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Effect of Water-deficit Stress on Cotton During Reproductive Development

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EFFECT OF WATER-DEFICIT STRESS ON COTTON DURING REPRODUCTIVE DEVELOPMENT

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ABSTRACT

Water deficit is a major abiotic factor limiting plant growth and crop productivity around the world. Cotton (*Gossypium hirsutum* L.) is considered to be relatively tolerant to drought and the effects of water stress on leaf physiology and metabolism have been extensively documented. However, information is lacking on the effect of water-deficit stress on the cotton flower. It was hypothesized that water-deficit stress would impair gas exchange functions which consequently would result in perturbation of carbohydrates of cotton reproductive units. To investigate this hypothesis growth room studies and field studies were conducted with the objectives being to document the physiological and biochemical changes that take place in cotton flowers and their subtending leaves when subjected to limited water supply. Additionally, the effect of the ethylene inhibitor 1-Methylcyclopropene under conditions of water stress was investigated as well as the response of leaf and ovary polyamine metabolism of two cotton cultivars differing in drought tolerance. Results indicated that water-deficit stress during flowering significantly compromised leaf gas exchange functions resulting in decreased stomatal conductance, photosynthesis, respiration and water potential. However, cotton reproductive units appeared to be less drought-sensitive compared to the leaves possibly due to higher water potential and glutathione reductase activity. Limited supply of water significantly affected carbohydrate metabolism of both leaf and pistil resulting in carbohydrate accumulation. Contrary to expectations, application of the ethylene inhibitor 1-MCP had no effect on leaf gas exchange function, however, it reversed the effect of water stress on pistil sucrose concentrations. Finally, water-deficit stress during flowering had a significant effect on polyamine metabolism of both leaf and pistil, resulting in increases in putrescine, spermidine

and spermine in drought-sensitive cultivars. The differential response of polyamine metabolism between drought-sensitive and tolerant cultivars suggests that polyamines could be effective tools not only in selection of drought-tolerant cultivars, but also in drought tolerance engineering, however further research is needed in order to elucidate the exact pathways of their action.

This dissertation is approved for recommendation
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DEDICATION

This dissertation is dedicated to my grandmother Vasiliki Kaplani.

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INTRODUCTION

Water deficit is the major abiotic factor limiting plant growth and crop productivity around the world (Kramer, 1983). It has been reported that approximately one third of the cultivated area of the world suffers from chronically inadequate supplies of water (Massacci et al., 2008). In all agricultural regions, yields of rain-fed crops are periodically reduced due to drought (Kramer, 1983), and the severity of the problem may increase due to changing world climatic trends (Le Houerou, 1996). Advances in irrigation technology help reduce the gap between potential and actual yield, however, yield differences remain in many areas around the world due to decreasing ground water supplies and increasing energy costs.

Cotton (*Gossypium hirsutum* L.), is a relatively drought tolerant crop since its wild, perennial ancestors originated in hot and dry areas (Lee, 1984). However, cotton modern cultivars that are cultivated as annuals appear to be less drought- tolerant since water-deficit stress occurring at any stage of development, but especially during flowering, results in significant compromises in the morphology, physiology, metabolism and ultimately yield (Grimes et al., 1969; Gerik et al., 1996; Pettigrew, 2004). Extensive research has been conducted in order to elucidate water-deficit stress effects on cotton, with the main body of it focusing on leaf physiology and metabolism nevertheless, no matter their great importance as yield determinants (Grimes et al., 1969) cotton's reproductive units have received limited attention. In order to elucidate the effect of water-deficit stress on cotton's reproductive units physiology and metabolism, a series of studies was conducted.

HYPOTHESES AND OBJECTIVES

STUDY I: Effect of water-deficit stress on carbohydrate and antioxidant metabolism of cotton flower.

HYPOTHESIS: Water-deficit stress during flowering would severely impair leaf gas exchange that would decrease carbohydrate content of the cotton flowers and increase glutathione reductase levels.

OBJECTIVE: Monitor gas exchange responses of cotton plants under conditions of limited water supply and evaluate the effect of those conditions on the carbohydrate and antioxidant metabolism of the cotton flower.

STUDY II: Effect of water-deficit stress on the physiology and metabolism of cotton flower and leaf in the field.

HYPOTHESIS: Water-deficit stress during flowering would severely impair leaf gas exchange that would decrease carbohydrate content and increase glutathione reductase as well as polyamine levels of cotton's flowers and their subtending leaves.

OBJECTIVE: Monitor and evaluate the alterations caused by water-deficit stress on the carbohydrate, polyamine and glutathione reductase metabolism of the cotton flower and its subtending leaf.

STUDY III: Effect of 1-MCP application and water-deficit stress on the physiology and carbohydrate metabolism of cotton flower and leaf.

HYPOTHESIS: Application of the ethylene inhibitor 1-Methylcyclopropene (1-MCP) would prevent ethylene production and result in alleviation of water-deficit stress consequences on the physiology and metabolism of cotton flower and subtending leaf.

OBJECTIVE: Evaluate the possible ameliorating effect of the anti-ethylene plant regulator, 1-MCP on cotton's first day flowers and their subtending leaves under conditions of limited water supply during reproductive development.

STUDY IV: Effect of water-deficit stress on polyamine metabolism of cotton flower and leaf.

HYPOTHESIS: Water-deficit stress would significantly affect polyamine metabolism of both first day flower ovaries and their subtending leaves.

OBJECTIVE: Investigate the changes in polyamine concentrations in first day flower ovaries and their subtending leaves under conditions of water deficit stress by using two cultivars differing in drought tolerance in order to determine whether polyamines are involved in drought tolerance.

REVIEW OF LITERATURE

Plant water deficits depend both on the supply of water to the soil and the evaporative demand of the atmosphere. In general, plant water stress is defined as the condition where a plant's water potential and turgor are decreased such that normal functioning of the plant is inhibited (Hsiao et al., 1973). Plant water deficit can be measured either by relative water content or leaf water potential and the deficit depends on the severity as well as the duration of the stress. Additionally, the genotype of the plant and the growth stage when the stress is imposed, determines the extent of the stress (Kramer, 1983).

Water availability and quality affect the growth and physiological processes of all plants, since water is the primary component of actively growing plants, ranging from 70-90% of plant fresh mass (Gardner et al., 1984). Due to its predominant role in plant nutrient transport, chemical and enzymatic reactions, cell expansion and transpiration, water stresses result in anatomical and morphological alterations as well as changes in physiological and biochemical processes and functions of the plants (Hsiao, 1973; Kramer, 1980).

EFFECTS OF WATER-DEFICIT STRESS ON MORPHOLOGICAL CHARACTERISTICS OF PLANTS

Water stress has long been considered to be one of the most important factors adversely affecting plant performance and yield development around the world (Boyer, 1982). Numerous studies have been conducted in the past to determine the effects of water stress on the morphology and development of cotton plants and it is known that water-deficit stress results in stunted growth because of reduced cell and leaf expansion, reduced stem elongation and reduced leaf area index (Jordan et al., 1970; McMichael and Hesketh, 1982; Turner et al.,

1986; Ball et al., 1994; Gerik et al., 1996). Leaf, stem and root growth rate are considered to be very sensitive to water stress since they are dependent on cell expansion (Hsiao, 1976; Hearn, 1994). Krieg and Sung (1986) reported that water stress caused a reduction to the whole plant leaf area by decreases in the leaf numbers rather than the leaf size. Both main-stem and sympodial branches had significantly less leaves, however the effect was less severe on the main-stem leaves. The authors attributed this decrease to reduced initiation of new leaves instead of leaf abscission due to senescence. However, Pettigrew (2004) reported that water-deficit stress resulted in a decrease in leaf size accompanied with an increase in the specific leaf weight (SLW). Significant increases in SLW due to water stress have also been indicated by Wilson et al. (1987). Significantly fewer nodes and lower dry weights of stems and leaves of water-stressed plants compared to those of the control were reported by Pace et al (1999), while McMichael and Quisenberry (1991) observed that plants grown under conditions of severe water stress decreased the shoot-to-root ratio and Malik et al. (1979) reported that root growth appears to be less affected by drought than shoot growth. Several researchers (Creelman et al., 1990; McMichael and Quisenberry, 1991; Ball et al., 1994; Pace et al., 1999) observed that seedlings of water-stressed cotton showed increased root elongation, accompanied however, with a reduction of root diameter.

A correlation between leaf abscission and low plant water potentials has been reported by many researchers (Addicott and Lynch, 1955; Bruce et al., 1965). McMichael et al (1972) identified a linear relationship between the rates of leaf abscission and the levels of the imposed water-deficit stress, reporting however, that leaf abscission occurred after the stress was relieved and not during the period of stress. This is in accordance with Addicott and Lynch

(1955), who speculated that formation of the separation layers is dependent on the plant's turgor. In addition to that McMichael et al. (1973) observed that younger leaves were not as prone to abscission as older ones.

