.8468	0.4794	0.6983
Stg Status*Year	0,7747	0.4482	0.2192	0.1005	0.2718	0.8820	0.4007	0.9163
Treatment*Year	4.9243	0.0525	3.3004	0.0611	2.3341	0.1932	3.4113	0.0592
Wax load*Stg status *Treatment*Year	0.4338	0.7046	2.5966	0.0564	2.9642	0.1405	4.6322	0.0694

RMSE, root mean square error; Stg, staygreen; **Bolded** values are significant at $\alpha = 0.05$

These observations suggest that WL has greater impact on grain number than the staygreen status alone.

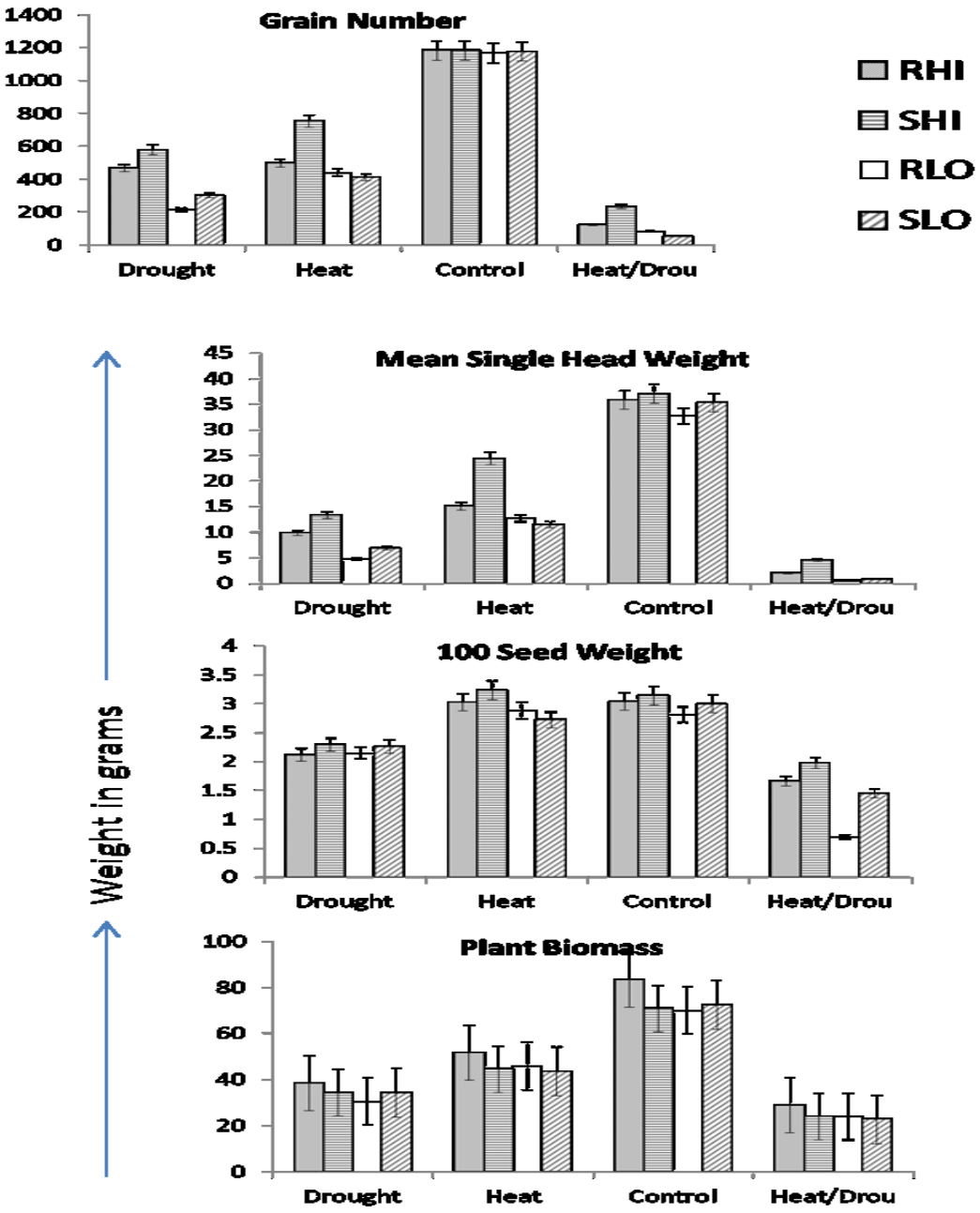

Figure 5. The effects of staygreen (Stg) and wax load (WL) on the yield of F12 recombinant inbred lines (RILs) derived from Tx642/Tx7000. The RILs were classified into four categories of those expressing: non-Stg and high-WL (RHI), Stg and high-WL (SHI), non-Stg and low-WL (RLO), and Stg and low-WL (SLO). Each bar graph represents the LSMean of 54 RILs (13 RILs X 2 reps X 2 plants per rep) in each treatment of well-watered and elevated temperatures (Heat, 42^{0}C d, 27^{0}C n); water deficit and normal temperature (Drought, 31^{0}C d, 27^{0}C n), well-watered and normal temperature (Control), and a combination of heat and drought (Heat/Drou). The experiments were set up in separate greenhouses in the spring 2012 and repeated in the spring 2013.

This was also seen in the mean GN and MSHW component graphs (Figure 5) where irrespective of staygreen status, high wax load was mostly associated with improved grain number in the water deficit and in heat treatments. There was no significant difference under a combination of the stresses.

The combined high wax and staygreen produced highly significant increase in both grain number and head weight compared to all the other phenotypic classes in nearly all the treatments. The staygreen high wax (SHI) had the least penalty on grain number and mean single head weight under water deficit and in the heat treatment. The staygreen low wax (SLO), showed a slight edge in grain number and head weight compared to the non-staygreen low wax (RHO) under drought, but not under heat or a combined heat and drought.

The differences in 100 seed weight were dramatic only under combined heat and drought conditions where the non-staygreen low wax genotypes (RLO) showed a significant reduction compared to the other genotypes (Figure 5b). These observations suggest that in grain sorghum, staygreen and high wax load together offer significant advantage in grain initiation but not necessarily grain filling. However, staygreen's effectiveness in improving grain filling can be enhanced by high wax load under stress. Also, it is possible to determine the differences in tolerance resulting from staygreen and wax irrespective of the timing of stress, though these differences were slightly more distinct when stress was delayed (40 DAE instead of 14 DAE).

3.4.2 The staygreen near-isogenic lines (NILs)

To further investigate the hypothesis that staygreen-wax load interact to provide improved grain yield performance under water deficit and heat stresses, the staygreen near isogenic lines (NILs), were used in similar experiments as described for the RILs. The results showed that the NILs responded differentially depending on the stress imposed.

The NIL Stg3, having a slightly lower wax load (Figure 6) compared to the other NILs, showed the highest treatment penalty on grain number and single head weight

(MSHW) under heat; Stg3 however experienced less reduction in both biomass and MSHW under drought (Figure 6).

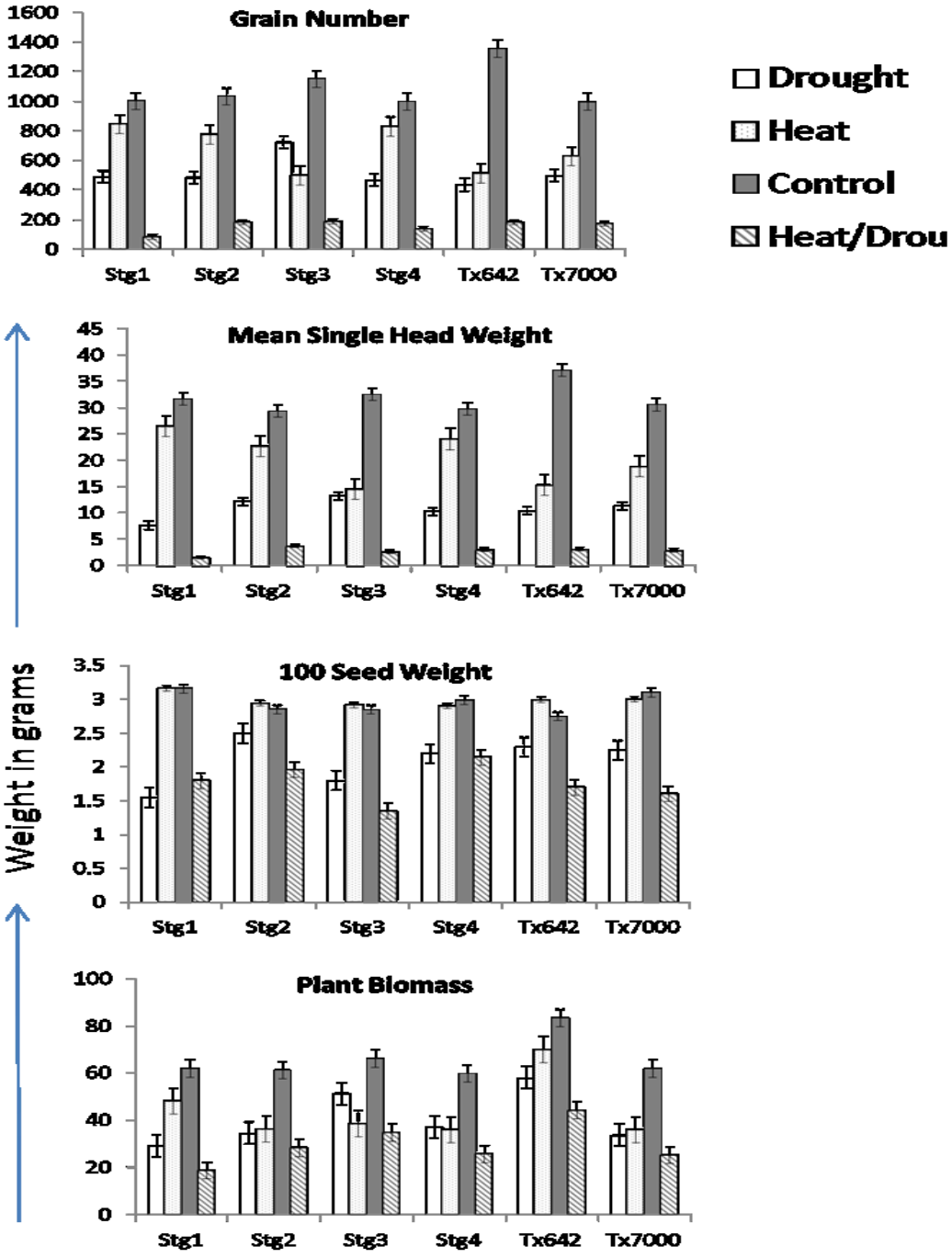

Figure 6. The yield performance of staygreen NILs, plant heights and respective wax load averaged at three data points from flag leaf emergence, flowering and 5 DAP. The data was taken in the late spring 2012 and late spring of 2013. The two-year results were very similar and were thus averaged.

The Stg1 showed the least reduction in GN and MSHW under heat treatment, while higher-WL Stg2 experienced the least combined penalty in the two traits under water deficit and in the heat treatment.

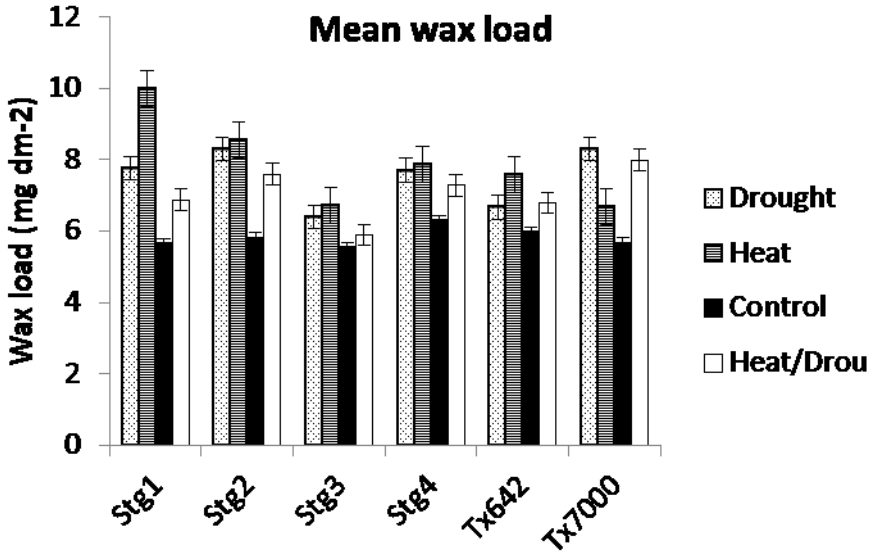

Figure 7. Mean wax load of Stg NILs and the parents. The growing conditions were: well-watered and elevated temperatures (Heat, 42^{0}C d, 27^{0}C n); water deficit and normal temperature (Drought, 31^{0}C d, 17^{0}C n), well-watered and normal temperature (Control), and a combination of heat and drought (Heat/Drou). Plants were thinned at 7 DAE and stress was imposed from 40 DAE in spring 2012 and from 7d after thinning in spring 2013. The yield parameters were determined as described in the methods and the relative penalties (PR) due to the trait X treatment interactions calculated relative to the values under the control treatment.

These results suggest that *Stg* loci have function-specific dominance under different conditions. The observations also suggest that the differences in wax load may be associated with the Stg allele specific response under stress

3.5 Discussions

In this study, we tested the *hypothesis* that high wax load is required for the functional staygreen trait and that this association is important in agronomic productivity under stress conditions. The *objective* was to determine if high wax load is required for the staygreen trait and whether this association is important in crop productivity under

stress conditions. Elevated temperature stress, water deficit stress and a combination of the two stresses were used to test this objective in the greenhouse. We screened 100 inbred lines for the staygreen trait and wax load and categorized these into four phenotypic classes: staygreen high wax (SHI), staygreen low wax (SLO), non-staygreen high wax (RHI) and non-staygreen low wax (SLO) as describe in the methods.

3.5.1 Staygreen and total compositional WL may interact to provide an improved tolerance to drought and heat stress, and a combination of both stresses

We found that staygreen variants with high wax load among the RILs generally had significantly higher grain number and higher mean single head weight (MSHW). The genotypes that had staygreen but low wax (SLO) showed a slightly higher GN and MSHW under drought but not in the heat treatment, suggesting that the staygreen trait may offer a slight advantage even with low wax load under drought although this advantage increases tremendously in the presence of the two traits (Figures 5).

A similar observation was made in the non-staygreen high wax RILs (RHI) which showed a small significant edge in the mean single head weight (MSHW) over the staygreen low wax RILs (RLO) under heat stress and under water deficit conditions, but not in a combination of both heat and drought treatments. The staygreen low wax RILs (SLO) had even lower MSHW under the high temperature treatment (Figures 5). High wax may improve reflectance of heat-generating irradiation, thereby reducing the damage to the photosystem II (Krause and Weis, 1991) and improve light absorption by pigments in juvenile leaves (Grassl et al., 2012; Merzlyak et al., 2008) and mature leaves at grain filling (Gous, 2015) under stress; these conditions are necessary to stay functionally green (Thomas and Howarth, 2000). The results show that wax on plant leaves may be required if the grain development processes are to be amenable to staygreen functionality under high temperature stress.

Compared to the statygreen low wax RILs (SLO) under drought and under heat, the RILs having high WL but are non-staygreen (RHI) also had on average higher mean grain number and mean single head weight under heat and in the water deficit treatment.

Both phenotypic groups had similar mean 100 seed weight (100SW). Taken together, these observations suggest that staygreen regulatory factors may play a role in the later seed development under normal and drought conditions but that this role is confounded by the heat stress-related responses. High wax load seems to augment the *Stg* factors to correct this anomaly. When combined, we saw a dramatic improvement in both seed set rate, head weight (Figure 6) and grain to biomass ratio (not shown).

In agreement with previous studies (Khush, 1995; Peltonen-Sainio et al., 2007), the mean single head weight seemed to be explained more by seed number than single grain weight. Wax load also seemed to offer greater stability to the staygreen trait under heat. However, the confounding effect of heat in the staygreen background and the early imposition of stress (see Figure 5) may have overshadowed the results in a combined heat and drought treatment.

These results also support the fact that the sorghum genotypes expressing post-anthesis staygreen can be distinguished from the non-staygreen types from seedling stages (Johnson et al., 2015; Pasini et al., 2014), but their stress tolerance may be influenced by the interaction between leaf wax and the staygreen. Thus staygreen factors may be linked to a continuum of stress signaling cascades, culminating in the well documented post-anthesis expression.