Water-deficit stress has also been reported to cause alterations in cell ultrastructure. Ackerson et al. (1981) observed that leaves of adapted plants contained larger starch granules while structure of the thylakoid membranes appeared to be damaged. In addition, Berlin et al. (1982) indicated that water-stress caused significant changes in the palisade cell walls, number and size of chloroplasts, as well as in the grana and stroma lamellae and the structure of mitochondria. In support of that observation, Bondada and Oosterhuis (2002) reported loss of chloroplast membrane integrity accompanied with an increase of leaf wax production. Changes in the chemical composition of epicuticular wax were also observed with wax from water-stressed plants containing higher more long-chain alkanes compared to the control (Oosterhuis et al., 1991). Meek and Oosterhuis (2010) also reported that water stress significantly increased leaf epicuticular wax content. Changes in leaf lipid content have also been observed with water-deficit stress, where both glycolipids and phospholipids were significantly decreased, with the phospholipids being less affected, while triacylglycerols increased (Pham Thi et al., 1985; Wilson et al. 1987).

EFFECT OF WATER-DEFICIT STRESS ON PHYSIOLOGICAL CHARACTERISTICS OF PLANTS

Photosynthesis, Stomatal Conductance and Chlorophyll Fluorescence

Photosynthesis plays a major role in the determination of crop productivity in all species and is considered to be directly affected by water stress. Photosynthetic rates of the leaves are

known to decrease as the relative water content and leaf water potential decrease (Lawlor and Cornic, 2002) however, there has been some controversy concerning the main physiological sites responsible for photosynthetic impairment under water-deficit conditions (Flexas and Medrano, 2002; Lawlor and Cornic, 2002; Chaves et al., 2002, Lawlor, 2002), i.e. stomatal or non-stomatal limitations. According to recent reports (Chaves and Oliveira, 2004; Flexas et al., 2004a), decreased CO_2 diffusion from the atmosphere to the site of carboxylation in the main cause for reduced photosynthetic rates under most water-stress conditions. Reduced CO_2 diffusion has been attributed to either stomatal closure and/or reduced mesophyll conductance (Flexas et al., 2002; Warren et al., 2004).

Significantly positive correlations between leaf water potential and stomatal conductance under conditions of water-deficit stress have been reported (Socias et al., 1997), however diverse reports exist for cotton. Harris (1973) and Bielorai et al. (1975) reported that in potted experiments stomatal conductance was significantly decreased under conditions of water-deficit stress, whereas Ackerson et al. (1977) reported that leaf stomatal conductance of field-grown cotton was slightly affected and leaf stomata did not completely close even under very low water potentials, and they speculated that light intensity is probably more of a controlling factor than leaf water status. Those differential responses were attributed to the different ways of stress imposition as well as the different growth conditions in each study, while Jordan and Ritchie (1971) suggested that stomatal closure in field-grown plants is prevented in order for the plants to maintain water flux. In addition, Ackerson et al. (1977) observed differences between measurements taken at different times of the day (morning vs. afternoon) as well as between leaves of different age which was in accordance with previous

reports (Jordan and Ritchie, 1971; Jordan et al., 1975). Wullschleger and Oosterhuis (1990) also reported that both moderate and severe water stress significantly decreased leaf stomatal conductance whereas bract stomatal conductance remained unaffected. Other factors such as abscisic acid (ABA) concentration, ambient CO_2 concentrations and nutrient deficiencies have been shown to have an effect on leaf stomatal conductance under limited water conditions. Radin and Ackerson (1981) in potted experiments with different CO_2 concentrations and nitrogen rates indicated that water-deficit stress significantly decreased both stomatal and mesophyll conductance compared to the control. They also reported that nitrogen deficiency significantly increased stomatal sensitivity to the intercellular CO_2 concentrations at low water potentials, a result which was similar to the effect of ABA application. They concluded that behavior of stomata is closely controlled by ABA concentrations under conditions of water deficit. Similar responses of stomatal conductance were reported for phosphorus-deficient cotton plants (Radin, 1984).

In experiments with different cotton genotypes (Pettigrew, 1993) found that okra and superokra leaf type plants had lower stomatal conductance values than normal leaf type isolines at high water potentials and this was attributed to the lower abaxial stomatal density of okra leaf types (Wells et al., 1986). Similar findings were reported by Karami et al. (1980) and Nepomuceno et al. (1998) who also noticed that super okra was able to maintain higher leaf and turgor potentials at lower osmotic potentials compared to the normal leaf plants under water deficit.

Changes in the photosynthetic apparatus under drought through metabolic impairment are far more complicated than those resulting from stomatal function inhibition, and they are

predicted to occur under conditions of severe drought stress. Gimenez et al. (1992) reported that capacity of ribulose 1,5-bisphosphate (RuBP) regeneration could be a metabolic process that could be a limiting step in photosynthesis under water-deficit stress, while Medrano et al. (1997) speculated over the activity of ribulose 1,5-bisphosphate carboxylase/oxidase (Rubisco). Additionally, adenosine 5-triphosphate (ATP) synthesis or ATP-synthase activity could be severely inhibited resulting in a decrease in photosynthetic rates (Younis et al, 1979; Tezara et al. 1999). Leaf photochemistry (Cornic and Massacci, 1996) and permanent photoinhibition (Bjorkman and Powles, 1984) have also been suggested to be affected under limiting water conditions. Recently, Flexas et al. (2006) observed that photosynthetic rates are mostly limited by decreased stomatal conductance as well as reduced mesophyll conductance that ultimately result in a general metabolic impairment due to lower carbon substrate concentrations. However, the extensive research, a conclusion over which photosynthetic metabolic process is most sensitive under water-deficit stress has yet to be made.

In cotton, several reports have indicated that water stress causes a reduction in photosynthesis rates due to a combination of stomatal and non-stomatal limitations (Pallas et al., 1967; McMichael and Hesketh, 1982; Turner et al., 1986; Sung and Krieg, 1986; Genty et al., 1987; Ephrath et al., 1990; Faver et al., 1996, Lacape et al., 1998; Leidi et al., 1999). Marani et al. (1985) reported reduced photosynthetic rates under conditions of water stress which they attributed to decreased leaf expansion and hence, leaf area as well as to the leaf age of the canopy and the increased senescence rates due to reduced supply of water. However, Constable and Hearn (1981) with field experiments in Australia, observed that net assimilation rate was not affected by irrigation treatments. In partial agreement with them, Pettigrew

(2004) reported that leaf photosynthetic rates increased in the morning for water-stressed field-grown cotton plants in the Mississippi Delta before decreasing in the afternoon. Those different responses however, could be attributed to the different stages of growth that water-deficit stress was imposed, the different genotypes, the different leaf ages and position of leaves in the canopy. As Karami et al. (1980) reported, photosynthesis during the reproductive stage was less sensitive to water stress compared to vegetative stage while young leaves had higher photosynthetic rates compared to older ones at the same leaf water potentials. In addition, Wullschleger and Oosterhuis (1990) reported that moderate and severe water-deficit stress resulted in a significant decrease in leaf photosynthesis, however bract photosynthetic rates remained unaffected regardless the severity of stress.

Pettigrew (2004) also speculated that the higher photosynthetic rates could be attributed to the hydraulic conductivity of the soils that allowed the plants to rehydrate during the night, hence enabling their photosynthetic apparatus to operate more efficiently during the morning while he suggested that the higher PSII quantum efficiency (ΦPSII) that was observed could be attributed to the higher chlorophyll content per unit leaf area that was observed. Similar results were reported from Massacci et al. (2008) who observed that photosynthetic electron transport was enhanced under conditions of water stress due to an increased efficiency in the open PSII reaction centers. They also observed that photorespiration increased at the onset of water stress in order to prevent an inhibition of the photosynthetic apparatus and over-production of damaging reactive oxygen species. Massacci et al. (2008) attributed this to an increase in photorespiration rates in order to prevent an inhibition of the photosynthetic apparatus and over-production of damaging reactive oxygen species. Genty et al. (1989) also

reported that PS II quantum efficiency (ΦPSII) is positively correlated with the quantum efficiency of CO_2 fixation. They also noted that photon receptors were not impaired under conditions of water stress. Similarly photon distribution and PS II photochemistry was not affected, however electron transport through PSI was inhibited. In contrast, Enahli and Earl (2005), Inamullah and Isoda (2005), and Kitao et al. (2007) observed that quantum efficiency of PS II (ΦPSII) decreases under conditions of water stress. Additionally, Enahli and Earl (2005) in their study, where water stress levels varied from moderate to severe, observed that even though photosynthetic rates remained unaffected under moderate stress rates significant decreases were observed in the velocity of carboxylation of Rubisco and at the CO_2 concentration at the site of carboxylation. Those responses became more prominent under severe water-deficit stress where both photosynthetic rates as well as concentration of CO_2 at the site of carboxylation decreased. Upon relief from the water stress, CO_2 concentrations returned to control levels however, photosynthetic rates remained low indicating metabolic and non-stomatal inhibition, which is in contrast with Pettigrew (2004). The explanation for these contrasting results has been suggested to lie in the heterogeneity of the photosynthetic apparatus across the cotton leaf (Wise et al., 1992). However, Massacci et al. (2008) indicated that leaf patchiness is significantly decreased under conditions of water deficit.