3.5.2 The Stg alleles may be independently inherited, interact differentially with wax load and show varying degrees of tolerance to stress

Given the fact that the *Stg* loci are widely distributed in the genome (Subudhi, 2000), the observations made among the RILs and the staygreen NILs, led us to a similar conclusion as reached by an earlier study that the staygreen genomic regions may function separately and show varying degrees of dominance (Yoo et al., 2007). The individuals sampled among the RILs showed a wide variation in seed number, biomass, and head weight but not in the 100 seed weight. The staygreen allelic loci may exist in different combinations in the RIL groups in this study from Tx642 / Tx7000, producing the observed differences in tolerance to heat and drought.

3.6 Conclusions

By imposing drought and heat stress long before anthesis recombinant inbred lines (RILs) earlier selected for having high wax at anthesis and expressing the stygreen trait and post-anthesis, showed improved grain yield potential compared to the RILs with low wax and expressing or not expressing the styagreen trait. These results also support the previous findings that the sorghum genotypes expressing post-anthesis staygreen can be distinguished from the non-staygreen types from seedling stages. However, we found that the degree of their stress tolerance may be influenced by the interaction between leaf wax and the staygreen. Thus wax load may be an important factor linked to a continuum of stress signaling cascades culminating in the well documented post-anthesis staygreen expression. We also concluded that the Stg loci are differentially effective against elevated temperature stress, water deficit cues and a combination of both stresses

CHAPTER IV

PLANT-WATER RELATIONS: INVESTIGATING THE TEMPORAL AND CONDITIONAL ASSOCIATION BETWEEN TRANSPIRATION AND LEAF WAX

4.1 Overview

Plant-water relation remains a confounding enigma in crop-based modeling. The considerable gap in understanding of how the interactions between the many physiological traits affect water-use and the actual yield partly defines this inadequacy. The *objective* of this study was to determine how plant surface waxes affect plant evapotranspiration (ET) over time and the significance of the crosstalk between wax and the staygreen alleles in the ET patterns during the critical reproductive phase and the late grain filling stages. We also determined whole plant water-use efficiency in order to understand the differences in water-use efficiency of the different staygreen alleles. We used staygreen near-isogenic lines (Stg NILs) from the Stg Tx642 in the non-staygreen Tx7000 background to partition the effects of Stg1, Stg2, Stg3 and Stg4 alleles on water-use and wax load (WL). Five replicates of each NIL in two repeated experiments were treated to normal control (C), water deficit (D) and elevated temperature (H) conditions in different greenhouses. Stress was imposed from 7 days after germination / emergence (DAE) up to physiological maturity to determine the differences in integrated water-use efficiency (WUE). Wax load (WL) was determined at flag leaf emergence at (FLE), 50% pollination and thereafter at 5d interval to 25d after complete pollination (DAP). Canopy temperature differentials (TD) and quantum yield of photosystem II (ϕPSII) were measured every 2d from FLE to 35 DAP. Evapotranspiration (ET) was measured every 2d from 7 DAE to 35 DAP. We found that WL and ET were temporally antagonistic with high WL alternately corresponding to lower ET in a non-linear, polynomial manner. The polynomial fit defined the TD ranges which correlated strongly with ϕPSII between FLE and 5 DAP and with the amount of seed set. Both the grain-based and biomass-based integrated water-use efficiency based on grain and biomass (WUE_{grain} and $WUE_{biomass}$) aligned with patterns unique to each genotype. These findings suggest

fundamental relationships which may improve prediction-based crop modelling in breeding under sub-optimal growth conditions.

4.2 Introduction

We have shown that during flowering, plant canopy cooling is influenced by wax load. We have also shown that staygreen (*Stg*) loci differentially interact with leaf wax to influence tolerance to different stresses. In this study, we investigated how plant evapotranspiration (ET) and leaf wax crosstalk to modulate leaf internal temperature, water-use and the potential of plant productivity.

The focus on increasing high water-use efficiency (WUE) stems from the need to produce more biomass and grain yield with a smaller amount of water where scarcity of water is a limiting factor. Epidermal cells synthesize epicuticular waxes which are localized in the cuticular matrices and on the cuticle surface. The benefits of leaf wax to plants include protection against excessive water loss (Mondal et al., 2015). Waxes also have embedded phenolic lipids that function in defense against bacteria and fungi (Sobrado, 2008). Plant transpirational demand is temperature dependent, and under drought can lead to elevated leaf temperatures. Increased epicuticular leaf wax mediates the leaf-air interface and may lower potential desiccation of a plant (Kirkham, 2005; Monneveux et al., 2007; Seibt et al., 2008) by acting more as a physical barrier, and by reducing infrared radiation. Using leaves detached from field-grown sorghum, Jordan et al. (1984) found that WL greater than 0.067g m^{-2} could provide an effective barrier to water loss through the cuticle of sorghum leaves under most conditions (Jordan et al., 1984).

Optimum gas exchange allows the leaf photosynthetic machinery to access carbon dioxide (CO_2) but high leaf temperature reduces the diffusion and solubility of CO_2 in the leaf mesophyll cells and air spaces (Chen et al., 1994; Fock et al., 1979). High ambient temperature may also lead to higher transpiration TE under soil water deficit (Blum, 2005b), leading to increasingly negative water potentials in the mesophyll intercellular spaces. This condition may culminate in a reduced diffusion of CO_2 onto

the photosynthetic apparatus. In this regard, it is obvious that the internal temperature status of a plant is a key determinant of CO_2 influx-dependent photosynthetic performance. But improvements in canopy temperature are still hindered by a lack of well-defined relationships between physiological traits that have a direct bearing on it such as the interaction between increased leaf wax and transpiration.

4.3 Materials and method

4.3.1 Germplasm

We used staygreen NILs (Stg1, Stg2, Stg3 and Stg4) derived from the post-anthesis staygreen parent Tx642, and generated in the post-flowering non-staygreen Tx7000 background.

4.3.2 Treatments and design

The materials were sown in five replications (pots) with two plants per 3-galon-pot in four greenhouse conditions, in College Station, Texas, with mid anthesis occurring in late summer of 2012 and 2013. The pots were lined with a layer of nursery mesh cloth to allow free flow of air. They were then filled with potting mix (MetroMix 900TM forest peat moss, Schulenburg, Texas). A 4cm space was left between the soil surface to the brim of the pot to accommodate added water and to avoid spillage. Soil-filled pots were initially weighed before watering, and then reweighed after watering to pot-capacity to obtain the weight of water required to saturate the soil without dripping. For estimates of evaporation from the soil, 5 reps of pots filled with soil (but with no plants) were watered in the same manner as the planted pots and randomly distributed in rows between the planted pots, in each treatment. Additionally, 3 other randomly distributed pots filled with water were used as open-pan evaporation checks per treatment. All the pots were reweighed every 2d before adding water until 35 days after pollination.

The treatment conditions were, high temperature (heat, H): $45/29^0$C (d/n), relative humidity (RH) 55 – 60; 1.5L of water every 2d for 40d after germination, and

2L every 2d thereafter until physiological maturity. Water deficit (drought, D): 31/29^{0}C (d/n), 1L every 2d until 40 DAE, and 0.5L thereafter until physiological maturity. Combined heat and drought (HD): 45/29^{0}C (d/n), RH 55 – 60 and watering the same as the water deficit treatment. Well-watered, normal temperatures (control, C): the conditions were similar to the D treatment except that watering was the same as in the H treatment.

4.3.3 Phenotypic measurements

Data collected included stomatal conductance (Gs), PSII quantum yield (ϕPSII), canopy temperature depression (TD), total leaf cuticular wax (WL), and plant evapotranspiration (ET). These data were collected from flag leaf emergence (FLE) to 25d after pollination (DAP). Wax load (WL) was determined at flag leaf emergence at (FLE), 50% pollination and thereafter at 5d intervals to 25d after complete pollination (DAP). Canopy temperature differentials or depression (TD, the ambient air temperature minus canopy temperature) and quantum yield of photosystem II (ϕPSII) were measured every 2d from FLE to 35 DAP. Evapotranspiration (ET) was measured every 2d from 7 DAE to 35 DAP.

The quantum yield of electron transfer of photosystem II (ϕPSII) is a quantity of chlorophyll fluorescent light computed as the efficiency of the open reaction centers in light. It was measured using a fluorometer (Fluopen FP100, Photon System Instruments, Czech Republic). It is highly correlated with the overall photosynthetic capacity (Fracheboud et al., 1999). Stomatal conductance, the measure of the rate of passage of mainly CO_2 entering the leaf or water vapor exiting through the stomata, was measured using a porometer (SC-1 Leaf Porometer System, *encecoglobal.com*). The leaf wax was quantified using a standard chloroform-based colorimetric method (Ebercon et al., 1977) and canopy temperature was determined as described previously (Balota, 2008a).

The mean above ground ET was determined every 2d by subtracting the pot weight on the second day before watering, from the previous weight after watering.

Plant evapotranspiration per pot in each treatment was calculated as$(\frac{\mathrm{PE}}{r1} - \frac{\mathrm{SE}}{\mathrm{r2}})/2$, where:

PE = pot evapotranspiration (the difference in planted pot weight between two consecutive measurements for every genotype); **SE** = soil evaporation (the mean difference in unplanted pot weights between two consecutive measurements of the soil-filled pots without plants); **r** = the number of reps per treatment (r1 for planted pots, r2 for soil-filled pots without plants); divided by 2 because there were two plants per pot (similar transpiration was assumed for each of the two plants in each pot). The ET readings per rep were also kept separately.

Whole plant water (moisture) content at maturity (WC_{ma}) was determined to check its correlation with the GLA (green leaf area). Whole plant fresh weight (Y) was taken at harvest, two plants per rep (pot). The heads were then cut off weighed, threshed and seeds dried to about 12% moisture. The remaining head straw (after threshing) were bagged separately and dried in an air convection oven at 60^0C for 72 hours. Twenty random sample weights were monitored every 3 hours for 12 additional hours until no further change in weight was observable. The fresh stovers were carefully chopped and bagged in small batches, and similarly dried in the oven until no further weight change was observable. All weights before and after drying were summed per sample per rep and recorded thus:

$WC_{ma} = (P_a+S_a+V_a)-(P_b+S_b+V_b)$; where:

P_a, P_b = panicle weight without seed before and after oven-drying, respectively;

S_a, S_b = seed weight at harvest and after drying to 12% moisture, respectively;

V_a, V_b = stover (without panicle) before and after oven-drying, respectively.

Y and $(P_a+S_a+V_a)$ were similar.

For the combined heat and drought treatment, we have only shown data for WUE, yield and above-ground plant water content at maturity (WC_{ma}).

The dry weight of shoots $(P_b+S_b+V_b)$ was the taken as the constant weight achieved during oven-drying of the stovers plus the weight of the dried grain. It was the same as the fresh weight at maturity, that is $P_a+S_a+V_a$, minus moisture content at

maturity, WC_{ma}) The methods described in earlier studies (Kemanian, 2005; Kirkham, 2005; Xin et al., 2008) were used to determine water-use efficiency based on grain (WUE_g, $\frac{\text{Mean weight of dry grain per genotype}}{\text{The total weight of water supplied to the genotype}}$, and based on above-ground biomass (WUE_b, $\frac{\text{Mean dry weight of above ground biomass per genotype}}{\text{The total weight of water supplied to the genotype}}$. Root biomass was assumed to be similar for all the NILs.

4.3.4 Data analysis

Where clustering was used, a hierarchical clustering based on WARD's data standardization algorithm (Ward, 1963) in SAS-JMP (described earlier) was used. Data was analyzed using R, SAS-JMP and Excel. Analysis was done in stages. In a combined regression analysis, we first determined the significance of treatment, sampling stage, year and genotype (assumed to be fixed estimators) on the variation of wax load (WL) and plant evapotranspiration (ET). Log-transformed WL and ET were the dependent variables. We used the significant terms (estimators) in determining the effects of WL and TE on canopy temperature depression (TD) in a full factorial regression with WL and TE as the random predictors and TD the dependent variable. These were blocked within treatments, with the sampling stage as the fixed effect. Where random effects were involved, analysis was run using the restricted maximum likelihood (REML) with unbound variance components in SAS-JMP. Year had no significant effect on both WL and ET, and so was left out in further analyses. The LSMeans were separated using Tukey HSD and the Studentized t-test in ANOVA. Relevant statistical assumptions were considered, in arriving at the inferences.

In order to demonstrate the trend in the variation of WL and TE over the sampling stages (phenology), quantitative changes between two adjacent sampling stages were used. We quantified the actual change of WL in relation to the actual change of ET which served to show both the qualitative trend (increase or decrease) and the magnitude of the change for each parameter over the same phenological time scale. To achieve this we constructed non-linear polynomial fits of these quantitative changes (for

each genotype in every treatment) to optimize the power of the derivative of the curve functions [f'(x)]. The curve functions represent the behavior of TD in response to TE and WL as the plants developed. For convenience, the number of (representative) phenological phases for ET and TD, were reduced by winsorization (Dixon, 1960) of two flanking means on either side of the target representative stage. This was done to match the fewer WL measurements. We also determined the correlation between WL, ET, TD and ϕPSII (the functional quantum yield of photosystem II) for each sampling stage and treatment. Inferences were also made on the net effects of these interactions on yield and water-use efficiency.

4.4 Results

We evaluated the hypothesis that leaf waxes and plant evapotranspiration interact to modulate canopy temperature and that this interaction is antagonistic. The objective was to determine if the amount of wax on the leaf surface and the plant water loss through transpiration are associated and how this association affects the canopy temperature as the plant develops. Five main aspects of these relationships were investigated. 1. How the variation in wax load (WL) and in plant evapotranspiration (ET) are affected by treatment, year, genotype, and the phenological stage of sampling. 2. The effects of wax load and plant evapotranspiration on the changes in canopy temperature. Canopy temperature change was determined in terms of canopy temperature depression (TD), that is, the ambient air temperature minus leaf temperature. Higher positive TD means lower canopy temperature and the cooler the plant than the surrounding air, and vice versa. 3. The temporal pattern of the variation of WL and ET, and TD. 4. How the variation in TD affect photosynthesis during the reproductive processes and during grain filling. 5. The implication of these interactions on water-use efficiency based on grain (WUE_g) and on biomass (WUE_b).

The data were obtained under high temperature stress (Heat), severe water deficit (Drought), a combination of both stresses (Heat & Drought) and normal watering at normal temperature (Control). Wax load was determined only at flag leaf emergence

(FLE), at flowering (FLG) and at 5 days after pollination (DAP). Analysis for the combined heat and drought has been left out of the first four aspects which required integration of the WL values beyond FLG stage. The treatments were administered in separate greenhouses, in the late spring of 2012 and repeated in late summer of 2013. We used 2 plants per pot x 5 reps in each treatment in each year for all the genotypes (staygreen sorghum near isogenic lines Stg1, Stg2, Stg3 and Stg4, their recurrent parent Tx7000, and the *Stg* source parent Tx642).

4.4.1 Determining the significance of treatment, sampling stage, year and genotype on the variation of wax load and plant evapotranspiration

Table 3. Combined analysis of the effects of genotype, year, sampling stage and treatment on leaf wax and plant evapotranspiration.

Model	Wax Load		Plant Evapotranspiration	
R2	0.934763		0.988564	
Observations	754		722	
RMSError	0.346569		0.157184	
Prob > F	<.0001		<.0001	
Effect Tests	**F Ratio**	**Prob > F**	**F Ratio**	**Prob > F**
Year	1.07836	0.3873	1.3926	0.1274
Genotype	104.3283	<.0001	277.6243	<.0001
Year*Genotype	0.8075	0.5446	0.6462	0.5857
Stage	376.0621	<.0001	1348.858	<.0001
Year*Stage	1.179	0.316	0.2608	0.903
Genotype*Stage	31.9249	<.0001	261.8374	<.0001
Year*Genotype*Stage	1.0786	0.3571	0.338	0.9995
Treatment	79.0798	<.0001	391.201	<.0001
Year*Treatment	1.2834	0.278	2.5164	0.1133
Genotype*Teatment	47.9766	<.0001	149.364	<.0001
Year*Genotype*Treatment	0.511	0.8827	0.6264	0.7558
Stage*Treatment	78.3207	<.0001	183.5643	<.0001
Year*Stage*Treatment	0.6843	0.7672	0.4105	0.9417
Genotype*Stage*Treatment	28.8171	<.0001	271.1032	<.0001
Year*Genotype*Stage*Treatment	1.0418	0.3962	0.4003	1

In the combined analysis (Table 3), wax load (WL) and plant evaptranstiration (ET), the dependent variables, were regressed against in a full factorial model against genotype, year, phenological stage of sampling (Stage) and treatment. Only Year and its interactions had no significant effect on WL and ET variability. Genotype, stage, treatment and their interactions were highly significant, explaining 93.4% of variability of WL and 98.9% of variability of ET. Year and all its interactions have therefore not been included in further analysis.

4.4.2 Determining the effects of wax load and pant evapotranspiration on canopy temperature depression

We determined the general effect of wax load (WL) and plant evapotranspiration (ET) on canopy temperature depression (TD) across all genotypes in each treatment. Wax load and ET were used as the random predictors, and the stage of sampling (Stage) as the fixed effect, in a in a mixed model using a restricted maximum likelihood (REML) method in SAS-JMP (Table 4).

Of particular interest are the variance components for the random effects, which represent the variation of TD explained by the random terms (Table 3). For instance, the total variation of TD predicted by all the random terms including the error variance (residual) were 0.73, 1.81, and 1.48 under control, heat and under drought, respectively. The proportion of the variation of TD predicted by each of the random term can be better understood in terms of the percent of totals under each treatment, that is, the ratio of the variance component for the effect to the variance component for the total as a percentage.

For example, WL with a variance component of 0.8 under both control and drought predicted 10.57% of the variation in TD in the control treatment, but only 5.55% in the drought treatment. Its variance component was 0.17 under heat, which accounted for 9.62% of the predicted TD in the same treatment. Plant evapotranspiration was the best predictor of TD variation under control (26.49%). It predicted a slightly lower (7.59%) but not significantly different TD variation in the heat treatment, compared to

that predicted by WL (9.62%) in the same treatment. The interactions WL (Wax) x ET, and Stage x WL x TD, accounted for the highest predicted TD under stress (25.5 and 26.5%, respectively, under heat) and (23.13 and 23.06%, respectively, under drought).

Table 4. By treatment analysis of the effects of wax load, plant evapotranspiration and the sampling stage on canopy temperature cooling for all the genotypes. Data presented to two decimal places for the random effects.

	Control					Heat					Drought				
Random Effect	**Var Ratio**	**Var Comp**	**Std Error**	**Pct of Total**	**Δ**	**Var Ratio**	**Var Comp**	**Std Error**	**Pct of Total**	**Δ**	**Var Ratio**	**Var Comp**	**Std Error**	**Pct of Total**	**Δ**
Wax	0.71	0.08	0.04	10.57	c	1.10	0.17	0.04	9.62	bc	0.37	0.08	0.01	5.55	d
Stage*Wax	0.74	0.08	0.05	10.96	c	0.76	0.12	0.04	6.62	c	0.70	0.15	0.01	10.48	cd
ET	1.79	0.19	0.03	26.49	a	0.87	0.14	0.04	7.59	c	0.86	0.19	0.05	12.96	bc
Stage*ET	0.56	0.06	0.05	8.33	cd	1.77	0.28	0.59	15.44	b	0.65	0.14	0.08	9.75	cd
Wax*ET	0.84	0.09	0.02	12.43	bcd	2.92	0.46	0.02	25.50	a	1.54	0.34	0.00	23.13	a
Stage*Wax*ET	1.11	0.12	0.01	16.41	b	3.04	0.48	0.14	26.50	a	1.53	0.34	0.02	23.06	a
Residual		0.11	0.03	14.81	bc		0.16	0.01	8.73	c		0.22	0.01	15.06	b
Total		0.73	0.23	100			1.81	0.88	100			1.48	0.18	100	
Fixed effect	**R2**	**F Ratio**	**P-value**			**R2**	**F Ratio**	**P-value**			**R2**	**F Ratio**	**P-value**		
Stage	0.44	5.10	**0.0002**			0.79	6.65	**<.0001**			0.63	13.52	**<.0001**		

Var Comp - variance component, the variation of TD explained by the random effect;
Var Ratio - ratio of the variance component for the effect to the variance component for the residual. It compares the effect's estimated variance to the model's estimated error variance;
Std Error - the standard error for the variance component estimate;
Pct of Total - percent of total, the ratio of the variance component for the effect to the variance component for the total as a percentage;
Stage – sampling (phenological) stage; ET – evapotranspiration from the plant;
Δ Percent of totals not connected by the same letter within a treatment are significantly different using the Tukey's HSD.
Bolded p-values are significant at $\alpha = 0.05$

These observations suggest that compared to wax load, plant evapotranspiration was generally a better predictor for canopy temperature under control and drought treatments, while wax load showed an improved prediction power over ET under heat stress. They also show that the interaction terms provide the best prediction under stress, which together with the main effects explained 85.19, 91.27 and 84.94% of the total TD variations under control, heat and under drought, respectively. The large error variance (proportion of the residual) may have been due to the non-inclusion of the genotype effect. The fixed effect, Stage, also showed a significant fit in all the treatments.

4.4.3 The temporal changes of WL and ET, and TD

Here, the objective was to determine how wax load (WL) and plant evapotranspiration (ET) change over phenological sampling stages, and the resulting behavior of canopy temperature depression (TD). Because ET and TD are more instantaneous on a measurement scale than wax load, we determined the net quantitative changes in the traits between every two adjoining phenological intervals to allow for a uniform interpretation. Trend lines were fitted at the midpoint of each interval, and the vertical scale was harmonized to allow for the plotting of the three traits side by side (Figure 8).

The results show that the genotypes responded in unique patterns that depended on treatment and the sampling phenological stage. In most cases, however, the net changes in WL and TD showed antagonistic patterns, where the net decrease in WL corresponded to a net increase in ET over a period of time. The net change in TD corresponded to the net change in either WL or ET (e.g., WL for Stg1, Stg3, Stg4 and Tx7000 between FLE to FLG, and ET for Stg1 and Tx7000 between 20 to 25 DAP). On other occasions, TD showed a compromise response between WL and ET (e.g., Stg2, Stg3, Stg4 and Tx642 under water deficit and Stg1 under heat, all between FLE to 5 DAP). There were also rare occasions when TD showed a surprising change opposite to those of both WL and ET (e.g., Tx7000, water deficit, FLE to 5 DAP).

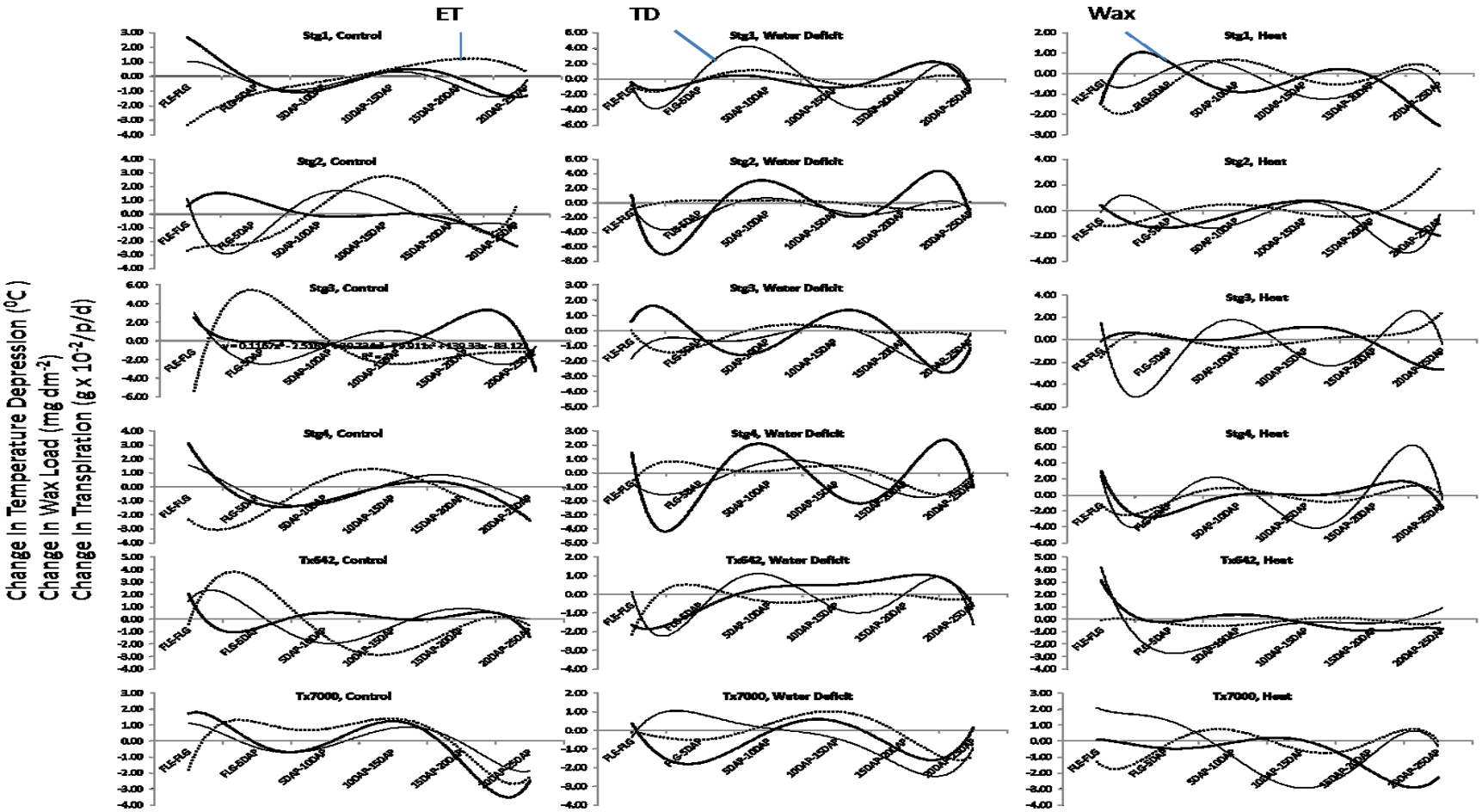

Figure 8. The relationship between the quantitative increase or decrease in transpiration (asterisk broken lines), wax load (continuous bold lines) and temperature depression (regular continuous lines) as they varied with phenology (horizontal axis). The vertical scale has been harmonized to accommodate all the three parameters. Data points below the vertical zero (origin) represent negative changes of the corresponding magnitude on the vertical scale. The data points above the origin represent positive changes. The graphs were fitted as 5th degree polynomials with the highest R^2, using the differences in mean values of two adjacent phenological data points of LFE, flag leaf emergence; FLG, 50% flowering; DAP, days after full head pollination up to 25 DAP. The values of the changes are computed bins (e.g., FLE-FLG, means FLE to FLG), and the mid-point plots are at the center of each bin. g/p/d, grams per plant per day.

These results provide an idea of how of how plant waxes may affect evapotranspiration and canopy temperature at different growth stages under different stress treatments.

4.4.4 Determining the effects of the mean transpiration and mean wax load on TD and ϕPSII between FLE and 5DAP, and between 20DAP and 25DAP

As the changes in ET, WL and TD in Figure 6 shows, these physiological factors show trends that are common to all the genotypes, as well as some signature responses specific to a genotype as it interacts with treatment and phenology. The objective here was to determine if there are signature relationships between WL, TE and TD that may define the quantum efficiency of photosynthesis, during reproductive periods and at late grain filling in sorghum. Two development cluster periods, FLE to 5 DAP, and from 20 DAP to 25 DAP (Table 5) were used. The FLE to 5 DAP represented the reproductive and early seed set phase, to capture early effects on seed formation. The 20 DAP to 25DAP depicted parts of the late grain filling stages, to delineate the late effects on grain maturation.

Canopy temperature depression (TD) and the quantum efficiency of photosynthesis (determined by the efficiency of photosystem II – ϕPSII) were generally lower at the later developmental stages compared to the reproductive phase suggesting reduced photosynthesis was signaled by low sink demand as the grains matured. It also points to the intrinsic relationship between TD and ϕPSII at this stage (Table 5).

Table 5: The mean values of wax load (WL), evapotranspiration (TE), temperature depression (TD) and efficiency of photosystem II (ϕPSII) during the reproductive stages (FLE to 5 DAP) and the late grain filling stages (20 DAP to 25 DAP).

	Control				Drought				Heat			
	FLE to 5 DAP				FLE to 5 DAP				FLE to 5 DAP			
Genotype	WL	ET	TD	ϕPSII	WL	ET	TD	ϕPSII	WL	ET	TD	ϕPSII
Stg1	5.5 d	227.4 cd	1.8 ef	0.7 ab	5.7 cd	56.8 gh	2.1 e	0.7 ab	7.4 a	125.4 f	4.3 bc	0.7 ab
Stg2	5.5 d	230.5 cd	1.5 f	0.7 ab	6.7 b	45.2 h	4.9 b	0.7 ab	7.1 ab	104.2 g	6.0 a	0.8 a
Stg3	5.2 de	353.1 ab	2.3 e	0.7 ab	6.0 c	73.3 g	3.8 bc	0.6 bc	5.7 cd	163.4 e	3.0 de	0.7 ab
Stg4	6.1 b	230.5 cd	2.3 e	0.7 ab	6.1 b	42.9 h	2.5 e	0.6 bc	6.3 bc	131.3 f	4.0 c	0.7 ab
Tx642	5.1 de	327.7 b	1.8 ef	0.7 ab	5.5 d	127.8 f	2.4 e	0.4 de	5.8 cd	127.9 f	3.6 cd	0.6 bc
Tx7000	5.7 d	192.5 de	2.2 e	0.7 ab	6.5 b	72.5 g	3.2 d	0.7 ab	6.1 c	116.9 fg	4.9 b	0.7 ab
Average	5.5 d	260.3 c	2.0 ef	0.7 ab	6.1 c	69.8 g	3.2 d	0.6 bc	6.4 bc	128.2 f	4.3 bc	0.7 ab
	20 to 25 DAP				20 to 25DAP				20 to 25 DAP			
Stg1	4.9 e	201.0 d	0.6 g	0.5 cd	4.9 e	15.1k	0.9 gh	0.1 fg	4.9 e	58.4 gh	2.6 de	0.4 de
Stg2	4.7 ef	392.1 a	1.9 ef	0.7 ab	7.3 a	30.2 hj	1.9 ef	0.6 bc	5.8 cd	221.9 cd	3.4 cd	0.5 cd
Stg3	6.5 b	57.0 gh	0.5 h	0.5 cd	4.8 e	17.7 jk	1.1 g	0.0 g	5.5 d	214.3 d	1.4 f	0.4 de
Stg4	4.5 f	77.3 g	1.7 ef	0.7 ab	5.2 d	33.0 hj	1.2 fg	0.5 cd	6.2 b	54.3 gh	3.2 d	0.5 cd
Tx642	5.5 d	29.1 hjk	1.0 g	0.6 bc	6.0 c	20.1 jk	1.5 f	0.3 e	5.5 d	40.0 h	1.7 ef	0.4 de
Tx7000	4.7 e	260.5 c	1.7 ef	0.5 cd	4.8 e	70.1 g	0.8 g	0.3 e	2.8 g	52.9 gh	1.5 f	0.3 e
Average	5.1 de	169.5 e	1.2 fg	0.6 bc	5.5 d	31.0 hj	1.2 f	0.3 e	5.1 de	107.0 g	2.3 e	0.4 de

Stg, staygreen; WL, wax load; ET, plant evapotranspiration; TD, temperature depression; ϕPSII, efficiency of photosystem II; FLE, flag leaf emergence; DAP, Days after full pollination

Comparison is made within the same trait, between different genotypes, across the treatments. Trait values not connected by the same letter are significantly different at = 0.5

High temperature stress also produced some dramatic differences. While all the Stg NILs and Tx642 had statistically similar WL at the 20DAP to 25DAP cluster, the WL of Stg1 and Stg2 at the FLE to 5DAP stage were significantly higher than the other NILs, who had similar quantities to both Tx642 and Tx7000. Stg2 had the highest mean TD and lowest mean TE between FLE to 5DAP. Stg2 tied with Stg3 for the highest mean ET in the 20DAP to 25DAP range. The Stg3 NIL also showed the least TD in the later stage.