Concerning genotypic differences, okra and super okra leaf type plants exhibited higher photosynthetic rates at similar low water potentials compared to the normal leaf type plants in greenhouse and field experiments (Karami et al., 1980; Nepomuceno et al., 1998; Pettigrew, 2004).

Respiration

Respiration is the process by which a plant obtains energy by reacting oxygen with sugars (glucose) to produce water, carbon dioxide and adenosine 5-triphosphate (ATP). Dark respiration (in contrast to photorespiration and photosynthesis) occurs during the day and night, and its rates during the day vary between 25 and 100% of the respiratory activity during the night (Krômer, 1995). Of the CO_2 fixed each day by net photosynthesis about 30-70% is released back to the atmosphere through dark respiration (Atkin et al., 1996) with 50-70% of whole plant respiration occurring in leaves (Atkin, 2007). Respiration rates of well watered plants depend largely on photosynthesis (Noguchi, 2004). Nevertheless this may not be the case under conditions of water deficit stress and evidence suggests that reduced plant growth and not limitations in photosynthates is the main reason for the decreased respiration rates observed in plants under conditions of water stress (Wilson et al., 1980). However, Flexas et al. (2005, 2006) pointed out that the percentage of daily fixed carbon that is respired is expected to be higher in water-stressed plants mainly because of the inhibitory effect that water deficit has on photosynthesis. Respiration pathways in plants can be separated in glycolysis, which occurs both in the cytosol and the plastid, the tricarboxylic acid cycle located in the mitochondrial matrix, and the electron transport chain which is in the inner mitochondrial membrane (Fernie et al., 2004).

According to Atkin et al. (2009) the responses of respiration rates to water deficit are variable and with no clear pattern depending on the type and the age of tissue (mature or still actively growing), the duration and severity of stress, changes in activity of respiratory enzymes, changes in hormonal and osmolyte accumulation, substrate availability and ATP

demand. De Vries et al. (1979) conducted studies in maize (*Zea mays* L.) and wheat (*Triticum aestivum* L.) and observed that while respiration rates remained unaffected at low or moderate water stress, they decreased at severe water stress. A similar pattern was also observed by McCree et al. (1984) in sorghum (*Sorghum bicolor* L.) and Boyer (1970) and Ribas-Carbo et al. (2005) in soybean (*Glycine max* L.). However, Boyer (1970, 1971) in studies with sunflower (*Helianthus annuus* L.), found a decrease in respiration rates when drought stress was imposed, similarly to Palta and Nobel (1989), Gonzalez-Meler et al., (1997), Escalona et al., (1999) and Haupt-Herting et al., (2001) while Lawlor and Fock (1977) as well as Loboda et al. (1993) reported no change. Ghashghaie et al., (2001) working with sunflower noticed an initial decrease in leaf respiration rates, however at higher intensity of stress respiration rates increased even above the control. Suggestions for these differential responses in respiration's response under water deficit stress conditions include differences in species, tissues, conditions or experimental techniques that were used. Additionally, the complicated interactions that take place with other environmental factors, as well as the existence of a stress threshold under which changes in respiration rates occur have been proposed as the causes of these discrepancies in plant respiration under water stress conditions (Flexas et al., 2005). However, there is a general consensus that respiration rates are significantly less sensitive to water stress compared to photosynthetic rates (Boyer, 1970) and a biphasic pattern in respiration rates should expected with a progressive decrease of respiration at initial stages of water stress, followed by increase below the threshold of water stress intensity (Flexas et al., 2005).

Limited data exist in cotton on the effect of water-deficit stress on respiration rates. Pallas et al. (1967) reported a biphasic response of cotton leaf respiration rates where they

initially decreased with increasing severity of the water stress and eventually increased at more severe stress. Wullschleger and Oosterhuis (1990) conducted studies and reported that boll respiration remained unaffected under moderate water stress and significantly decreased once the stress became more severe.

ATP Content

Adenosine 5-triphosphate (ATP) is a multifunctional nucleoside consisting of adenine, ribose and three phosphate groups. It constitutes the molecular currency of intracellular energy transfer for plant metabolism. Photosynthesis and respiration are the main plant processes through which ATP is produced, and specifically through the pathways of (a) photophosphorylation (cyclic and non-cyclic) in the chloroplasts, (b) glycolysis in the cytosol, and the most important pathway (c) oxidative phosphorylation in the mitochondria (Raymond and Pradet, 1983).

Measurements of ATP in water-stressed tissues show considerable variability. Flexas and Medrano (2002) reported a decrease in ATP content of leaves with a relatively small decrease in relative water content, however Tezara et al. (1999) observed that ATP content was not depleted completely even at very low relative water content and when photosynthesis had stopped. Sharkey and Seeman (1989) found no differences in the ATP content of mildly-stressed bean (*Phaseolus vulgaris*, L.) leaves, while Meyer et al. (1992) indicated that ATP content progressively decreased as the relative water contents decreased. Recently, Lawlor and Tezara (2009) speculated that drought stress might also result in an increased ATP content through the respiratory pathway in order to compensate for reduced rates of chloroplast ATP

synthesis. Pandey et al. (2002) conducted studies to determine the effect of water-deficit stress on the photosynthetic metabolites on cotton during the reproductive stage. They reported that water-stress resulted in a decrease in leaf ATP content while, nicotinamide adenine dinucleotide phosphate (NADP) content was increased. Leaf 3-phosphoglyceric acid (3-PGA) and pyruvate content remained unaffected from the water stress treatments.

Carbohydrate Metabolism and Translocation

As mentioned above photosynthesis has been shown to be greatly and adversely affected by water stress. Considering that photosynthesis is the fundamental function through which plants fix carbon and produce carbohydrates it is expected that water stress would also affect carbohydrate metabolism.

An early study by Eaton and Ergle (1948) showed that cotton leaves under water stress exhibited large reductions in starch concentrations, variable effects on sucrose accumulation and increased hexose sugars. Similar results were reported by Parida et al. (2007). In their experiment, water stress was imposed at the flowering stage on two cotton cultivars differing in drought tolerance and they concluded that leaf total soluble carbohydrate and leaf hexose concentrations were increased, while leaf starch contents decreased in both drought-tolerant and drought sensitive cultivars. Increase in hexose and depletion of starch leaf concentration have also been reported in soybean (*Glycine max* (L.) Merr.) (Huber et al., 1984; Liu et al., 2004) and pigeonpea (*Cajanus cajan* L.) (Keller and Ludlow, 1993). In contrast, Ackerson (1980) observed that higher quantities of starch were accumulated in water-stressed cotton leaves compared to those of the control. Additionally, he reported that acclimated young cotton

leaves had the ability to export sucrose, whereas non-acclimated plants did not, at the same low leaf water potential and he speculated that translocation of photosynthates was greatly inhibited under conditions of water stress. In support of this observation, Timpa et al. (1986) reported that drought stress caused no change in leaf sucrose concentrations of non-flowering cotton strains, while glucose levels were significantly higher in the drought-stressed leaves compared to those of the control, indicating that the source sink-relationships are affected by drought. Impairment of the photoassimilate translocation mechanism under conditions of water-deficit stress has been reported for other crops as well, such as sugarcane (*Beta vulgaris* L.*)* (Hartt, 1967), maize (*Zea mays* L.) (Boyer and McPherson, 1977), wheat (*Triticum aestivum*, L.) (Johnson and Moss, 1976) while Liu et al. (2004) made a similar observation for soybean source-sink relationships and reported that sucrose and starch leaf concentrations decreased significantly under water stress and resulted in a decrease in the rate of sucrose export from the leaves.

Sung and Krieg (1979) conducted experiments, with different leaf-type cotton genotypes and water stress at different stages of development, to study the effect of water stress on the rate of assimilate export from the leaf as disappearance of labeled ^{14}C from the leaf. They reported that translocation of assimilates was reduced under much lower water potential values compared to photosynthesis concluding that photosynthesis is more sensitive to water deficit stress than translocation which is in accordance with Wardlaw (1967), who also concluded that main consequence of water stress on translocation is on the availability of photosynthate. Sung and Krieg (1979) however, observed that water-deficit stress altered the pattern of assimilate export for the upper canopy leaves allocating more photosynthate to

vegetative growth and fruits while the water stress had no effect on the export pattern of the lower canopy leaves.

Guinn (1976) however, did not notice any difference in carbohydrate accumulation in 4 day old bolls in cotton plants that had been subjected to water stress compared to those that had been properly watered. Heitholt et al. (1994) additionally, did not observe any differences in sugar concentrations and boll retention of receptacles and ovaries of 5 day old cotton flower buds, flowers, and 2 day old bolls, however the plants were not subjected to water stress. Similarly, Liu et al. (2004) failed to correlate pod-abortion of water-stressed soybeans with pod carbohydrate concentrations.

Zinselmeier et al. (1995, 1999) observed that an accumulation of sucrose in young water-stressed maize ovaries occurs simultaneously with the cessation of ovary growth and an additional decrease in hexose concentration, and they speculated that the ratio of hexose to sucrose could play an important role in ovary development. An inhibition of invertase activity due to drought stress could also result in an increase in ovary sucrose content (Schussler and Westgate, 1991; Liu et al., 2004). This was also noted by Weber et al. (1998) for legume seed development. Further explanation of carbohydrate metabolism in flowers and developing bolls during drought stress is needed.

Plant Mechanisms In Response To Water-Deficit Stress

1. **<u>Antioxidants:</u>**

Drought stress has been reported to induce an oxidative stress due to inhibition of photosynthesis (Smirnoff, 1993) resulting from the production and accumulation of toxic

oxygen species such as peroxide radicals, hydrogen peroxide and hydroxyl radicals (Foyer et al., 1997). The accumulation of reactive oxygen species originates mainly from the decline in CO_2 fixation which leads to higher leakage of electrons to O_2 (Foyer et al., 1997), while other factors for formation of free radical forms can be fatty acid β-oxidation (del Rio et al., 1998), membrane associated oxidases (Desikan et al., 1996) and photorespiration (Faria et al., 1999).

Accumulation of reactive oxygen species under conditions of water-deficit stress is mainly dependent on the balance between their concentrations and concentrations of antioxidants (Mittler et al., 2004). Production of antioxidants is greatly affected by the type of environmental stress, the severity and the duration of the stress as well as the type of the tissue and its ability to compensate for the energy imbalance (Mittler et al., 2004). Main sites of reactive oxygen species formation are the chloroplasts, where a decrease in CO_2 fixation accompanied by over-reduction of the electron transport is the main mechanism for production of reactive oxygen species. Similarly, over-reduction of the electron transport chain is mitochondria constitutes them as another site of significant reactive oxygen species production (Davidson and Schiestl, 2001), while in peroxisomes, oxidation of glycolate to glycoxylic acid results in production of H_2O_2 (Mittler et al., 2004).

These reactive oxygen species produced during water-deficit stress can damage many cellular components including lipids, proteins, carbohydrates and nucleic acids (Monk et al., 1987). Membrane lipid peroxidation and protein oxidation constitute the simplest criteria of assessing the extent of oxidative damage in the tissue (Noctor and Foyer, 1998; Mittler 2002). Efficient antioxidant systems in the plant can minimize the level of oxidative stress and protect the tissues (Rhizsky et al., 2004; Wang et al., 2005). Such antioxidants systems can be enzymatic

or non-enzymatic. The major antioxidant species in the plants are superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (AP), and glutathione reductase (GR), along with carotenoids and α-tocopherol (Gaspar et al., 2002). Additionally, polyamines and flavonoids have been shown to provide some protection from free radical injury (Bouchereau et al., 1999), while polyamine catabolism has also been reported to regulate reactive oxygen species accumulation as well as formation of reactive oxygen species scavenging enzymes (Moschou et al., 2008). In addition, the photosynthetic system through the xanthophyll-zeaxanthin cycle can also contribute in the relief of oxidative stress. The levels of these antioxidants systems, however, have shown increases, decreases or no effect, depending on the species, duration of drought stress and the specific antioxidants investigated (Reddy et al., 2004).

Mahan and Wanjura (2005) performed field studies to identify changes in antioxidant metabolism in cotton. They observed that even though the glutathione amount and form changed during the season, in both years of their study, the changes were not in response to the water-stresses that were imposed and they concluded that cotton has a limited ability to alter glutathione metabolism in response of drought stress. In contrast, ascorbate peroxidase activity was increased in water-stressed plants compared to the well-watered plants while no significant change was also reported in the levels of malondialdehyde (MDA), an indicator of cell-membrane damage, leading them to speculate that the oxidative stress was alleviated before membrane damage could occur. However, Kawakami et al. (2010) reported that glutathione reductase of potted grown plants was not affected by water-deficit stress, whereas superoxide dismutase of water stressed plants was significantly decreased compared to the control. Light et al. (2005) in experiments with transgenic cotton plants overexpressing *Nt*107, a

tobacco gene with both glutathione-s-transferase (GST) and glutathione peroxidase (GPX) activities, reported that GPX activity failed to increase compared to the wild plants and suggested that the antioxidant machinery in cotton may be disrupted by the expression of *Nt*107.

2. Proteins:

Plants have been shown to accumulate specific stress-associated proteins in order to survive adverse environmental conditions (Vierling, 1991; Ingram and Bartels, 1996). Heat shock proteins and late embryogenesis abundant (LEA)-type proteins are two major types of stress-induced proteins that are produced upon the induction of drought stress and are considered to play a role in cellular protection during the stress (Ingram and Bartels, 1996; Zhu et al., 1997).

Heat shock proteins have been observed to be produced at any stage of crop development and under different environmental factors such as water-deficit stress (Bray, 1993), UV-radiation (Dohler et al. 1995), or heavy metal accumulation (Neumann et al. 1994). Their molecular weights and proportions differ among species, and they are considered as molecular chaperones essential for the maintenance of protein homeostasis and prevention of denaturation (Vierling, 1991), even though the mechanism by which they contribute to drought tolerance is still not certain. One hypothesis is that they are involved in energy dependent protein unfolding or assembly/disassembly reactions and they prevent protein degradation under adverse conditions (Pelham, 1986). Another hypothesis is that they are related to the protection and stabilization of particular organelles such as chloroplasts, ribosomes and

mitochondria. Additionally, some members of the heat shock proteins have been shown to aid in the maintenance and restoration of enzymes (Sun et al., 2001).

In arid and semi-arid regions, dryland crops may synthesize and accumulate substantial levels of heat shock proteins in response to elevated leaf temperatures due to decreased rates of transpiration. Burke et al., (1985) conducted experiments with field-grown cotton, where soil water deficits resulted in canopy temperatures of 40°C or greater for two to three weeks. At least eight new polypeptides accumulated in about half of the water-stressed leaves analyzed while no polypeptides were accumulated in the irrigated cotton leaves. In another study, Kuznetsov et al. (1999) imposed a short-term heat shock treatment to the plants at flowering, prior to water-deficit stress imposition and they observed that heat-treated plants accumulated greater quantities of two heat shock proteins (70 and 80kDa) as well as aminoacids (asparagine, proline and arginine especially). Additionally, larger osmotic adjustment values were observed and the authors speculated that heat shock proteins have a protective role in cotton under condition of water deficit stress. However, in a similar experiment with field-grown soybean (*Glycine max* (L.) Merr.) several heat shock proteins were observed in both irrigated and water-deficit stress plants (Kimpel and Key, 1985).

Late embryogenesis abundant (LEA) proteins, the second major type of stress-induced proteins, have been found in a wide range of plant species in response to desiccation or drought stress (Ingram and Bartels, 1996). Even though they were first identified in cotton seeds during their maturation and desiccation phases (Baker et al., 1988), it has since been recognized that they also accumulate in vegetative tissues under conditions of water stress (Bray, 1993). According to Bray et al., (2000) most LEA proteins exist as random coiled α-helices

and they are characterized by their high hydrophilicity index and glycine content (Garay-Arroyo et al., 2000). They are considered to act as water-binding molecules, participate in ion sequestration, and contribute in membrane stabilization (Ingram and Bartels, 1996).