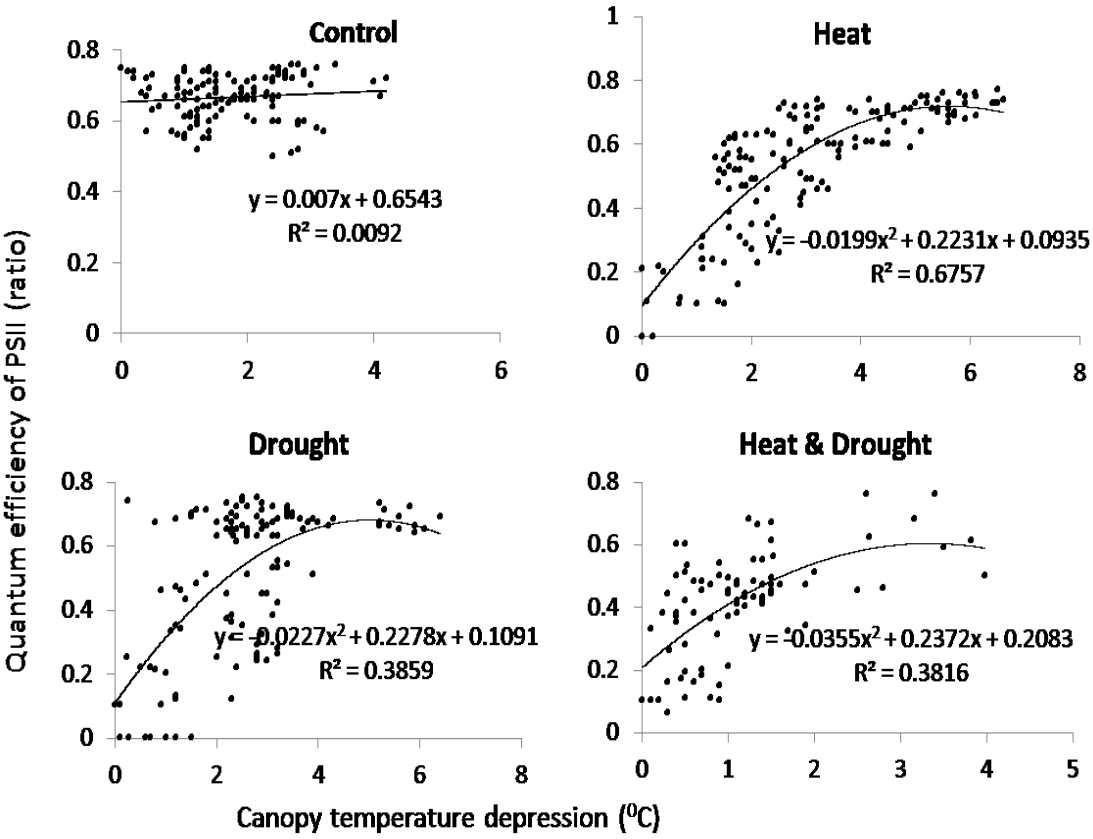

Figure 9. The relationship between ϕPSII and TD under control, heat stress, drought, and a combined heat and drought stress. Data were taken from all the genotypes and averaged for the two years (2012 and 2013). Sampling stages include flag leaf emergence (FLE), 50% pollination and 5 days after complete pollination (DAP) up to 25 DAP.

From the data presented, in all the stress treatments, increase in TD generally corresponded to an increase in ϕPSII up to ϕPSII ~0.7 to 0.8 where it plateaued (Figure 9). There was no significant relationship ($R^2 \sim 0$) under the non-stress treatment. Taken

together, these observations show that ET and WL may interact differentially in a genotype or condition-specific manner to influence TD. In that sense, both qualitative and quantitative (direction and magnitude) change must be taken into consideration.

4.4.5 The resulting water-use efficiency (WUE) dynamics

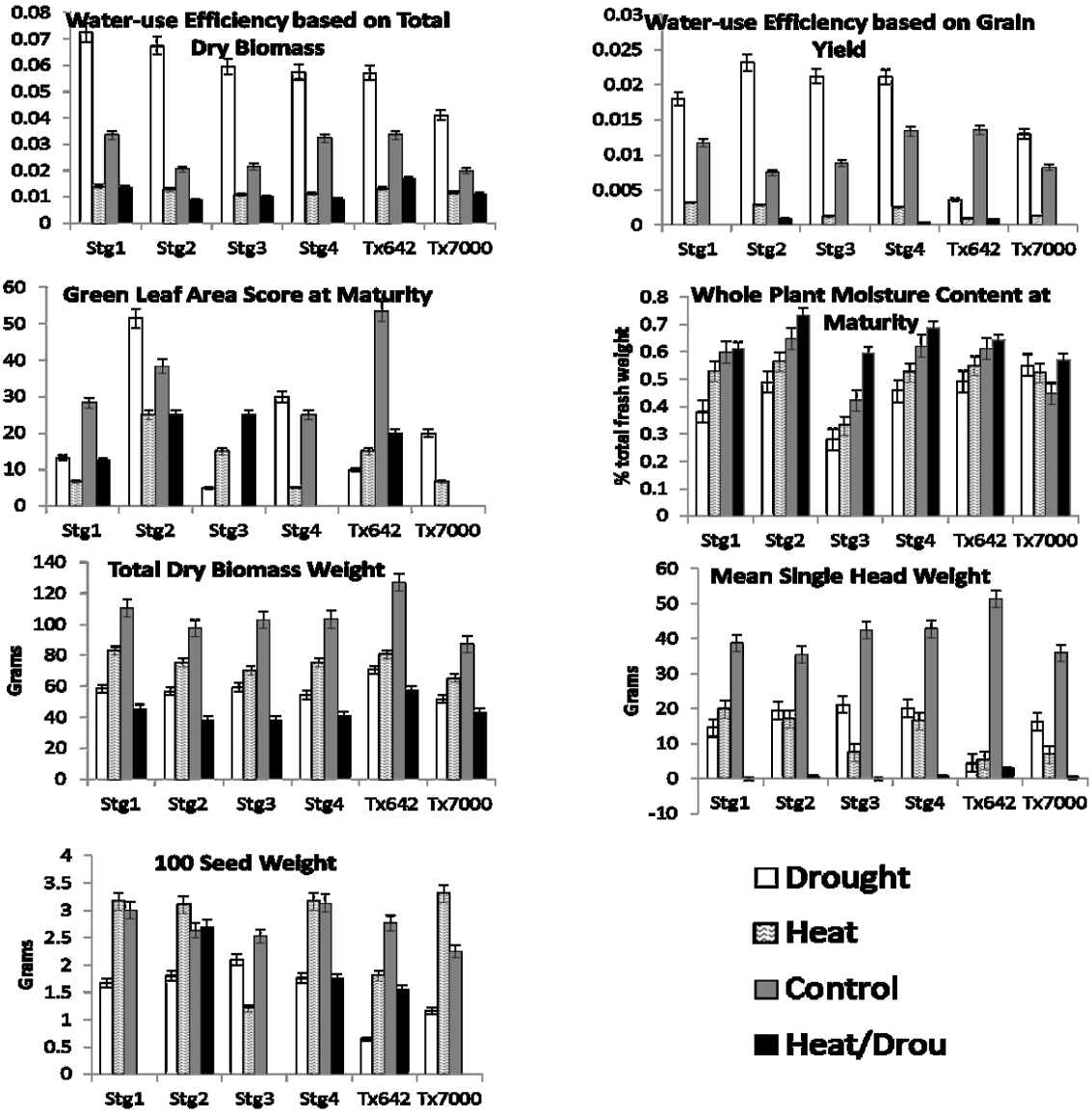

Figure 10. Water use, and yield parameters. A combined heat and drought (Heat-drought) has been included hear, though WL was not monitored up to 25DAP owing to the extreme stress levels leading much fewer plants surviving to maturity (an average of 2 reps unlike 5 reps in the other treatments).

There was no difference in WUE based on total biomass (WUE_b) under heat, and under heat and drought. However, Stg1 and Stg2 had higher WUE_b under drought. Stg3 also had the least water content percent by weight at physiological maturity (35 DAP, Figure 10), compared to the other NILs. Water content at maturity might be an important attribute in the integrity of functional staygreen, as seen in Stg1, Stg2 and Stg4 under heat stress, and Stg4 and Stg2 under drought.

There was no significant difference in total dry biomass under drought suggesting that later (after seed set) the mechanism for both biomass accumulation to the seed and to the non-grain component might be fixed earlier and may not depend on post-anthesis functional staygreen.

4.5 Discussions

Evapotranspiration (ET) has been extensively described as a necessary waste of water, while plants' surface waxes have been implicated in abiotic and biotic defense. Both traits have found a common role in which each has been associated with plant canopy temperature modulation. The objective was to determine if the amount of wax on the leaf surface and the plant water loss through evapotranspiration are associated and how this association affects the canopy temperature as the plant develops. This convergence is important to breeding outcomes (Condon, 2004) for water-use efficiency. We used Stg NILs generated in the Tx7000 non-staygreen background to partition out the effects of Stg1, Stg2, Stg3 and Stg4 on wax load (WL) and ET. Data was obtained in 2012 and 2013, with replicated experiments under heat stress, water deficit (drought), a combined heat and drought, and a normal (Control) conditions.

4.5.1 Accounting for wax load, evapotranspiration and stage of plant development may improve modeling for canopy temperature-based crop performance

Both wax load and plant evapotranspiration were significantly affected by genotype, sampling stage and treatment, and their interactions (Table 2). Year and its

interactions were not significant, probably due to the controlled conditions with less variable environment and seasons.

The strengths of the terms to predict TD were decomposed into the variance components (Table 3) for WL, ET, WL x ET, WL x Stage, and ET x Stage as the random predictors for all the genotypes combined. The proportion of the variation of TD predicted by each of the random term were compared using the percent of totals under each treatment, that is, the ratio of the variance component for the effect to the variance component for the total as a percentage, in each treatment. Plant evapotranspiration was the best predictor of TD variation under control (26.49%). It predicted a slightly lower (7.59%) but not significantly different TD variation in the heat treatment, compared to that predicted by WL (9.62%) in the same treatment. The interactions WL (Wax) x ET, and Stage x WL x TD, accounted for the highest predicted TD under stress (25.5 and 26.5%, respectively, under heat) and (23.13 and 23.06%, respectively, under drought). Together with the main effects, they explained 85.19, 91.27 and 84.94% of the total TD variations under control, heat and under drought, respectively. Given that canopy temperature can be used as a surrogate predictor of yield (Fan T., 2005), our results suggest that accounting for the interaction between WL and TE and the stage of plant development may tremendously modeling for crop performance under stress.

4.5.2 Wax quantitatively antagonize ET to influence the TD and, by extension, ϕPSII under well-coordinated physiological changes

The balance between WL and ET was more prominent in the reproductive periods under stress where a high mean WL corresponded to a reduced mean ET. A departure from this observation was seen in Tx642, which had similar mean WL as Stg3 under water deficit stress, yet showed a significantly higher ET. We observed that Tx642 had larger surface area due to higher leaf numbers compared to the NILs and Tx7000.

The net effect was that TD oscillated in a manner that was the compromise between ET and WL (Figure 8). At the level of photosynthesis, the ϕPSII also fluctuated according to the changes in TD. The plant TD also changed in response to ϕPSII. Taken

together, these observations suggest that the rate of photosynthetic requirements is phenologically dependent and may result in a negative feedback to either increase leaf temperature (lower TD) or reduce the leaf temperature (increased TD).

This could be seen where higher WL during reproductive periods resulted in reduced ET under water deficit, and under elevated temperature stress conditions. For instance, between FLE and FLG under water deficit, Stg2 with a WL of ~6.7mg/dm^2 and a TD 4.9^{0}C had a reduced ET by about 11.6g per plant per day (g/p/d) compared to Stg1 with WL 5.7mg/dm^2 and TD of 2.1^{0}C. Under the same conditions, Stg3 with WL 6.0mg/dm^2 lost 28.1g/p/d more than Stg2, but showed 1.7^{0}C cooler canopy (higher TD) than Stg1 (Table 4). Here, the higher TD by Stg3 resulted mainly from transpirational cooling.

A similar trend was seen under heat stress (although Stg1 showed a slight departure in comparison to Stg2): Stg1 had a mean WL of 0.3mg/dm^2 higher than Stg2, but still lost 21.2g/p/d more to transpiration. This was likely due to the propensity of Stg1 to optimize seed set potential under heat, which prompted increased demand for CO_2 and dry matter partitioning to the developing seeds. This may have required a less restricted transpiration (Blum, 2005b), and thus slightly more open stomata compared to those in Stg2 and the other NILs, under well-watered high temperature conditions. It can be seen that the TD by Stg1 also fell 1.7^{0}C lower than that of Stg2, accompanied by a ϕPSII penalty of 0.1 below that of Stg2. However, this penalty did not seem sufficient to result in lower grain yield of Stg1.

Among the Stg NILs and other RILs, we have observed that under sufficient supply of soil water, an elevation of leaf internal temperature to between 36 to 38^{0}C resulted in a ϕPSII of ≥0.7. For most of these genotypes, this is optimal photochemical yield required for floral development, fertilization and seed set. The genotypes with ϕPSII falling below 0.55 at the critical reproductive phase suffer severe seed set failure. The overall effect of TD and ϕPSII on seed set, therefore defines one of the main roles of the interplay between wax accumulation and transpiration in grain set.

A closely related concept was outlined based on simulated summer heat conditions using potted plants (Newell, 1996), where the decrease in photosynthetic rate appeared to be primarily due to high leaf temperatures beyond some threshold value, while the decrease in stomatal conductance was attributed mainly to high Δw (leaf-to-air water vapor concentration differences). Leaf temperatures exceeding the critical threshold value still triggered depressed stomata, even when Δw was artificially maintained at a constant level. Together with our findings, it can be deduced that by keeping leaf temperature below a critical value, the role of wax accumulation may be expanded to include allowing for near-normal photosynthesis (may be among C4) without the need for a wide stomatal aperture for transpirational cooling under heat stress. This is normally neglected in many prediction modelling studies and might partly explain why (Xu, 2004) found the predicted versus actual gas exchange-based WUE was much stronger in cotton (a C3), compared to sweet corn (a C4).

4.5.3 Waxes constitutively synthesized may have an expanded role beyond stress mitigation at the reproductive phase (FLE to FLG)

Sorghum seems to constitutively increase its WL during reproductive development. The increase in ϕPSII performance from FLE to FLG also seemed to accompany increase in WL. Even under water deficit, the plants were able to accommodate the slight penalty on TD due to ET decrease, probably to conserve the scarce moisture required for floral and early seed set efficacy. As was observed, a consistent minimum threshold WL appeared to be ~ 6mg/dm^2. It was evident that the genotypes that failed to reach this threshold in any condition were also among the least successful in seed formation and seed set under those conditions (Figure 10). This lends credence to our speculation that increased wax is linked to mechanisms that maintain floral integrity irrespective of the conditions.

4.5.4 Wax may stabilize fluctuation in ET to improve PSII performance and grain set under high temperature

The PSII performance under water deficit (drought) and high temperature treatments across all the genotypes showed a greater correlation with canopy depression (TD) than under normal (Control) conditions (Figure 9). This was probably because optimized ϕPSII does not solely rely on the stomatal aperture-dependent CO_2 influx (stomatal aperture is expected to be optimal under normal growth conditions) and may function normally with diminished gas exchange in some sorghum types under water deficit and heat stress.

The rate of photosynthate conversion may in turn influence the number of the set seeds that develop to full maturity. For instance, under drought, Stg3 showed an average of ϕPSII = 0.6 between FLE to 5 DAP (caused by a slump to 0.4 at FLG, Table 4), and dropped from a high of 0.63 at 10 DAP to 0.04 at 25 DAP, but still produced grain yield equivalent to Stg2 and Stg4 under drought treatment. Stg1 showed a similar drop but had a lower grain yield (Figure 10) under drought. Both Stg1 and Stg3 remained green, but with reduced functional photochemical conversion beyond 25 DAP, compared to Stg2 and Stg4 which continued their ϕPSII functionality at a high rate under severe water deficit. This also agrees with a rarely acknowledged fact that greenness alone does not define Stg functionality (discussed elsewhere).

A study in maize indicated that a heat-sensitive component in the photosynthetic apparatus was located downstream of the ϕPSII and before the carbon cycle (Sinsawat, 2004). High temperature (35^0C d/29^0C n) treatment promoted the growth of vegetative parts but reduced ear expansion in maize, due to impaired hemicellulose and cellulose synthesis through a reduction of photosynthate supply (Suwa, 2010). The plant biomass production may be enhanced and grain yield reduced by the high temperature treatment due to the effects on sink activity rather than source activity. For that reason, it may be no surprise that there were instances of significantly depressed grain development, yet very high biomass-based water-use efficiency under heat and drought.

4.6 Conclusions

We used staygreen near isogenic lines with different wax accumulation traits to determine how the variation in their wax load (WL) affects transpiration (ET) and the effects of their interaction on canopy temperature and photosynthetic efficiency. By extension, we also reported biomass and grain yield, responses in terms of whole plant water-use efficiency. We noted that ET and WL, changed during phenology in mostly antagonistic and polynomial manner. By accounting for the interaction between ET, WL and stage of sampling (phenology) the prediction accuracy for canopy temperature changes. This may have the potential of improving predictive modelling of canopy temperature-based crop performance.