3. Osmotic Adjustment and Compatible Osmolytes

Plants experiencing stressful conditions, such as drought tend to actively accumulate highly soluble organic compounds of low molecular weight, called compatible solutes as well as inorganic ions, i.e. K, in order to prevent water loss, maintain water potential gradients and re-establish cell turgor (Hsiao, 1973). This process is called osmotic adjustment and according to Boyer (1982) enables plants to: (1) continue normal leaf elongation but at a reduced rate; (2) adjust their stomatal and photosynthetic functions; (3) maintain the development of their roots and subsequently continue soil moisture extraction; (4) postpone leaf senescence; and (5) achieve better dry matter accumulation and yield production under adverse conditions. Osmotic adjustment has been reported in the leaves of a number of crops such as wheat (*Triticum aestivum* L.) (Morgan, 1977), maize (*Zea mays* L.) (Acevedo et al., 1979) sorghum (*Sorghum bicolor* L.) (Jones et al., 1978; Turner et al., 1978), rice (*Oryza sativa* L.) (Cutler et al., 1980), barley (*Hordeum vulgare* L.) (Matsuda et al., 1981), pearl millet (*Pennisetum americanum* L.) (Henson et al., 1982), sunflower (*Helianthus annuus* L,) (Turner et al., 1978) as well as cotton (*Gossypium hirsutum* L.) (Acevedo et al., 1979, Oosterhuis, 1986). Interestingly, cotton appears to have a greater ability to osmotically adjust to water stress compared to other major crops (Ackerson et al. 1977; Oosterhuis and Wullschleger, 1987). Additionally, Oosterhuis and Wullschleger (1987) observed that primitive landraces and wild types of cotton exhibited

higher osmotic adjustment compared to commercial cultivars. Oosterhuis and Wullshcleger (1987) investigated the osmotic adjustment of cotton roots under water deficit and demonstrated that cotton roots show a considerably larger percentage adjustment than the leaves reinforcing the ability of the plant to maintain a positive turgor and hence continue normal growth under water stress. A similar pattern was observed in cotton flowers (Trolinder et al., 1993) and bolls (Van Iersel and Oosterhuis, 1996) where both flowers and fruits were found to be less affected by the water stress imposed than the subtending leaves. These authors concluded that cotton flowers and bolls are largely independent of the xylem connections for their water supply and that the phloem is the most important factor in water transport to the flowers and developing bolls. Ackerson and Hebert (1980) observed that cotton plants that had been subjected to consecutive water-stress cycles exhibited increased osmoregulation compared to plants that had not been subjected to stress previously. They reported that photosynthetic rates were higher due to higher stomatal conductance at low water potentials, but the opposite was observed under high water potentials.

Osmolytes are organic compounds that exist in a stable form inside the cells and are not easily metabolized. In general, they do not have an effect on cell functions, even when they have accumulated in considerably high concentrations, i.e. more than 200mM (Hare et al., 1998; Sakamoto and Murata, 2002). Compatible solutes include sugars and sugar alcohols (polyols) (Yancey et al., 1982), amino acids such as proline (Aspinall and Paleg, 1981; Bonhert et al., 1995) and its analogues (Naidu et al., 1987), quaternary ammonium compounds (betaines) and tertiary sulfonium compounds (Rhodes and Hanson, 1993). Production of osmolytes is a general method in plants to maintain osmotic potential and cell turgor, as stated above,

however they also have secondary roles such as stabilization of membranes and maintenance of proper protein conformation at low leaf water potentials (Papageorgiou and Morata, 1995), protection of cells by scavenging for reactive oxygen species (Pinhero et al., 2001), as well as regulation and integration in the metabolism of stressed photosynthetic tissues (Lawlor and Cornic, 2002). Their synthesis and accumulation varies among plant species, as well as among cultivars of the same species, and they are most often confined to the chloroplasts and cytoplasmic compartments that according to Ain-Lhout et al., (2001) occupy less than 20% of the total volume of mature cells.

4. Polyamines

Polyamines (PAs) are low-molecular-weight organic polycations with two or more primary amino-groups (-NH_2), that are ubiquitous in bacteria, plants and animals. The diamine putrescine (PUT) and its derivatives, the triamine spermidine (SPD) and the tetramine spermine (SPM) are the most common polyamines in plants with SPD and SPM being synthesized from PUT and SPD, respectively, with addition of one aminopropyl moiety (Palavan-Unsal, 1995). The aminopropyl group, decarboxylated S-adenosylmethionine (dcSAM), is produced from S-adenosylmethionine (SAM) mediated by S-adenosylmethionine decarboxylase (SAMDC) (Bagni and Tassoni, 2001). The importance of PAs in plants is due to their participation in a multitude of plant metabolism functions, including photosynthesis, enzyme activation and maintenance, cell proliferation, division and differentiation, morphogenesis and embryogenesis as well as organogenesis (Evans and Malmberg, 1989; Galston et al. 1997; Kakkar and et al., 2000). In addition, PAs are involved in the correct conformation of nucleic acids, gene expression and

translation, hormone action mediation, modulation of cell signaling, membrane stabilization and ion channel regulation, as well as heat shock proteins and macromolecular synthesis. Besides all the aforementioned functions, PAs also act as second messengers in leaf senescence and apoptosis and more importantly for biotic and abiotic stresses (Kumar et al., 1997; Walden et al., 1997; Malmberg et al., 1998; Bouchereau et al., 1999; Liu et al., 2000; Alcazar et al., 2006; Kusano et al., 2008; Groppa and Benavides, 2007; Konigshofer and Lechner, 2002; Ioannidis and Kotzabasis, 2007).

The first observation of PAs involvement in plant response under adverse conditions was reported in potassium deficient barley plants that over-accumulated PUT (Richards and Coleman, 1952). Further research revealed that the ADC pathway is more active compared to the ODC pathway under stress conditions (Smith and Richards, 1964; Flores and Galston, 1984). Since then, extended investigation in a number of plant species has shown that changes in PA production is a common plant response to a variety of abiotic stresses, including salinity, chilling, heat and drought as well as biotic stresses (Bouchereau et al., 1999; Alcazar et al., 2006b; Groppa and Benavides, 2008). Additionally, recent advances indicate that the ratio of PA catabolism to PA anabolism is an important factor in PA stress tolerance (Moschou et al., 2009).

Despite the extensive research on other crops, limited information exists for cotton (*Gossypium hirsutum* L.) with the only reports being on the distribution of polyamines in the cotton plant (Bibi et al., 2011) polyamine content just prior to rapid fiber elongation (Davidonis, 1995), the effect of heat stress on PAs (Bibi et al., 2010), and the occurrences of uncommon polyamines (norspermidine, norspermine, pentamine, and hexamine) (Kuehn, et al., 1990).

EFFECT OF WATER-DEFICIT STRESS ON YIELD

Water deficit is a major cause for significant compromises in plant development and productivity around the world (Boyer, 1982). In many crops, reproductive development is the most drought-stress-sensitive period after seed germination and seedling establishment has been concluded (Saini, 1997). In cotton however, there is still debate about the most sensitive period to water-stress period during development in relation to yield, even though water sensitivity during flowering and boll development has been well established (Constable and Hearn, 1981; Cull et al., 1981a,b; Turner et al., 1986). According to Reddell et al. (1987) the early flowering period is the most sensitive to water stress, whereas Orgaz et al. (1992) concluded that water stress during peak flowering had the most detrimental effects on cotton yield. On the other hand, a number of reports (Radin et al., 1992; Plaut et al., 1992; de Cock et al., 1993) state that boll development, specifically well after the end of effective flowering, is the most water-deficit sensitive period for cotton. Additionally, in an earlier experiment Harris and Hawkins (1942) reported that delaying irrigation at fruiting could prevent yield decreases due to excessive vegetative growth. Similar results were observed from Singh (1972) who withheld irrigation until wilting was reached in the morning during the pre-flowering season and reported increased number of flowers and bolls per plant as well as increased yield. However, Stocton et al. (1961) and Lashin et al. (1970) observed that increased irrigation resulted in increased flowering. Guinn et al. (1981) concluded that a moderated water-deficit stress early in the season could be beneficial to the plants since it would mildly retard growth, however either delaying or limiting water supply could lead to negative results and these practices should be approached with caution.

Cotton is not the only crop where different opinions exist concerning the extent of sensitivity of each growth stage to water-deficit stresses. A similar confusion has also been observed in grain crops such as wheat, rice, barley and maize where all stages of reproduction (meiosis, anthesis, pollen fertility, fertilization, gametophyte fertility and zygote development) are considered to be adversely affected by water stress (Saini and Westgate, 2000). Cotton however, provides an extraordinary challenge due to its indeterminate growth pattern which results in an inability to distinguish distinct growth stages. That inability in combination with the variable ways water stress affects cotton plants, explains the lack of understanding concerning the effects of water stress on cotton seed set and development.

According to Grimes et al. (1969) there is a positive correlation between the yield and the number of bolls produced, however, the biochemical or metabolic functions affecting boll retention have not been adequately investigated. The majority of studies have concentrated on the consequences of water stress on dry matter, boll number and weight, as well as lint yield and their correlations to leaf photosynthesis and plant water relations, without any focus on the biochemical and metabolic processes of the reproductive units themselves. Guinn et al. (1976, 1981, 1984, 1988, 1990) focused mainly on the hormone metabolism of water-stressed cotton fruiting forms and specifically on the responses of abscisic acid (ABA), indole-3-acetic acid (IAA) and ethylene. They observed that water stress increased ethylene evolution from young bolls as well as their ABA content while it decreased the concentrations of free IAA. However, they were unable to conclude that any of the above hormones was the single responsible factor causing boll abscission and ultimately yield reduction. Research in other crops however, has indicated that ABA is a cause of pollen sterility in barley, wheat and rice

(Saini and Westgate, 2000). McMichael et al. (1973) also reported a strong, linear correlation between boll shedding rates and decreasing pre-dawn plant water potentials. However, they speculated that boll abscission was also controlled by endogenous factors that were dependent on plant water status, such as increased ethylene production (McMichael et al., 1972).

Lint yield is generally reduced under water-stress because of reduced boll production, primarily due to the production of fewer flowers and bolls (Stocton et al., 1961; Grimes, 1969; Gerik et al., 1996) but also because of increased rates of boll abortion when the stress is extreme and occurs during reproductive growth (Grimes and Yamada, 1982; McMichael and Hesketh, 1982; Turner et al., 1986). In addition, Pettigrew (2004) reported that the distribution of the bolls, both vertically and horizontally, was affected by water-deficit stress, with the water-stressed plants retaining more bolls at first position and producing less bolls above node n=11 compared to the control. He speculated that the reduction observed in lint yield production was due to the loss of these fruiting positions as well as reduced lint per number of seeds.

Fiber properties have been reported to be insensitive to water-deficit stress (Bennet et al., 1967; Marani and Amirav, 1971, Hearn, 1976, 1995), unless the water-deficit stress is extremely severe. Leaf water potentials of -2.8 MPa have been shown to reduce fiber length (Bennet et al., 1967). Water-deficit stress has also been reported to cause a significant reduction in fiber micronaire (Eaton and Ergle, 1952; Marani and Amirav, 1971).Timing of water-deficit stress is also a significant factor, since Marani and Amirav (1971) showed that stress early in the flowering season, had no effect on fiber quality but stress, however when the stress occured shortly after flowering it significantly decreased fiber length.. Since the extension

of the cotton fiber is a process primarily dependent on turgor (Dhindsa et al., 1975) and carbohydrate supply, the reductions in plant water status and photosynthesis that occur under conditions of water-deficit stress would result in decreases in fiber growth. This was supported by Cosgrove et al. (1993) who reported that increased volume of growing plant cells depends on the water uptake by the vacuole. However, lint yield is a function not only of fiber qualities but also a function of number of fibers/seed and number of seeds/unit area (Lewis et al., 2000). According to Rabadia et al. (1999) a strong correlation exists between plant water content and accumulation of dry matter of the developing fiber and seed which implies that rapid water uptake is required in order to support seed growth. Additionally, the number of motes (unfertilized ovules) has also been demonstrated to increase under conditions of water-stress deficit (Saranga et al., 1998) leading to further yield reduction.

EFFECT OF WATER-DEFICIT STRESS ON COTTON WATER USE EFFICIENCY

Water use efficiency (WUE) is an important parameter connecting plant biomass production with water consumption. Physiologically WUE is defined as the ratio between photosynthetic and transpirational rates, while agronomically is defined as the ratio between dry matter produced and quantity of water used. Due to the nature of its definition, high water use efficiency implies that high yield is produced under limited water conditions, hence water use efficiency has always been an alluring trait to determine and correlate with drought tolerance. However, measurements of water use efficiency are difficult to obtain and often variable. Soil and atmosphere environmental factors, such as solar radiation, temperature, humidity, CO2 ambient concentrations as well as soil type and structure and soil water

availability significantly affect water use efficiency measurements (Lin and Ehleringer, 1982; Constable and Rawson, 1980; Reich et al, 1985; Zure and Jones 1984; Reddy et al., 1995). Additionally, water use efficiency is dependent upon plant characteristics such as leaf size and position and plant structure along with management practices, such as row spacing and plant density (Rosenow et al. 1983; Krieg, 2000). Water use efficiency's variable and sometimes unreliable values are additionally attributed to the limited amount of information acquired since most water use efficiency measurements are based on single leaf measurements at certain times during the day or during the growing season. Hence, whole plant water use efficiency evaluations are mostly based on the total dry matter production and water consumption, measurements that are even more difficult to accurately measure. Farquhar et al. (1982b) reported that in C_3 plants carbon isotope discrimination is associated with the ratio between the intercellular CO_2 concentration (Ci) and the ambient CO2 concentration (Ca). Ehleringer et al. (1989, 1993) observed in a number of crops that the Ci/Ca ratio controls the $\delta^{13}C$ discrimination ratio. Water use efficiency was reported to correlate positively with the $\delta^{13}C$ discrimination ratio, providing in that way a more reliable technique for its evaluation. However, limitations of this technique have been reported since any change in Ci concentration has an effect on $\delta^{13}C$ discrimination.

Concerning cotton, a number of studies has been conducted in order to evaluate its water use efficiency. Eaton and Belden (1929) as well as Gustein (1969) reported that Acala (*G. hirsutum*) cultivars had lower water requirements compared to Pima (*G. barbadense)* cultivars under various environmental conditions. Rawson and Constable (1980) in greenhouse experiments reported that water use efficiency of individual leaves was dependent on their age

and the leaf position on the plant. In support of this observation, Wullschleger and Oosterhuis (1989) in field-grown cotton experiments observed that differences in water use efficiency on main-stem and sympodial leaves at node 10 were dependent on the leaf age as well as on the position along the branch. Quisenberry et al. (1976, 1991) conducted studies with primitive and modern cultivars with indeterminate and determinate growth habits respectively, and observed that intraspecific variation in water use efficiency was present in cotton. They also reported that primitive cultivars, characterized by indeterminate growth patterns, had much higher water use efficiencies compared to the modern determinate cultivars concluding that water use efficiency was positively correlated with the indeterminate growth habit. Radin (1992) observed that a positive correlation existed between photosynthetic rates and water use efficiency values of field grown plants and suggested that water use efficiency is dependent mainly on photosynthesis in the field. After the introduction of the carbon isotope discrimination technique, a number of studies were conducted in order to evaluate cotton's water use efficiency and it's correlation to drought tolerance and yield production. Leidi et al. (1993, 1999) in field experiments in Spain, reported a positive correlation between carbon isotope discrimination and yield, however the results were inconsistent across the years, which is in contrast with Gerik et al. (1996) who observed a consistent positive relation between carbon isotope discrimination and yield. Yakir et al. (1990) reported that no correlation existed between carbon isotope discrimination and plant productivity and its results were supported by similar findings from Saranga et al. (1998, 2005). However, Lu et al. (1996) reported a positive association between carbon isotope discrimination and stomatal conductance. Saranga et al. (2008) observed that no correlation existed between carbon isotope discrimination and yield

production under water-deficit stress conditions and concluded that water use efficiency could be used as an indicator for selection of drought tolerant genotypes but it had to be combined with other physiological parameters for more accurate results.

AMELIORATION OF WATER-STRESS DEFICIT

Alleviation of water-deficit stress through management practices such as early planting and irrigation has been known to farmers for a long time. Recent technological advances have provided scientists with a better understanding of the physiology of crops, thereby enabling them to make predictions and schedule management practices to minimize yield loss due water stress.

Plant growth regulators

Amelioration of water-stress deficit through the use of plant growth regulating substances has been suggested as a potential solution to water-deficit stress. Glycine betaine, a quaternary ammonium compound that is naturally accumulated in higher plants, has been shown to protect functional enzymes and lipids of the plant photosynthetic apparatus and maintain electron flow through thylakoid membranes (Xing and Rajashekar 1999; Allakverdiev et al. 2003). Foliar application of glycine betaine has been reported to enhance drought tolerance and yield in maize (Agboma et al., 1997a), tomato (Makela et al., 1998), tobacco (Agboma et al., 1997b), and wheat (Diaz-Zarita et al., 2001). In cotton however, there are contrasting results since Gorham et al. (2000) found that glycine betaine aids to drought

tolerance in Pakistan cotton while Meek et al. (2003) reported that foliar application of glycine betaine in Arkansas had no significant effect on yield.

Salicylic acid, a plant hormone that has been shown to increase the production of antioxidants, has also been observed to induce drought tolerance and improve yield in wheat (Singh and Usha, 2003; Waseem et al., 2007) and sunflower (Hussain et al., 2008c, 2009). Application of salicylic acid however, has yet to be tested in cotton.