CHAPTER V
THE BLOOMLESS PHENOTYPE: ALLELIC DIVERSITY AND INHERITANCE

5.1 Overview

Ethylmethanesulfonate (EMS) is a well-known chemical mutagen capable of producing high frequency base substitution in the genome. When used at different concentrations, EMS offers an improved saturation for possible mutations in the genes associated with a target trait. The bloomless trait in sorghum has been traditionally defined as recessive with a simple inheritance pattern. However, it is clear from previous studies that the allelic diversity of this trait has not been fully exploited. The objective of this study was to evaluate the allelic diversity and inheritance of the bloomless trait using EMS-induced bloomless mutant populations derived from Tx623 sorghum inbred line. The mutant lines were obtained from the USDA-ARS, in Lubbock, Texas. These were advanced were advanced to M5 at the Texas A&M AgriLife facilities in College Station, Texas. Of these,11 true breeding bloomless lines were intercrossed in different combinations between the mutants and between the mutants and three wild-type (bloom) lines. At least 6 different sources of bloomless were found, with significant overlap between the segregation patterns. The bloomless trait was recessive in most cases, but showed dominance in a few crosses. The finding of a source of dominant bloomless is an obvious departure from what has been widely reported. Some of the segregation patterns also suggested possible complex allelic and intragenic interactions that produced much unexpected outcomes. We propose more detailed studies of these materials to ascertain some of these findings.

5.2 Introduction

The bloom phenotype is the wildtype and most common trait in sorghum and many other grasses. Bloom is expressed in the form of epicuticular wax exudate (Peterson et al., 1982) which forms a heavy glaucous covering on the plant surface. The

bloomless plants lack this appearance. The genes regulating this trait are believed to be diverse given the plethora of known and unknown genes in wax w biosynthesis beginning with fatty acid (FA) synthesis in the plastids (Kunst and Samuels, 2009; Lee and Suh, 2014) to FA elongation and the transit of wax constituents. Only a few studies have implicated the fatty acid (FA) chain length to the physical wax architecture or morphology on the plant leaves (Lee and Suh, 2014). However, considering the many unknowns in the biosynthetic pathways of plant waxes (Kunst and Samuels, 2009), it is likely that several yet to be identified alleles may be responsible for the bloomless phenotype in sorghum.

A single gene inheritance, as well as two- or three-gene models has been experimentally shown to fit the inheritance pattern of the bloomless trait. Some allelism tests (Jenks et al., 1992; Jenks et al., 1994) reached the conclusion that bloomlessness is controlled by a single nuclear gene. They had used the M4 progeny of P954035, from Purdue Sorghum Improvement Center, which had been mutagenized by EMS or diethyl sulfate. These studies agreed that the limited number of *bm* mutant alleles (24) reported so far could not have represented complete saturation of possible gene mutations for the bloomless trait. Burrow et al. (Burow, 2008) also reported a single recessive nuclear gene inheritance pattern using F2 progeny from a cross between an irradiation-induced bloomless parent and a bloom wild-type.

An earlier study by Peterson at al. (Peterson et al., 1982), however, established that two independent loci could be conditioning the bloomless phenotype while sparse bloom is conditioned by three or four genes. Some bloom X bloomless crosses failed to satisfy the two-gene (9:7 or 9:3:4) inheritance model, partly attributable to too many bloom in a cross with one parent and too many bloomless in a cross with another parent. They suggested this deviation could be due to a general preponderance of the bloomless type apportioned to chance. An interesting pattern of inheritance was observed in a sparse-bloom (h) X bloomless cross. The bloom phenotype resulted from one locus containing at least one *Bm* allele and the other at least one wild type *H* (*Bm_H_*). The sparse-bloom phenotype resulted from a *Bm* allele in at least one locus and a

homozygous *hh* on the other locus (*BmBmhh* or *Bmbmhh*), and the bloomless phenotype resulted from one locus being homozygous for *bm* regardless of the alleles at the other locus (*bmbmHH*, *bmbmHh, or bmbmhh*).

The two studies (the one-gene and the two-gene patterns) both assumed a pathway with one gene or two genes in a simple sequence. However, as suggested by Peterson et al. (1982), more than two genes could be involved in regulating the sparse-bloom and potentially, the bloomless. The failure of some bloom X bloomless crosses to fit the two-gene model also called for further investigations. We sought to find different sources of alleles responsible the bloomless and to use these in determining the inheritance of the bloomless phenotype. Populations of EMS induced bloomless progeny derived from the highly bloom Tx623 parent, were used. The mutagenesis was achieved at three different concentrations of EMS. Based on the expected high frequency of base substitutions, these concentrations were envisaged to improve the mutation saturation for most of the loci associated with the bloomless loss-of-function.

5.3 Materials and methods

Table 6. The sorghum germplasm used in crosses

Mutant#	Phenotype	Origin
M401	Bloomless	EMS Tx623 USDA-ARS , Lubbock
M1688	Bloomless	EMS Tx623 USDA-ARS , Lubbock
M1789	Bloomless	EMS Tx623 USDA-ARS, Lubbock
M144	Bloomless	EMS Tx623 USDA-ARS , Lubbock
M965	Bloomless	EMS Tx623 USDA-ARS , Lubbock
M1028	Bloomless	EMS Tx623 USDA-ARS, Lubbock
M1587	Bloomless	EMS Tx623 USDA-ARS , Lubbock
M1427	Bloomless	EMS Tx623 USDA-ARS , Lubbock
M567	Bloomless	EMS Tx623 USDA-ARS, Lubbock
M883	Bloomless	EMS Tx623 USDA-ARS, Lubbock
M9868	Bloomless	EMS Tx623 USDA-ARS , Lubbock
Wild-type		
Tx7000	Bloom	Texas A&M Foundation Seed s, College Station
Tx623	Bloom	Texas A&M Foundation Seed s, College Station, and USDA, Lubbock
Stg4	Bloom	Texas A&M Foundation Seed s, College Station

All mutant parents were true breeding at M5, all derived from Tx623.

A population of 118 mutant (M4) lines derived from the ethylmethanesulfonate (EMS)-mutagenized Tx623 was obtained from the USDA-ARS in Lubbock, Texas. These were planted and selfed in the Texas A&M University Research Fields (TAMURF) near College Station, TX. Eleven true-breeding M5 bloomless mutant lines (Table 6) were selected and seeds pooled within a line.

The mutants are normally discernable as having reduced or non-observable glaucousness on the abaxial sheath (Burow, 2008; Jenks et al., 2000) compared to their wild-type parent. A complete lack of the chalky substance on the leaf sheath and stem (nodes and internodes for older plants) is considered bloomless. We also found that the bloomless are easily tested by the complete absence of a whitish coating on the finger when swiped on the leaf sheath, mature leaves from 3 weeks after emergence, and the stem (nodes and internodes) of older plants. The bloom type will leave a conspicuously heavy, chalky coating on the finger when swiped on the leaf sheath) and fully formed leaves and is clearly observable from about 3 weeks after emergence, and the stem (in plants older than 3 weeks. Sparse bloom types have a visible light smear of chalkiness on the leaf sheath and stem but do not leave an easily discernible mark on the finger.

5.3.1 Allelic variation

To determine allelic variation in the bloomless material, the individual bloomless mutants were intercrossed between themselves in the TAMURF near College Station in spring 2013. Most of the mutants were also segregating for male sterility and these were used as the females in the mutant to mutant crosses. Hand emasculation was used on mutant M401 and M567 and any other fertile individual selected as a female. Otherwise, M401 was mostly used as the pollinator parent, being highly bloomless and a good pollen source. The F1 hybrids were simultaneously planted in the greenhouse in College Station and in the field in Weslaco in fall 2013, and selfed. The resulting F2 seed were planted head-to-pot in the greenhouse in winter 2013/14, and head to row in TAMURF at College Station in spring 2014. Segregation counts were taken for each F2 row. Since there was no significant difference in the segregation ratios of bloom : bloomless in the

two environments (greenhouse and field), we concluded that the expression of bloomlessness is independent of environment. For further analysis, the data obtained from the bi-parental families in the greenhouse were combined with the data obtained from the corresponding bi-parental families in the field.

A bloomless phenotype appearing in the F1 from a cross between two bloomless individuals would suggest the two alleles are of the same gene. Mutations in different genes were expected to produce the wild-type (bloom) F1 hybrids by complementation, and a segregation into bloom and bloomless in the F2 families.

5.3.2 Inheritance and gene action

Of the 11 bloomless mutants in Table 6, seven mutants were selected and used as pollinators in the cross with the wild-type bloom Tx623. The F1 were grown in the greenhouse in fall 2013 and the resulting F2 seed were planted in 3 reps of Tx623 X bloomless mutant family rows in TAMURF at College Station in spring 2014. Each row had between 40 and 122 plants. Another set of F2 seeds from the same families as those planted in the field (spring 2014) were planted in single reps in the greenhouse in fall 2014 with at least 37 plants per pot. The bloomless mutant M1789 was also crossed into two other wildtype bloom female parents, Tx7000 and Stg4. The segregation patterns were evaluated as described for the mutant X mutant families. The F2 progeny segregation was used to determine the inheritance of the bloomless trait in the study material, and also the gene action.

5.4 Results and discussion

5.4.1 Allelic variation

The true-breeding bloomless lines were intercrossed between themselves in different combinations. Two main groups of bloomless sources were determined. In the first main group were those producing all-bloomless F1 hybrids in a cross with the other bloomless lines, and remained all bloomless in F2 (Table 7). These were by convention

considered as containing the same mutational source of the bloomless trait. The second main group consisted of the complementing pairs and produced at least some bloom in the F1 (Table 8). Of these, the bloomless parents producing uniquely different phenotypic patterns in a cross with the other mutant parents are shown in Table 8.

Table 7. Crosses not producing segregating progeny

	F1	F2
M401 X M401	All bloomless	All bloomless
M401 X M567	All bloomless	All bloomless
M401 X M1587	All bloomless	All bloomless
M401 X M1688	All bloomless	All bloomless
M401 X M1789	All bloomless	All bloomless
M401 X M1427	All bloomless	All bloomless
M401 X M1028	All bloomless	All bloomless
M401 X M965	All bloomless	All bloomless
M401 X M144	All bloomless	All bloomless
M401 X M883	All bloomless	All bloomless
M401 X M9868	All bloomless	All bloomless
M1587 X M1587	All bloomless	All bloomless
M1028 X M1028	All bloomless	All bloomless
M1789 X M1789	All bloomless	All bloomless
M1789 X M1688	All bloomless	All bloomless
M1789 X M965	All bloomless	All bloomless

The bloomless mutant M401 was crossed to all other 10 bloomless mutants

If two recessive genes are allelic, they should fail to complement each other in the F1 hybrids. Thus, the number of complementation groups reveal the number of different loci (Demerec and Ozeki, 1959). On this basis, 4 crossing sub-groups can be delineated from the data presented for the complementing group. Some members of one group, however, overlap into other groups which is not unique in such analysis (Mehta et al., 2005). This suggests the type of complex gene interactions to be expected.

The *first* is the *sub-grou*p (Table 8) containing bloom F1 that segregated into a single mutant (bloomless) phenotype with the F2 ratio of 9 bloom : 7 bloomless, suggesting the bloomless phenotype resulted from epistatic interaction of two genes (loci) in the same pathway. Such a relationship has also been known to occur between a regulatory gene and the gene it regulates (Griffiths, 1999). In this subgroup were the crosses M1789 X M1028, M1789 X M1587, M144 X M1587, and M1789 X M965 (one variant).

Table 8. Crosses between mutant lines

Cross	F1 phenotype	F2 phenotypic ratios			Test for 9:7 or 9:3:4		**Gene model satisfied
		Bloom	Sparse-bloom	Bloomless	X^2	P value	
M1789 X M144	Bloom	43	0	16	6.631	0.0100	12:4
M1789 X M965 Δ	Bloom	54	0	44	0.052	0.8188*	9:7
	Bloomless	0	0	16			?
M1789 X M1028	Bloom	56	0	39	0.281	0.5961*	9:7
M1789 X M1589	Bloom	105	0	79	0.05	0.8236*	9:7
M1789 X M14273	Bloom	83	0	6	NF	NF	15:1
M1789 X M9868	Bloom	47	0	22	3.948	0.0469	9:4
M144 X M1589	Bloom	72	0	53	0.093	0.7609*	9:7
M965 X M9868	Bloom	15	0	53	NF	NF	3:13
M14273 X M9868	Bloom	27	8	Lethals	NF	NF	9:3:0
M144 X M965	Boom	64	12	23	3.685	0.1585*	9:3:4
M965-1 X M965-2	Bloom	32	7	12	1.113	0.5731*	9:3:4

Δ Showing mutant phenotype at F1, together with the wild-type phenotype. Several lethals at F2.

** Most of the different crossing combinations resulted in the gene models represented in this table, irrespective of the test models.

NF, not feasible, given the F1 phenotype.

P value answers shows that if the theory that generated the expected values were correct, the discrepancy between the observed and expected values should be low. A small P value is

In the *second subgroup* were those producing bloom F1 and segregated into 15 bloom : 1 bloomless ratio at the F2 (M1789 X M883), suggesting that these are duplicate genes (Yook et al., 2001) resulting in the single mutant phenotype. A *third subgroup* segregated at F2 into three phenotypes, bloom, sparse-bloom and bloomless, suggesting a two-step, two-gene mutations, with the first mutant locus resulting in a sparse-bloom, and the second mutant locus terminating in the bloomless phenotype. Examples here were F2s from M144 X M965 and from a cross between two alleles of M965 (M965-1 X M965-2). In a cross with a male sterile M1789, one other allele of M965 produced both bloom and bloomless at F1. The F1 bloom segregated into bloom and bloomless at F2, while the F1 bloomless failed to segregate at F2. These observations suggest that entry M965 may be harboring several alternative mutations for the bloomless trait.

In the *fourth subgroup* the F2 segregation pattern did not fit the typical two gene model. These included M1789 X M9868 (9 bloom:4 bloomless, and M1789 X M144 (12:4, which might not be the same as 3:1). The cross M965 X M9868 produced 3: 13, at F2. The selfing data (not shown) for entries M1427, M883 and M9868 produced bloom F1 and segregating F2 when crossed with a few other bloomless (Table 8). A special case was the production of the lethal bloomless seedlings in the cross M883 X M9868. Taken together, it possible that these mutants harbor several different alleles responsible for the bloomless trait.

5.4.2 Inheritance of bloomless trait

Some of the above representative mutants were used as pollen sources in a cross with male sterile Tx623 individuals. From the first main group, M401 was used and from the subgroups in the second main group, the following were used: subgroup 1: M1789, M1028, M1587; subgroup 2: M883, M1789; subgroup 3: two alleles of M965, and subgroup 4: M883 (Table 9.

Some of the of the F2 segregation ratios classed with the common one- and two-gene models involving a recessive trait while in many cases, the two-gene models were uncommon. Three *m401* alleles satisfied the 3:1 one gene inheritance with the *m401*

being recessive to the *Tx623* allele, confirming that the bloomless trait in the M401 entry is conditioned by a recessive allele of one gene. Mutant M401 also failed to complement with all the other mutants in this study, even with those that complemented with others. This suggests that *m401* may be one of the many mutation events in the genes responsible for the bloomless trait in the other mutant entries.

For instance, one mutation in M1789 may be similar to that in M401, while the other mutation in the second gene in M1789 is unique. Hence *M401* and *M1789* do not complement, while M401 produces 3:1 in a cross with Tx623. Similar single gene mutations such as in *m401* are the most widely reported in past studies (Burow, 2008; Jenks et al., 1994; Peterson et al., 1982)

The non-conventional inheritance patterns were confirmed in the crosses with the wild-type bloom parents (Tables 8). In some cases, the mutant phenotype showed up in the F1. For instance, the cross between Tx623 with a variant of M965 produced F1 bloomless, and Tx623 X M1028 produced F1 sparse-bloom. In a cross with the wild-type, a mutant phenotype appearing in the F1 hybrids would be due to a possible dominant mutation (Griffiths, 1999). Of peculiar interest was the bloomless F1 hybrid from the Tx623 X M965, segregating at F2 in the ratio 7 bloom : 9 bloomless; the heterozygous bloomless lines segregated in a similar manner in the F3, implying a dominant mutation with a complex intralocus allelic interaction (Demerec and Ozeki, 1959; Hideki, 1992; Yook et al., 2001)

Table 9. Crosses between mutants and the wild-type lines

Cross	F1 phenotype	F2 phenotypic ratios			Test for 9:7 or 9:3:4 ratio		Test for 3:1		Gene model satisfied
		Bloom	Sparse-bloom	Bloomless	X^2	P value	X^2	P value	
Tx623 X M401-1	Bloom	278	0	110	37.389	0.0001	2.323	0.1275*	3:1
Tx623 X M401-2	Bloom	197	0	71	32.433	0.0001	0.318	0.5726*	3:1
Tx623 X M401-3	Bloom	47	0	12	13.140	0.0003	0.684	0.4083*	3:1
Tx623 X M1789	Sparse-bloom	0	4	19	NF	NF	NF	NF	3:13
Tx623 X M965	Sparse-bloom	0	417	31	NF	NF	NF	NF	15:1
Tx623 X M965	#F1=bloomless	18	0	27	0.257	6121	5.400	0.0201	7:9
Tx623 X M1028	Bloom	69	0	9	32.886	0.0001	7.538	0.0060	14:2
Tx623 X M1028	Bloom	14	34	0	NF	NF	NF	NF	4:12
	#Sparse-bloom	10	30	0	5.714	0.0168	0	1*	4:12
Tx623 X M1589	Bloom	2	46	32	NF	NF	NF	NF	?
Tx623 X M567	Bloom	27	2	0	NF	NF	NF	NF	15:1
Tx623 X M14273	Bloom	34	0	0	NF		NF	NF	?
Stg4 X M1789	Bloom	142	0	42	32.375	0.0001	0.464	0.4959*	3:1 and 13:3
Tx7000 X M1789	Bloom	72	0	15	24.842	0.0001	2.793	0.0947*	3:1 and 13:3

#Seeds from a single mutant head . The corresponding ratio is reversed starting with the mutant phenotype

** Most of the different crossing combinations resulted in the gene models represented in this table, irrespective of the test models.