PGR-IV is a plant growth regulator that contains giberrelic acid (GA) and indolebutyric acid (IBA) that has been reported to increase root growth, nutrient uptake, boll retention and lint yield of well-watered cotton (Hickey, 1992; Oosterhuis, 1995; Oosterhuis and Zhao, 1994). However, in a 4-year field study, foliar application of PGR-IV was shown to increase yield under water-deficit stress conditions (Livingston et al., 1992). Zhao and Oosterhuis (1997) conducted growth chamber experiments and indicated that application of PGR-IV before the onset of water stress could result in enhanced photosynthesis and dry matter accumulation. The increase in photosynthesis was attributed by the authors to either an increase in the nutrient absorption or improved carbohydrate translocation (Oosterhuis, 1995).

1-Methylcyclopropene, an ethylene inhibitor (Binder and Bleecker, 2003), has also been demonstrated to have a positive effect on stomatal resistance of water-stressed cotton leaves but with no significant changes in yield (Kawakami et al., 2010). On the contrary, da Costa and Cothren (2011) reported that 1-MCP had no effect on gas exchange, chlorophyll content and dry matter partitioning of water-stressed cotton plants. Accordingly, the increase in the number of reproductive nodes that was observed in 1-MCP treated water-stressed plants did not result in higher yield since 1-MCP caused higher fruit abscission (da Costa and Cothren 2011).

It would appear that the use of PGRs has the potential to ameliorate water-deficit stress in cotton production. However, there is insufficient information on the use of these chemicals for such a purpose, specifically, how they influence the metabolism to offset the adverse effect of drought and help maintain yield potential.

Selection for drought tolerant genotypes

Drought tolerance is a quantitative trait, which means that is controlled by more than one genes and has a complex inheritance. Since cotton originates from areas that are often exposed to water-deficit stress, considerable genetic variability in drought tolerance exists (Saranga et al., 1998; Pettigrew and Meredith, 1994; Quisenberry et al., 1981). Past research focused on physiological traits such as photosynthesis and stomatal conductance (Leidi et al., 1993; Nepomuceno et al., 1998; Jones et al. 1999), transpiration rates (Quisenberry et al., 1982; Leidi et al. 1993), canopy temperature (Hatfield and Quisenberry,1987; Jackson et al., 1988), Highest Specific Leaf Weight (Morey et al., 1974; Kumar et al., 1987, Lopez et al., 1995), excised leaf water loss (Roark et al., 1975; Quisenberry et al., 1982), leaf turgor maintenance (Quisenberry et al. 1983), leaf carbon isotope discrimination (Yakir et al., 1990; Saranga et al., 1998, 1999; Leidi et al., 1999), leaf and root osmotic adjustment (Wullschleger and Oosterhuis, 1987; Nepomuceno et al., 1998; Saranga et al., 2001), leaf fluorescence (Burke, 2007; Longenberger et al., 2009), water use efficiency (Quisenberry and McMicheal, 1991; Saranga et al., 1999), biomass accumulation (Quisenberry et al., 1981; Hatfield et al., 1987) , root growth and root-to-shoot ratio (Quisenberry et al., 1981; Cook, 1985; McMichael and Quisenberry, 1991), cell membrane stability (Rahman et al., 2008) and fruiting habit (Burke et al., 1985a;

Sharp and Davies, 1989; Lopez et al., 1995). However, none of the above physiological traits has so far been correlated positively and consecutively with drought tolerance. Molecular studies have also been conducted for identification of quantitative trair loci (QTLs) responsible for improved cotton production under water limiting conditions (Saranga et al., 2004, 2008) while use of genetic engineering and transgenic plants has been shown to result in helpful correlations (Lv et al., 2007; Parkhi et al., 2009).

SUMMARY

Water-deficit stress has a significant effect on cotton's growth and development, by primarily affecting the plant's structure, leaf morphology and cell ultrastructure. Physiological processes such as stomatal conductance, photosynthesis and respiration are consequently impaired with further implications on the metabolic functions such as carbohydrate and energy production as well as carbohydrate translocation and utilization. Even though cotton possesses mechanisms to anticipate the negative effects of water-deficit stresses (accumulation of antioxidants, osmolytes and heat shock proteins) their protective capacity depend not only on the extent of the stress, but also on the timing of the stress as well as the progression rate of the stress occurs (sudden or gradual). Yield reductions and fiber quality compromises are inescapable when water-deficit stress conditions override the plant's protective mechanisms.

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CHAPTER I
EFFECT OF WATER-DEFICIT STRESS ON METABOLISM OF THE COTTON FLOWER

ABSTRACT

Flowering in cotton (*Gossypium hirsutum* L.) is a sensitive stage to water-deficit stress, but the effects on metabolism are not well understood. The objective of this study was to monitor gas exchange responses of cotton plants under conditions of limited water supply and evaluate the effects on the carbohydrate and antioxidant metabolism of the cotton flower. Growth chamber experiments were conducted in 2008 and 2009. A widely used cultivar, ST5288B2F, was planted in 1L pots filled with a horticulture mix arranged in a completely randomized design. The growth chamber was set for normal day/night conditions of 32/24^{o}C and optimum quantities of Hoagland's nutrient solution were applied until flowering. Eight weeks after planting, plants were separated in two groups; one group received optimum quantities of water (control), while water supply was withheld from the other group (water-stress) until visual wilting point was reached, after which 50% of daily water use was supplied. Water-deficit stress resulted in a significant decrease in leaf stomatal conductance. Leaf photosynthetic and respiration rates were similarly decreased compared to the control. Ovary and style water potential of water-stressed leaves were significantly higher compared to the water potential of water stressed leaves, indicating that cotton flowers are fairly resistant to changes in the water status of the plant. However, carbohydrate concentrations of water-stressed pistils (ovary and style) were significantly increased compared to the control and a similar pattern was observed on the levels of glutathione reductase of water-stressed pistils. In conclusion, water-deficit stress during flowering resulted in significant decreases in leaf gas exchange functions as well as leaf water potential. Cotton pistils appeared to be less sensitive since they were able to maintain water potential similar to the control under limited water

supply and increase glutathione reducactase levels. However, pistil carbohydrate metabolism was significantly affected resulting in accumulation of both hexose and sucrose indicating a perturbation in sucrose cleaving and hexose utilizing enzymes that could potentially have as a consequence a compromise in fertilization and seed set efficiency.

INTRODUCTION

Water-deficit stress is a major abiotic factor compromising plant growth and productivity in more than 30% of cultivated areas around the world (Boyer, 1985; Masacci et al., 2008). Cotton (*Gossypium hirsutum* L.) a perennial with indeterminate and complex growth pattern has been described as relatively drought tolerant due to its origin from hot and arid areas (Lee, 1984). As a result cotton plants are able to mitigate the negative effects of water-stress through a variety of defense mechanisms such as leaf and root osmotic adjustment (Oosterhuis and Wullschleger, 1987; Nepomuceno et al., 1998), accumulation of compatible osmolytes (Kuznetsov et al., 1999), heat shock protein production (Burke et al., 1985a) or high water use efficiency (Ackerson et al., 1977b). Nevertheless, due to its domestication and cultivation as an annual crop, the effectiveness of those mechanisms has been highly compromised (Quisenberry and Roark, 1976), ultimately resulting in significant yield losses (Basal et al., 2005) since cotton's physiological and metabolic functions are rendered vulnerable against water stress. Past research has indicated that cell expansion, plant height and leaf area index are significantly decreased under limited water supply (Pace et al., 1999) while additionally, significant decreases in leaf photosynthetic rates due to stomatal or non-stomatal limitations have been observed (McMichael and Hesketh, 1982; Turner et al., 1986; Faver et al.,

1996, Leidi et al., 1999). Furthermore, variable results have been reported on leaf respiration activity of water-stressed plants, with Pallas et al. (1967) describing a biphasic response with an initial decrease in leaf respiration rates at moderate water stress and an increase under more severe stress, while similarly variable results have been described for leaf carbohydrate levels (Eaton and Ergle, 1948; Ackerson et al., 1981; Timpa et al., 1986; Parida et al., 2007). On the other hand, differential responses have been reported for different antioxidants with leaf glutathione reductase activity either increasing (Burke et al., 1985b) or remaining unaffected from limited water supply (Mahan and Wanjura, 2005; Kawakami et al., 2010). Conversely, ascorbate perosidase activity was noted to be higher in water-stressed leaves (Mahan and Wanjura, 2005) while the opposite was observed for superoxide dismutase (Kawakami et al., 2010).