NF, not feasible, given the F1 phenotype.

P value answers shows that if the theory that generated the expected values were correct, the discrepancy between the observed and expected values should be low. A small P value is evidence that the gene model suggested has not been satisfied.

*Significant P value under the test model

Another Tx623 X M965 produced all sparse-bloom F1 segregating into 15 sparse-bloom : 1 bloomless, alluding to a duplicate gene action with the double homozygous mutant being bloomless. The mutation in this latter case was recessive. These two divergent observations support the earlier assessment (M965-1 X M965-2, Table 7) that there may be different site mutations on the *M965* gene conferring on the alleles varying degrees of dominance. These could also be two closely linked loci or two unique mutations on the same locus. Further analysis is suggested.

A 15 bloom : 1 bloomless ratio was observed among the F2 from the backcross TX623 X M567. This case suggested a duplicate gene action with the negatively interacting gene products (Yook et al., 2001) resulting in the single mutant phenotype. Such inference is based on the idea that some genes may be present more than once in the genome, such as those controlling fruit shape in *Capsella bursa-pastoris* (Griffiths, 1999). Further confirmatory tests are necessary to verify this assessment.

The cross TX623 X M1028 also returned a similar twist of mutant and wild-type phenotype at F1 but with sparse-bloom as the mutant (Table 6). The F2 of either produced similar ratios of 4 bloom : 12 sparse-bloom, suggesting a 'dominant' mutation or low penetrance of the bloom phenotype in the heterozygotes. This also hints to haploinsufficiency in one of the genes, forcing varying levels of low expressivity of the bloom phenotype. Expressivity describes the variability in the mutant phenotypes observed in individuals with a particular phenotype.

The M1789 as the male parent satisfied the ratios 3 bloom : 1 bloomless, and 13 bloom : 3 bloomless in the crosses with the Tx7000 as well as in a cross with Stg4. The 3:1 ratio suggests a single gene action. This presents a problem since M1789, in a cross with Tx623, failed to satisfy a similar gene model. It might be that the expression of *m1789* is affected by the wildtype genetic background. Alternatively, the 13:3 ratio may be consistent with the concept of inter-loci suppression, with a recessive suppressor in one locus acting on a recessive mutation in the second locus (Fay, 2006; Hawley and Walker, 2009; Hwang and Sternberg, 2003) and abrogates an otherwise mutant phenotype. The 'good corrects evil' proposition was first forwarded by (Crick and Orgel,

1964). The concept implies that one mutant subunit mutually corrects the other faulty mutant subunit in the same locus to restore a functional complex (Fay, 2006; Hideki, 1992; Kramer and Johnson, 1993). More detailed studies are necessary to clarify these conundrums.

5.5 Conclusion

Many different dihybrid phenotypic combinations were observed from the crosses between the individual bloomless mutants and between the bloomless mutants and the wild-type bloom lines, Tx623, Tx7000 and Stg4. The results pointed to different alleles responsible for the bloomless phenotype. Among these were possible dominant mutant alleles for the bloomless trait. Even though only a small number of mutant lines were used, our results point to potentially large diversity in the alleles that can influence the production of the bloomless sorghum. Our observations have agreed with both the one-gene and the two gene models. However, based on the observed complex interactions between some of the alleles, loci and gene products, we have proposed that more detailed studies and validation steps may be required to ascertain the different inheritance patterns.

CHAPTER VI
INVESTIGATING THE PHYSIOLOGICAL AND YIELD POTENTIAL OF BLOOMLESS SORGHUM UNDER WATER DEFICIT

6.1 Overview

Bloom (*Bm*) is the wild-type phenotype in sorghum and is associated with resistance to biotic and abiotic stress. Bloomless (*bm*), a knock-out mutation of the bloom phenotype is expected to increase plant susceptibility to stress such as elevated heat and drought and as such compromise the photochemically-dependent reproductive processes. In this study, we tested the hypothesis that photosynthetic efficiency (PnE) during floral meristemic cell differentiation and development defines the difference in reproductive potential between *Bm* and *bm* under water deficit. The objective was to determine if the physiological response to stress differs between the *Bm* and the *bm* sorghum during reproductive stages and how such deference might influence tolerance to stress. We used ethylmethanesulfonate (EMS) mutagenized Tx623 to develop BC2F3 and BC1F5 progeny segregating for *Bm* and *bm*. Water deficit (WD) treatments were imposed from 40 days after emergence (DAE) until physiological maturity. Each treatment was set up in three reps and two separate greenhouse experiments, with spring and summer average sampling day temperatures at 41^0C and 44^0C, respectively. Intrinsic physiological responses were evaluated among the segregant *Bm* and *bm* near-isogenic lines (NILs) at flag leaf emergence (FLE), at heading, 50% anthesis and at 7days after pollination (DAP). Differences were determined in PnE in relation to canopy temperature depression (TD), stomatal conductance (Gs), transpiration (TE), vapor pressure deficit (VPD), CO_2 influx-dependent net photosynthesis (Pn), leaf cuticular wax load (WL), and grain yield parameters. Compared to the *Bm* which had in which PnE showed a linear relationship with seed number (GN) under water deficit, the *bm* types showed exponential relationship and also a greater R^2 in both treatments. There was no significant difference in the mean WL between *Bm* and *bm*. In some cases, antagonistic

PnE and Pn was observed in the *Bm*. On average, genotypes that maintained higher PnE, irrespective of Pn during the reproductive phase had higher GN per head among the *Bm.* The *bm* required both high Pn and high PnE. Surprisingly, most of the *Bm* had reduced (Pn) in the well-watered, high temperature (WW) conditions than their *bm* counterparts. Taken together, these observations suggest that the *bm* mutation is associated with altered heat receptors important in signaling the regulation of stomatal aperture. These alterations may be linked to a modification in the C4 pathway that increases their overdependence on CO_2 influx and open stomata under heat and drought.

6.2 Introduction

The leaf pigment absorb light in the photosynthetically active radiation region (PAR, 400 – 700nm) but not in the near infrared region (NIR, 700 – 1200), thus reducing the reflection of PAR but not of NIR (Araus et al., 2002). Excess heat can hinder pollen maturation and disrupt the synchronization of the pollen and ovule maturation (Selmani and Wassom, 1993). Thus, heat generating irradiation needs to be reflected to reduce heat-related damage to the reproductive processes of a plant. Waxiness (wax load and architecture) of leaves is known to improve this reflectance (Kunst and Samuels, 2009; Mijitaba, 2004; Mondal et al., 2015). This reflective property is also affected by the chemical composition of surface waxes (Pfündel et al., 2008) and is an important determinant of total canopy reflectance. Mutations conferring the bloomlessness may be associated with the disruption of surface wax morphology (Jenks et al., 1992; Jenks et al., 2000), fatty acid chain length (Jenks et al., 2000; Lee and Suh, 2014), and possibly composition (Seo and Park, 2011) which together can influence the photosynthetic efficiency by the degree to which the incident heat-generating irradiation is reflected. It is still not clear how the bloomless wax conformation affects the association between temperature-related stress and the activities of the photosynthetic efficacy.

The efficiency of photosystem II can give a measure of the rate of linear electron transport and an indication of overall photosynthesis. It is especially critical to have well-functioning photosystems during the reproductive processes (Plaut et al., 2004).

The success of floral meristems initiation from the vegetative apex as a signal of inflorescence development is very sensitive to abiotic and biotic stress (Aspinall and Husain, 1970; Husain and Aspinall, 1970; Jordan, 1983), which when severe leads to impaired germinal cell differentiation and development (Dolferus, 2011; Ji et al., 2010). Of these, the rate of floral meristemic cells initiation is more sensitive to severe water deficit (Moss, 1971; Saini, 1997) and high tissue temperatures (Fischer, 1979) . This may be worsened if the reproductive potential is fixed by the total number of floral meristem cells formed within a predetermined short period (Jordan, 1983), such as in sorghum.

Some of the effects of germinal cells failure include complete absence of floral parts, aborted but fully formed florets (Jordan, 1983), reduced fertilization or failed seed establishment at anthesis (Boyer and McLaughlin, 2007; Dolferus, 2011; Ji et al., 2010; Moss, 1971; Parish et al., 2012). The failure of sorghum heads to fully exert (emerge) might also be associated with susceptibility to pre-anthesis water deficit stress (Inuyama, 1976; Jordan et al., 2012). The overall result is reduced seed set, grain number and grain weight. Thus, it is *hypothesized* that the relationship between photosynthetic efficiency and yield factors can be used to delineate the difference in tolerance between the bloom sorghum type and the bloomless sorghum mutants.

Fischer (1979) also found an association of reduced kernel number in barley with failure of photosynthetic production and allocation to spike during pre-anthesis periods when spike dry matter is accumulated. Thus the periods before anthesis are also important in assessing the reproductive significance of stress.

A higher radiation uptake and higher radiation-use-efficiency should lead to an increase in availability of sugars for transportation during floral development and anthesis (and grain filling), and a higher grain number/ yield (Araus et al., 2008). Overheating reduces the PSII efficiency. Thus, the relationship between sink strength and the strength of the PSII can be used to surrogate the effects of surface reflective integrity of the *bm* mutation.

6.3 Materials and methods

6.3.1 Germplasm development

Germplasm with a common parent but expressing both bloom (*Bm*) and bloomless (*bm*) are useful materials for comparative genomics and in determining the physiological effects of the genes responsible for the phenotype.

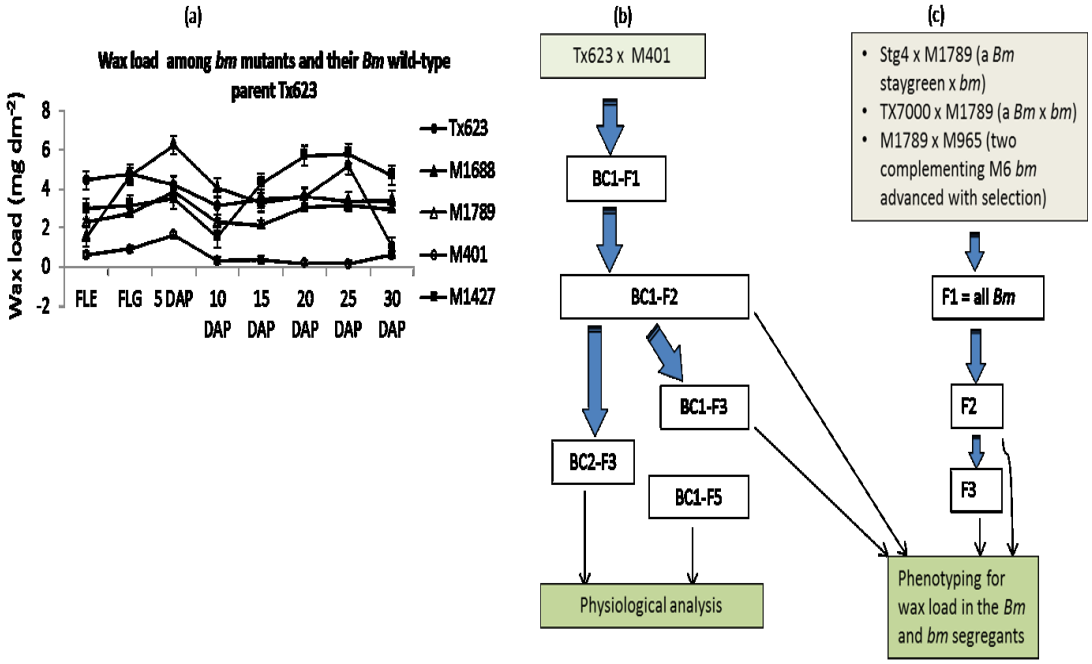

Figure 11. Development and selection of germplasm for physiological analysis. **(a)** Selection of *bm* mutant M401 as the male parent in a backcross to the *Bm* wild-type recurrent parent Tx623. Wax load (WL) among the *bm* mutant lines showing marked differences at phenological phases. Each data point is WL averaged for10 plants per row by 4 field reps, for every sampling stage shown. Data were taken under well-watered field conditions at day-time temperatures averaging between 41^{0} to 44^{0}C (these 2014 and 2015 late spring and late summer temperatures are considered in this study as elevated temperatures above the 29^{0} to 32^{0}C normal, 2014 spring temperatures at planting and earlier growth phases). FLE, flag leaf emergence; FLG, 50% flowering per head and at least 70% plants in row flowering; DAP, days after pollination. **(b)** The development of backcross populations from Tx623 X M401. The BC1-F2 and BC1-F5 Bm and bm near-isogenic lines were used in physiological evaluation. BC1F2 and BC1F3 were used to check the differences in WL between Bm and bm lines. (c) Staygreen (Stg4) and Tx7000, in crosses with the bloomless M1789, provided additional screening background for wax load. The F2 progenies of two complementing bloomless mutants have also been included.

The M4seeds from EMS-mutagenized Tx623 were provided by the USDA-ARS in Lubbock, Texas. These were selfed to M5 in College Station, Texas in 2013, to

confirm purity and to select for true-breeding *bm* lines. The true breeding *bm* lines were advanced to M6 in the Texas A&M AgriLife Fields Labs (TAMUFL) near College Station, Texas. Four of these lines were evaluated for wax load at flag leaf emergence (FLE), 50% pollination (flowering – FLG), and at 5d intervals from 5d after pollination (DAP) to 30 DAP (Figure 11a). The mutant M401 which showed the least WL over the sampling periods was selected as the backcross pollen donor to their wildtype parent Tx623. These were advanced to BC2-F3 and BC1-F5 (Figure 11a) over different cycles in the greenhouse and in the field between summer 2013 and spring of 2015. Deriving progeny from a cross of mutant to its wild-type parent ensured fewer segregating loci conditioning the target phenotype (Abe et al., 2012). The earlier generations BC1F2 and BC1F3 populations (from Tx623 X M401) were used in wax load comparison between the bloom and bloomless.

Along with the BC1F2 and BC1F3 populations (from Tx623 X M401), bloom – bloomless segregating F2s were also developed between the bloomless M1789 as the male parent and two bloom wildtypes, Stg4 and Tx7000 as the females. Segregating F2s were also produced between two complementing bloomless mutants M1789 X P0965. These F2s provided independent genetic backgrounds to assess the WL segregation resulting from the *bm* mutation.

For other physiological analyses, seeds from twelve heterozygous bloom BC2-F2 (from Tx623 X M401) and twelve heterozygous BC1F4 bloom plants (from Tx623 X M401) were sown heat-to-pot in 5 replicates (total 250 to 500 plants from each head).for a total of 120 pots. The *Bm* and *bm* segregants were identified at 3 weeks after emergence. There were no segregating bloomless plants used in this experiment.

6.3.2 Treatments and design in the greenhouse

The seeds from each BC2F3 head and each BC1F5 head were planted head-to-pot divided into 5 replicates (pots). These were planted in three-gallon pots filled with potting mix (MetroMix 900^{TM} forest peat moss, Schulenburg, Texas). The pots were thinned to 4 plants per pot at the end of the third week after emergence. At this point, all

the bloomless and all the bloom could clearly be distinguished. In thinning, all the bloomless plants were rogued from 12 segregating pots, leaving only 4 of their bloom sibs in each pot per rep for a total 5 reps per treatment. In another 12 pots, 4 bloomless sibs were left in each pot per rep for a total of 5 reps per treatment. The phenotypic values of the BC2F3 plants and the values of BC1F5 plants were combined within each rep of the bloom types and within each rep of the bloomless type.

Each treatment (well-watered and water deficit) was set up in a complete randomized design, and the two treatments set up in the same green greenhouse but separated by a 10m horizontal space between them. In each treatment, all the pots were randomly shifted every 1 week to remove any nearest-neighbor bias. The pots were normally watered until 40 DAG and continued at 2 kilogram (kg) every 2d for the well-watered (WW) until physiological maturity. The amount of water was reduced to 1kg every 4 days after 40 DAG for the water deficit (WD). The treatment temperatures were 41^0C, d and 27^0C, n in the late spring of 2015, and 44^0C, d and 27^0C, n in the late summer of 2015 for both the WD and the WW. These high temperatures are also considered in this study as heat stress. The notation WD therefore combines water deficit and heat stress, while WW represents well-watered treatment under heat stress.

6.3.3 Physiological phenotyping

For wax load analysis, six random F2 bloom and six random F2 bloomless from the Tx623 X M401- BC1F2 segregants were sampled at 50% flowering. Each of the six heads from the sampled bloom plants was bagged (to facilitate selfing). The BC1F3 seeds were planted head to row and the resulting BC1F3 sibs (Figure 11b) were sampled for WL in a similar manner as for the BC1F2 sib segregants. Wax load (WL) for the BC1F3s were averaged for 10 bloom plants and separately for 10 bloomless plants in each segregating head-row and 4 reps per head-row. The same number (as used in the BC1F3s above) of F2 segregant bloom and bloomless individuals from Tx7000 (*Bm*) X M1789 (*bm*) and Stg4 (*Bm*) X 1789 (*bm*) were similarly sampled for WL. These latter

populations provided additional genetic backgrounds to test the hypothesis that WL is independent of *Bm* or *bm* phenotype (Figure 11c)

For every plant sampled, wax load was quantified using five 7mm-diameter discs obtained from the flag leaf of each plant. Standard the chloroform and colorimetric method (Ebercon et al., 1977) and a conversion formula described earlier (Paynter, 1981; Swinehart, 1962; Vose et al., 1995) were used to determine WL in mg/dm^2. Wax load were averaged separately for all the bloom and for all the bloomless in each segregating row.

The mean intrinsic photochemistry and gas exchange parameters were determined in the greenhouse in late spring 2015 and late summer 2015, under well-watered conditions and under water deficit in both seasons (see further details under treatments). For these experiments the Tx623 X M401 BC2F3 and B1F5 near isogenic sibs were used. Data were taken in 7d intervals from FLE up to 7 DAP. Gas exchange was measured using CI-340 Handheld Photosynthesis System, CID BioSciences, USA. Two readings were recorded per rep. The quantum efficiency of photosystem II was measured using Fluorpen FP100, Photon System Instruments, Czech Republic. The CI-340 Photosynthesis System also integrated other parameters including canopy temperature differentials (TD), vapor pressure deficit (VPD), intrinsic transpiration (E) and stomatal conductance (Gs).

The yield parameters determined including the total dry grain weight per panicle (HW), mean single grain weight (SGW), grain number per panicle (GN) and above ground dry biomass weight (BMW). Also determined were the peduncle length (PL - to represent the extent of head extrusion from the sheath), and the plant water content at maturity (WC_{ma}).

Whole plant water (moisture) content at maturity (WC_{ma}) was determined to check its correlation with the dry biomass weight. Whole plant fresh weight (Y) was taken for two plants per rep (pot) at harvest. The heads were then cut off weighed, threshed and seeds dried to about 12% moisture. The remaining head straw (after threshing) were bagged separately and dried in an air convection oven at 60^0C until there

was no further change in weight. The fresh stovers were carefully chopped and bagged in small batches, and similarly dried in the oven until no further weight change was observable. All weights before and after drying were summed per sample and per rep and recorded thus:

$WC_{ma} = (P_a+S_a+V_a)-(P_b+S_b+V_b)$; where:

P_a, P_b = panicle weight without seed before and after oven-drying, respectively;

S_a, S_b = seed weight at harvest and after drying to 12% moisture, respectively;

V_a, V_b = stover (without panicle) before and after oven-drying, respectively.

Y and $(P_a+S_a+V_a)$ were similar.

The dry weight of shoots $(P_b+S_b+V_b)$ was taken as the constant weight achieved during oven-drying of the stovers plus the weight of the dried grain. It was the same as the fresh weight at maturity, that is $P_a+S_a+V_a$, minus moisture content at maturity, WC_{ma}. After, each head was carefully hand-threshed on clean stainless steel bench and cleaned of all chaff, the grain counts per head were taken using a digital seed counter (Seed Counter – Data Count & Fill S25, Data Detection Technologies Ltd, Israel, www.data-tech.co.il).

6.3.4 Analysis

Multivariate and pairwise correlations were fitted to determine the interdependence between the physiological phenotypes and between the physiological phenotypes and the yield-related phenotypes. The means were separated by treatment (and by genotype for wax load) using ANOVA in SAS-JMP. Where appropriate, a two factor regression fit was used.

6.4 Results

6.4.1 Comparing wax load among Bm and bm (F2, BC1F3, BC2F2)

The obvious identifying phenotype of the bloomless trait is the lack of whitish, fluffy appearance of the surface wax found in the bloom type. Since wax load (WL) is

known to affect canopy temperature and plant water balance (Kunst and Samuels, 2009; Seo and Park, 2011), we determined if wax load affected by the bloomless trait. Comparing the WL between the bloom and the bloomless segregants was a first step in determining probable underlying effects of the bloomless deviation from the bloom wild-type.

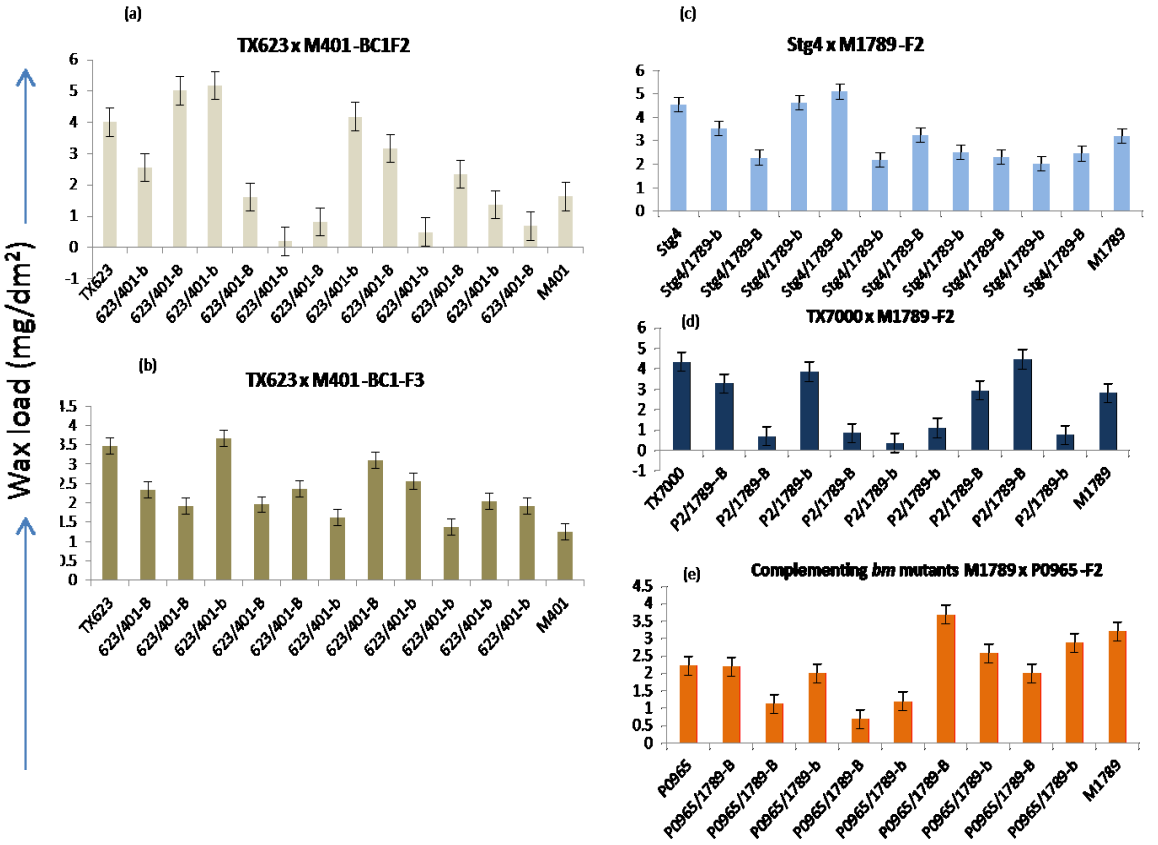

Figure 12. Random segregation for WL among *Bm* and *bm* in different backgrounds. **(a)** Six F2 segregants (each represented by bloom, -B and bloomless, -b) randomly selected from the Tx623 X M401- BC1F2 planted head to row. **(b)** BC1F3 advanced from the *Bm* BC1F2, and WL was averaged for 10 bloom plants and 10 bloomless plants in each segregating head-row and 4 reps per head-row. **(c)** and **(d)**, Tx7000 (*Bm*) X M1789(*bm*) and Stg4 (*Bm*) X M1789 (*bm*) provided independent genetic backgrounds to test the hypothesis that WL is independent of *Bm* or *bm* phenotype; Also shown is that WL segregation can be transgressive. **(e)** P0965 (*bm*) X M1789 (*bm*) F2 segregants (-B for bloom, and –b for bloomless); adjacent -B and –b are sibs from the same segregating F1 *Bm*.

From flag leaf emergence to 30d after pollination, comparisons between four true breeding bloomless mutant lines, M1688, M1789, M401, M1427, and their original bloom parent, Tx623 (Figure 11a,) showed that wax load may not be dependent on

bloom or bloomlessness. To verify this, we used the wildtype bloom parent Tx623 and the bloomless M401 (which showed the consistently lowest WL among the 4 bloomless mutants, Figure 11a) to develop segregating backcross populations as shown in Figures 12 (b) and 12 (b). Segregating F2s were also developed between a male bloomless M1789 and two wildtype females Stg4 and Tx7000, and between two complementing *bm* mutants M1789 X P0965 (Figure 12c). These latter crosses provided additional genetic backgrounds to assess the WL segregation between the mutants and the wildtype-expressing individuals. All the results showed that there were no clear WL patterns associated with the bloom or the bloomless types. Some *bm* types had significantly higher WL than their *Bm* counterparts and vice versa. There was also a reduced variation in WL with increased selfing (compare Figure 12a and 12b).

In developing Tx623 X M401, BC2F3 and the BC1F5 near isogenic lines (NILs), selection was made for progeny showing uniform height, leaf angle, leaf width, stem diameter and bloom or bloomless traits. These NILs were used to determine how photosynthetic efficiency differentially affects plant productivity among *Bm* and *bm* sorghum

6.4.2 The correlation among physiological factors and among agronomic factors

The data is summarized in Table 10 and in Table 11. Notably, increase in wax load (WL) corresponded to a non-significant increase in both vapor pressure deficit (VPD) and intrinsic transpiration (E) among the bloomless under water deficit (WD). The bloomless also showed non-significant but antagonistic relationships between WL and both VPD and E in the well-watered, high temperatures (WW) treatment. Among the bloom variants, increased WL corresponded to a non-significant but reduced VPD under WD, and significant and reduced VPD and E in the WW. High VPD was associated with reduced E and vice versa, for both phenotypic groups. These observations suggest that, even though the variability of wax load among the bloom may not be significantly different from the variability of WL among the bloomless, the way

wax affects water balance in the two phenotypic groups (bloom and bloomless).may be different.

Among the bloomless under WW, reduced canopy temperature (increased TD) was significantly associated with increased photosystem efficiency (PnE; r = 0.73), the net photosynthesis (Pn – based on CO_2 assimilation; r = 0.95), stomatal conductance (Gs; r = 0.79) as well as WL (r = 0.61) and E (r = 0.86). But it showed a non-significant positive relationship with E (r = 0.23) among the bloom. The Gs showed significant positive correlation with Pn for both the bloom and the bloomless under both treatments. However, genotypes which maintained high efficiency of photosystem II (PnE) also showed corresponding high TD among both the bloom and the bloomless in the WD. This latter case was true only among the bloomless in the WW treatment.

Table 10. The correlation analysis of plant water use, photochemistry and yield factors among bloom and bloomless sorghum under water deficit.

Water deficit

	TD	VPD	Hflag	DBW	WCma	PnE	Pn	E	WL	Gs	PL	GN	HW	SGW
TD		-0.7412*	-0.0482	0.4474*	0.5403*	0.6908*	0.8982*	0.6884*	0.6995*	0.8414*	0.0348	0.8020*	0.6684*	0.1484
VPD	-0.4956*		-0.1679	-0.1140	-0.5589*	-0.2608	-0.5931*	-0.5170*	-0.3611	-0.5999*	-0.1682	-0.4274*	-0.3922	-0.3387
Hflag	-0.1674	0.0528		0.1799	0.0100	-0.1481	0.0169	0.0899	0.4609*	0.3265	0.5994*	-0.1447	-0.2811	0.0893
DBW	0.4503	-0.1429	-0.0106		0.0535	0.3669	0.5052*	-0.0585	0.2119	0.4033*	-0.1449	0.6288*	0.6620*	-0.3270
WCma	0.0488	0.0726	0.0843	0.0687		0.0229	0.4530*	0.4033*	0.4528*	0.4024*	-0.1345	0.2187	0.2092	0.0151
PnE	0.9736*	-0.5021*	-0.2319	0.2988	0.1582		0.6861*	0.4024*	0.5098*	0.5460*	0.1013	0.6980*	0.5215*	0.1153
Pn	0.9266*	-0.6147*	-0.2219	0.3558	0.1990	0.9108*		0.6590*	0.4092	0.7483*	0.1367	0.5076*	0.8635*	-0.0524
E	0.5145*	-0.8054*	-0.0421	0.1586	0.0697	0.5124	0.6304*		-0.6014*	0.8587*	0.2287	0.5392*	0.4561*	0.1137
WL	0.7702*	0.4003	0.3001	0.4042	0.4920*	0.8097*	0.5262*	0.3914		0.5953*	0.3003	0.5003*	0.4411*	0.0211
Gs	0.7738*	-0.5859*	-0.0466	0.0882	0.1837	0.7306*	0.6950*	0.6531*	-0.3780		0.1449	0.6812*	0.6185*	0.1109
PL	0.4638	-0.3066	0.9289*	-0.1481	-0.1869	0.5153	0.3567	0.2182	0.2231	0.7364*		0.2098	-0.0303	-0.1559
GN	0.4550*	-0.5057*	-0.1612	0.0809	0.1088	0.5671*	0.6319*	0.2477	0.4401*	0.4397	0.7950*		0.9672*	0.0232
HW	0.2038	-0.3016	-0.2285	0.1321	0.1510	0.3514	0.2039	0.2119	0.4929*	0.3936	0.5763	0.9472*		-0.2316
SGW	-0.011	-0.1593	0.3956	-0.3109	-0.1618	0.1712	0.0183	0.0052	0.3742	0.0823	0.9286*	0.3858	0.1812	

Bloom

Bloomless

TD: temp depression VPD: vapor pressure deficit Hflag: height to flag leaf DBW: dry biomass weight Wcma: water content at maturity PnE: PSII efficiency Pn: net photosynthesis E: transpiration WL: wax load Gs: stomatal conductance PL: peduncle length GN: grain number HW: head weight SGW: single grain weight *Significant at α=0.05

Table 11. The correlation analysis of plant water use, photochemistry and yield factors among bloom and bloomless sorghum under well-watered and high temperature conditions.

Well-watered

	TD	VPD	Hflag	DBW	WCma	PnE	Pn	E	WL	Gs	PL	GN	HW	SGW
TD		-0.4590*	-0.3558	0.3569	0.1440	0.2207	**0.5269***	0.2273	**0.3220**	**0.5845***	0.2812	0.1554	0.1893	0.0817
VPD	-0.8443*		**0.5266***	**-0.3368**	**-0.0056**	**0.1751**	**-0.3045**	**-0.8314***	**-0.6101***	**-0.6213***	**-0.3165**	**-0.3308**	**-0.0195**	**0.1536**
Hflag	-0.2450	**0.0183**		-0.1641	-0.1637	0.1185	-0.2053	-0.3189	0.3621	-0.1519	-0.0353	-0.0579	-0.0441	0.0070
DBW	**0.5727***	**-0.2934**	-0.1042		**-0.0309**	**0.3116**	**0.5985***	**0.2711**	**0.3781**	**0.5023***	**0.0852**	**0.5960***	**0.6250***	**0.3799**
WCma	0.4381	**-0.6176***	0.0592	**-0.0131**		0.2123	0.0558	-0.1297	0.1721	-0.1870	0.2299	0.0753	0.0516	-0.2702
PnE	**0.7342***	**-0.5404***	-0.2743	**0.4595**	0.1218		**0.3598**	**-0.1451**	**0.5105***	**0.0198**	**0.1693**	**0.6705***	**0.2533**	**0.0200**
Pn	**0.9476***	**-0.7384***	-0.1546	**0.6176***	0.2840	**0.7996***		0.4159	0.3998	**0.4534***	0.1445	0.4340	**0.6547***	0.3130
E	**0.8635***	**-0.9412***	-0.1677	**0.1778**	**0.5412***	**0.5958***	**0.7500***		**0.4002**	**0.6804***	**-0.0511**	**0.5720***	**0.3370**	**-0.0362**
WL	**0.6108***	**-0.0498**	0.2310	**0.5113***	0.3383	**0.4899***	0.0112	**-0.2301**		0.0341	0.3023	0.3831	**0.4621***	0.1609
Gs	**0.7941***	**-0.9324***	0.1443	**0.2263**	0.4294	**0.6025***	**0.7059***	**0.8912***	0.2804		**-0.1199**	**0.5720***	**0.3923**	**0.2425**
PL	0.3534	**-0.6150***	0.2134	**0.1268**	**0.6991***	**0.2111**	0.2625	**0.5428***	0.1872	**0.5413***		-0.2626	-0.3861	**-0.4626***
GN	**0.7646***	**-0.5882***	-0.1429	**0.3912**	0.2735	**0.8749***	**0.8324***	**0.5882***	0.4013	**0.5676***	0.1719		**0.8820***	**0.5228***
HW	**0.7439***	**-0.5559***	-0.1679	**0.2735**	0.1971	**0.8328***	**0.8029***	**0.5882***	0.4222	**0.5706***	0.0383	**0.9676***		0.1471
SGW	-0.2346	**0.2075**	0.6321	**-0.2756**	-0.2549	**-0.2090**	-0.2520	**-0.1868**	0.0014	**-0.0825**	-0.4408	**-0.0382**	**0.7758***	

Bloom

Bloomless

TD: temp depression VPD: vapor pressure deficit Hflag: height to flag leaf DBW: dry biomass weight Wcma: water content at maturity PnE: PSII efficiency Pn: net photosynthesis E: transpiration WL: wax load Gs: stomatal conductance PL: peduncle length GN: grain number HW: head weight SGW: single grain weight *Significant at α=0.05

These intriguing relationships between TD, Pn, PnE and Gs point to the possibility that most of the bloomless type preferentially need to keep their stomata open under both heat and water deficit to elicit photosynthetic activity that is comparable to their bloom counterparts.

Table 12. Mean separation of yield performance between the bloom and the bloomless near-isogenic lines under stress.

	Trait and Treatment				Least Sq Mean
GN	Bloomless,WW	A			318.125
	Bloom,WW	A			294.75
	Bloom,WD		B		135.375
	Bloomless,WD		B		96.75
BMW	Bloomless,WW	A			31.2975
	Bloom,WW	A			27.52125
	Bloomless,WD		B		12.0325
	Bloom,WD		B		11.44625
HW	Bloomless,WW	A			7.20875
	Bloom,WW	A			6.7775
	Bloom,WD		B		2.04625
	Bloomless,WD		B		1.47375
SGW	Bloomless,WW	A			0.022285
	Bloom,WW	A			0.021898
	Bloomless,WD	A	B		0.016022
	Bloom,WD		B		0.015809
WCma	Bloom,WW	A			64.91625
	Bloomless,WW	A		B	52.0775
	Bloom,WD			B	42.92875
	Bloomless,WD			B	40.05125

WW – well-watered, high temperatures; WD – water deficit, high temperatures; GN - grain number per panicle; BMW - single plant biomass weight; HW - total grain weight per panicle; SGW - single grain weight; WCma - water content at physiological maturity.

The relationships between photosynthetic factors, and those of grain and biomass yield were equally interesting. Increased PnE significantly correlated with improved grain formation (grain number – GN) among the bloomless under both treatments (r = 0.57 in WD; 0.87 in WW; Table 9 and Table 10). For the bloom, the PnE was similarly associated with GN under water deficit (r = 0.70) and in the well-watered, high temperature treatment (r = 0.67). The stomatal aperture- and CO_2 influx-dependent Pn had a significant correlation with GN among the bloomless under water deficit (r = 0.63) and in the well-watered, high temperature treatment (r = 0.83). Among the bloom Pn and GN had a reduced correlations in the water deficit treatment (r = 0.51) and under well-watered, high temperature conditions (r = 0.43) the water deficit. These observations suggest that during the reproductive processes, the daytime stomatal conductance-dependent photosynthesis is less critical in the number of seeds formed among the bloom compared to the bloomless mutants under heat and water deficit stress.

On a mean by mean basis (Table 12), there were no significant differences in the yield components between the bloom and the bloomless types within a treatment. There were significant differences between the two treatments for all the yield factors considered.

6.5 Discussions

Floral organs are active sinks that require adequate mobilization of photosynthetic assimilates. The purpose of this study was to determine if the bloomless trait (summed as the *bm* mutation) differentially affect plant photochemistry and related yield parameters in response to water deficit (and heat) stress. We used flag leaf emergence (FLE) to designate earlier stages of floral meristem differentiation and the flag leaves themselves to determine the differences in leaf-based photosynthetic parameters among bloom and bloomless sorghum. Flag leaf emergence is one of the obvious morphological markers signifying the beginning of reproductive processes (Lancashire et al., 1991; Tottman, 1987; Tottman et al., 1979). The flag leaf is also a significant source of photoassimilate for the developing florets (Austin et al., 1982;

Evans and Rawson, 1970a; Reekie and Bazzaz, 1987) and grain (Evans and Rawson, 1970b; Guitman et al., 1991; Sicher, 1993; Simpson, 1968; Verma et al., 2004), and has less variable vapor pressure differentials (Addington et al., 2004; Dai et al., 1992; Fletcher et al., 2007).

6.5.1 WL segregates independent of either the bloom or the bloomless trait

A screen of two reps of 108 and 157 field-grown Tx623/M401-F2 segregants showed that the *Bm* and the *bm* segregated for wax load (WL) independent of phenotype. Some of the *bm* segregants had WL which either compared to or slightly exceeded their high *Bm* siblings. The general classification of *bm* as a low-WL phenotype (Burow, 2008) can thus be misleading in limited genetic backgrounds. Given that the role of wax in canopy temperature balance has been suggested (Balota, 2008b; Bañon et al., 2004; Mondal et al., 2015; Yun-Ying et al., 2008), these results also suggest that beneficial wax-based stress tolerance selection may as well be exploited among some bloomless types. For instance, WL was positively associated with grain number but not necessarily improved single grain weight, in both *Bm* and *bm* under water deficit (Table 9), suggesting the role of wax during floral meristem differentiation and inflorescence under stress.

6.5.2 Temperature differentials among the bloom and the bloomless

Highly efficient photosynthesis requires adequate internal temperature regulation. As the floral meristem cells divide, differentiate and develop, they require steady supply of photoassimilates (Ji et al., 2010; Zhou et al., 2007) and well-regulated temperature (Craufurd and Peacock, 1993; Yun-Ying et al., 2008). Canopy temperature regulation was strongly associated with photosynthetic efficiency, supporting a general consensus that canopy temperature is an integrative trait which can be used to explain other physiological traits (Amani, 1996; Condon et al., 2008) and a surrogate to yield (Araus et al., 2002).

6.5.3 Photosystem II efficiency (PnE) and the net photosynthesis (Pn): the contrasting responses among the bloom and the bloomless under stress

High Gs intrinsically corresponded to high Pn in both treatments among the bloom and the bloomless. These observations agree with what might be expected. The net photosynthesis (Pn) is a function of CO_2 influx, as well as being a function of efficient sink for the photosynthates (Amani, 1996; Condon et al., 2008). The tandem increase or decrease in both Gs and Pn is expected. High PnE correlated strongly with high TD, high Pn and high Gs among both the bloom and the bloomless under water deficit and high temperature (WD). The correlation was not significant between the PnE, and Pn or Gs among the bloom under the well-watered, high temperature (WW) treatment. However, the mean change in PnE did not show significant association with either Pn or Gs among the bloom under the well-watered high temperature conditions. The lack of significant PnE correlation with both Pn and Gs among the *Bm* NILs under the WW treatment may be a departure from expectation. We speculated that this may have been due to the reduced Gs in response to the high daytime ambient air temperatures (41^0C and 43^0C). Based on these observations, we suggest that canopy temperatures fluctuations may be more critical among most of the bloomless types compared to their bloom counterparts.

Compared to the bloomless NILs, the bloom NILs showed consistently higher numerical but not statistically superior performance in the yield factors within a treatment. Many physiological and biochemical factors such as the osmotic status and membrane integrity, CO_2 concentration and light capture by open reaction centers of PSII (Genty et al., 1989; Saibo et al., 2009; Zhu et al., 2010) may conspire to regulate the photosynthetic output of green leaves. The photosystem efficiency of the bloomless types may be more dependent on continued diffusion of CO_2 into the leaves to maintain similar reproductive potential as their bloomless NIL counterparts under stress. Conversely, the bloom types may be more efficient in sequestering and mobilizing CO_2 in the bundle sheath when CO_2 conductance through the stomata is restricted under certain stress conditions. The role of photoreceptors and temperature sensors in stomatal

regulation has been widely documented (Hetherington and Woodward, 2003; Ilan et al., 1995; Yu et al., 2004). The bloomless mutations may also be associated with altered temperature feedback receptors on the membrane, thereby maintaining open stomata even under high temperatures.

6.6 Conclusions

Bloom and bloomless sorghum show dissimilar response to elevated temperatures and water deficit. The photosynthetic efficiency among the bloomless was more dependent on open stomata, whereas the bloom types could achieve similar photosynthetic levels with less stomatal gas exchange under high temperatures. Because of this, the bloomless types showed lower photosynthetic efficiency and lower grain number (compared to the bloom types) when stomatal opening was restricted under water deficit. We suggested that the bloomless mutation may be associated with altered temperature feedback receptors on the cell membrane. Also, the bloomless mutation may confer less efficient sequestration and mobilization of CO_2 in the bundle sheath.

CHAPTER VII
CONCLUDING SUMMARY

Strategies for resistance to stress employed by plants include modified leaf morphology and cuticular wax or trichomes, stomatal aperture, adaptive root architecture, and ability to resist senescence (staygreen). These traits may interact in complex and varied ways. However, there is still an insufficient understanding of how the interactions of these mechanisms help plants ameliorate complex cues due to stresses such as water deficit and heat stress. Both tissue water deficit and high ambient temperature and light lead to elevated internal leaf temperatures which can induce reproductive senescence and premature onset of vegetative senescence. This study attempted to define the intersect between leaf cuticular characteristics and the staygreen trait, and the effects this relationship may have in plant physiological performance, water-use and yield. It also defined allelic variations responsible for the bloomless (the non-glaucous) trait, and the effect of bloomlessness on the reproductive potential of sorghum under various stress regimes. The *central hypothesis* was that leaf-level stress reducing strategies, such as increased leaf wax, are intrinsically associated with reduced pre-anthesis and post-anthesis senescence and hence improved floral viability and seed set, respectively.

Objective 1 defined the relationship between wax and transpiration in regulating canopy temperature among staygreen NILs and non staygreen inbreds and how this affects yield potential under heat, drought and a combination of both stresses. There were two related hypotheses for this objective. The first was that the relationship between wax load (WL) and canopy temperature depression (TD) is significant in defining the yield potential and the difference between staygreen and non-staygreen genotypes, and that leaf surface waxes contribute to the staygreen phenotype by constitutively lowering canopy temperature and in this manner reduce injury to the photosystems which might induce senescence-related cascades. The second was that by acting as a cuticular physical barrier to cuticular water loss, leaf wax load is temporally

antagonistic to transpiration rate in modulating canopy temperature and in this way, high leaf wax increases water-use efficiency under water deficit and high temperature stress.

Generally, leaf surface wax and absolute temperature depression showed a positive correlation. However, we first demonstrated that accounting for duration to flowering and the inherent relative wax load improves the estimation accuracy of the WL to TD relationship. As a result, we have also shown that the wax to TD relationship is not always as linear as some studies have previously reported, but can be either a linear or a polynomial function depending on the amounts of wax load or the stress imposed. This also pointed to a possible threshold beyond which a further increase in wax load does not correspond to lowered canopy temperature. In any case, by inference, high WL phenotype on average contributed to lower seed set failure compared to the low WL phenotype, under heat, drought and a combination of both stresses

The 'concept' of differential tolerance to drought among the Stg NILs has been previously defined, but little has been known precisely how the variation in cuticular wax load can affect how each Stg locus contributes to the yield components under both heat and drought treatments. By imposing drought and heat stress long before anthesis, recombinant inbred lines (RILs) earlier selected for high wax at anthesis and expressing the staygreen trait post-anthesis showed improved grain yield potential compared to the RILs with low wax and expressing or not expressing the staygreen trait. These results also support the previous findings that some sorghum genotypes expressing post-anthesis staygreen can show greater stress tolerance from the seedling stages compared to the non-staygreen types. However, we found that the degree of their stress tolerance may be influenced by the interaction between leaf wax and the staygreen. Thus wax load may be an important factor linked to a continuum of stress signaling cascades culminating in the well documented post-anthesis staygreen expression. We also concluded that the Stg loci are differentially effective against elevated temperature stress, water deficit cues and a combination of both stresses.

Transpiration through stomata is the main cooling mechanism employed by plants and accounts for about 90% of water lost from the above ground plant tissues,

whereas cuticular water loss can be up to 10%. Cooling and water conservation are therefore two opposing but necessary processes under heat and drought stress conditions. We used staygreen near isogenic lines with different wax accumulation traits to determine how the variation in their wax load (WL) affects whole plant transpiration (evapotranspiration, ET) and the effects of their interaction on canopy temperature and photosynthetic efficiency. By extension, we also reported biomass and grain yield responses in terms of whole plant water-use efficiency. We noted that ET and WL, changed during phenology in mostly antagonistic and polynomial manner. Accounting for the interaction between ET, WL and stage of sampling (phenology) improved the prediction accuracy for canopy temperature. The main object was to highlight these seemingly subtle but important and largely ignored factors in crop productivity-based models.

Leaf and stem wax morphology or structural architecture are reportedly different between glaucous (bloom - Bm) and the non-glaucous (bloomless - bm) plants. The carbon chain-length of the precursor fatty acids may also be implicated in the final visible differences. However, there is little research on how these dissimilarities contribute the overall plant response to abiotic stress. Under *objective 2*, we determined if there is physiological and reproductive significance of the bloom compared to bloomless phenotype. The central hypothesis of this objective was that bloom offers physiological advantage over bloomless in modulating resistance to water deficit and heat. Bloom and bloomless sorghum showed dissimilar response to elevated temperatures and water deficit. The photosynthetic efficiency among the bloomless was more dependent on open stomata, whereas the bloom types could achieve similar photosynthetic levels with less stomatal gas exchange under high temperatures. Because of this, the bloomless types showed lower photosynthetic efficiency and lower grain number (compared to the bloom types) when stomatal opening was restricted under water deficit. We suggested that the bloomless mutation may be associated with altered temperature feedback receptors on the cell membrane. Also, the bloomless mutation may confer less efficient sequestration and mobilization of CO_2 in the bundle sheath.

In *objective 3*, we determined allelic diversity and significant gene interactions controlling the bloomless trait in Sorghum, in relation to wax production. Under this objective it was hypothesized that the genes controlling bloomless trait are simply inherited. Many different dihybrid phenotypic combinations were observed from the crosses between the individual bloomless mutants and between the bloomless mutants and the wild-type bloom lines, Tx623, Tx7000 and Stg4. The results pointed to different alleles responsible for the bloomless phenotype. Among these were possible dominant mutant alleles for the bloomless trait. Even though only a small number of mutant lines were used, our results point to potentially large diversity in the alleles that can influence the production of the bloomless sorghum. Our observations have agreed with both the one-gene and the two gene models. However, based on the observed complex interactions between some of the alleles, loci and gene products, we have proposed that more detailed studies and validation steps may be required to ascertain the different inheritance patterns.

NOMENCLATURE

NIL	Near-isogenic line
RIL	recombinant inbred line
TD	Temperature depression of plant canopy
SLO	Staygreen and low wax
SHI	Staygreen and high wax
RLO	Non-staygreen and low wax
RHI	Non-staygreen and high wax
SSF	Seed set failure
HW	Head weight
WT	Wild-type
WW	Well-watered
WD	Water deficit
GLA	Green leaf area
FLE	Flag leaf emergence
WL	Wax load
DTF	Days to flowering
FT	Flowering time
LR	Likelihood ratio
LSD	least square difference
LSMean	Least square mean
QTL	Quantitative trait locus
RFLP	Restriction fragment length polymorphism
SS	Sum of squares
SSR	Single sequence repeats
Stg	Staygreen
TD	Temperature depression

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