As it is shown above, cotton's plant growth as well as physiology and metabolism are greatly affected under conditions of water stress. Even though, considerable debate still exists on the developmental stage that cotton is most susceptible to drought (Singh, 1975; Reddell et al., 1987; Orgaz et al., 1992; Radin et al., 1992; Plaut et al., 1992; de Cock et al., 1993) cotton's high sensitivity to water stress during flowering and boll development is well established (Constable and Hearn, 1981; Cull et al., 1981a,b; Turner et al., 1986). Despite that, little attention has been given on the effects of water-deficit stress on the physiology and metabolism of cotton's reproductive units and especially flowers with the only information existing being on petal water potential (Trolinder et al., 1993) and flower hormonal balance (Guinn et al., 1990). In more detail, Trolinder et al. (1993) conducted field studies where plants were subjected to mild and severe limited water conditions. They reported that petal water

potential of water stressed plants was significantly higher compared to the leaves. In addition, it was observed that even though petal water potential varied in accordance with plant water status, due to the direct vascular connection between the petals and the plant stem, the water potential gradient the petals required for their expansion did not exist. They speculated that this inverted gradient could be attributed to metabolic reasons as for example a rapid solute breakdown. However, further investigation under conditions that restrained metabolic activity resulted in the same inverted gradient (Trolinder et al., 1993). Additionally, Guinn et al. (1990) conducted field experiments where plants were subjected to two cycles of water stress and flowers were collected the day of anthesis in order to investigate the effect of limited water supply on abscisic acid (ABA) and indoloacetic acid (IAA) concentrations. ABA levels of water-stressed flowers were increased compared to the control, while after irrigation ABA levels decreased. Conversely, water-deficit stress had a minimal effect on IAA concentrations, resulting in increased levels of conjugated IAA in water-stressed flowers, whereas free IAA concentrations of water-stressed flowers were similar to those of control. The authors speculated that the lack of an effect of water-deficit stress on the levels of free IAA was due to the small increase in ABA levels of the flowers.

Carbohydrate metabolism of plant reproductive units has been shown to be greatly affected under conditions of water-deficit stress resulting in soluble sugar accumulation (Westgate et al., 1989; Schussler and Westgate, 1995; Sheoran and Saini, 1996; Westgate et al. 1996; Lalonde et al. 1997; Setter et al., 2001a,b; Liu et al., 2003; 2004; Nguyen et al., 2010). Carbohydrate accumulation in water-stressed leaves has been reported to be an adaptation mechanism under stress conditions in a variety of crops (Bray, 1997), however in reproductive

units increases in carbohydrate concentrations have been reported to result in ovary abortion (Westgate et al., 1989; Zinselmeier et al., 1995, 1999; Schussler and Westgate, 1995; Andersen et al., 2002; Liu et al., 2003, 2004) or anther sterility (Lalonde et al. 1997; Sheoran and Saini, 1996; Westgate et al. 1996; Setter et al., 2001a,b; Nguyen et al., 2010; Fu et al., 2010). Furthermore, the ratio of hexose to sucrose concentrations has been reported to control cell division during early reproductive development (Weber et al., 1998). In support of that observation, a low hexose-to-sucrose ratio under conditions of water stress has been suggested as the reason for failure of kernel set in maize (Andersen et al., 2002) and pod abortion in soybean pods (Liu et al., 2004).

In addition to their role as substrate for growth of sink tissues, hexose and sucrose act as signaling molecules modulating a variety of metabolic functions in the plant (Koch, 1996; Couee et al., 2006; Gazzarini and McCourt; 2001) including the reactive oxygen species scavenging system (Couee et al., 2006; Cruz de Carvalho, 2008), while sucrose itself as a scavenger as well due to its antioxidant capability (Smirnoff and Cumbes, 1989). According to May et al., (1998) and Noctor and Foyer (1998), high levels of sugars are responsible for the maintenance of cellular nicotinamide adenine dinucleotide phosphate (NADPH) concentrations, which is an important precursor for the antioxidant substrates such as ascorbic-acid and glutathione in the ascorbate-glutathione cycle. Glutathione reductase catalyzes the rate limiting step of the ascorbate-glutathione cycle, and utilizes NADPH to reduce oxidized glutathione (GSSG) to two molecules of reduced glutathione (GSH) in order to regulate intracellular redox potential (Foyer and Noctor, 2005) and has also been shown to have a critical role in embryo and meristem development (Cairns et al., 2006; Reicheld et al., 2007; Frottin et a., 2009).

Furthermore, studies in other crops have associated high levels of reactive oxygen species with ovary or ovule abortion (Sun et al., 2004; Hauser et al., 2005), whereas higher concentrations of glutathione reductase have been reported to decrease male sterility in water-stressed rice anthers (Selote and Khanna-Chopra, 2004, Fu et al., 2010). In addition, associations between abscisic acid (ABA) and glutathione reductase have been reported with increasing ABA concentrations enhancing glutathione reductase activity in reproductive tissues (Guan and Scandalios, 2000; Jiang and Zhang, 2002; Hu et al., 2005). Guinn et al. (1990) reported increases in concentrations of ABA in water-stressed white flowers, but glutathione reductase levels were not.

Overall, it is apparent that the carbohydrate and antioxidant metabolism of reproductive units is greatly affected under conditions of limited water supply in other crops, however, no information exists for cotton. Hence, the objectives of our study was to monitor gas exchange responses of cotton plants under conditions of limited water supply and evaluate the effect of those conditions on the carbohydrate and antioxidant metabolism of the cotton flower. It was hypothesized that water-deficit stress during flowering would severely impair leaf gas exchange that would decrease carbohydrate content of the reproductive units and increase glutathione reductase levels.

MATERIALS AND METHODS

Cotton (*Gossypium hirsutum* L.) cv. ST5288B2F was planted in September 2008 at the Altheimer Laboratory, University of Arkansas into 1 L pots containing Sunshine potting media mix #1 (SunGro Distribution Inc., Bellevue, WA). The growth chambers (Conviron PGW36, Conviron Inc., Winnipeg, Canada) were equipped with incandescent and fluorescent lamps and

set for a 14-h photoperiod with a photosynthetic photon flux density (PPFD) of 800-850 µmol/m²s and a relative humidity of 60%. Cotton was grown under normal day/night temperatures of 32/24°C and all pots received half-strength Hoagland's nutrient solution daily to maintain adequate nutrients and water. At flowering (approximately 8 weeks after planting) plants were separated in two groups; control group received optimum quantity of water while irrigation was withheld from water-stress group until plants reached visual wilting point (5 mmol H_2O/ m²s) after which plants received 50% of their daily use of water for ten days.

Stomatal conductance measurements

Stomatal conductance measurements were taken the first, fifth and tenth day into the stress from the fourth uppermost main-stem leaf (n=15) from 11 :00 a.m. (five hours after the lights in the growth chambers came on) until 1:00 p.m. using a Decagon SC-1 Porometer (Decagon Inc. , Pullman, WA). Day 0 of the experiment was the day water-stressed plants had reached visual wilting point after which 50% of daily quantity water used was applied. Due to the small surface area of the cuvette (6.25mm^2), three measurements on various areas of the leaf were taken and then averaged. The results were expressed as mmol/m²s.

Gas exchange measurements

A Li-Cor Model 6200 portable photosynthesis system (LICOR Inc., Lincoln, NE) was used to determine photosynthetic and respiratory rates for the attached, fourth main-stem leaf (n=15) from the terminal of the plant. Measurements of photosynthesis and respiration were taken at 12:00 pm and 1:00 pm, respectively the fifth and tenth day of the stress and the results

were expressed as µmol/m²s. Incandescent and fluorescent lights were turned off in the growth chamber ten minutes before the measurements of respiration were taken and the plants were covered with an opaque cloth during the measurements.

Water potential measurements

Water potential values of leaves and pistils were determined with screen-caged thermocouple psychrometers (model 74 series, J.R.D. Merrill Specialty Equipment, Logan, UT) equipped with stainless steel sample chambers using the technique described by Oosterhuis (1987). Leaf and pistil tissues were excised and sealed in the sample chambers within 10 sec to avoid dehydration of tissues. Water potential was recorded after a 4-h equilibration period in an isothermal water bath that kept the temperature constant at 25°C. Water was condensed on the measuring junction by applying a 5 mA current for 15 sec, and the voltage output was monitored continuously with a micro-voltmeter and chart recorder.

Carbohydrate measurements

Soluble carbohydrate content was measured according to a modification of the Hendrix (1993) protocol. White flowers were sampled at 12:00 pm from control and water-stressed plants during the 10-day stress period. The pistils were excised and were oven dried for 3 days at 50°C and then ground with a mortar and pestle. Forty mg of the ground tissue were extracted 3 times with 80ºC aqueous ethanol (800 ml ethanol /L) and the samples were centrifuged after each extraction at 5000 rpm and finally the fractions were pooled. Active charcoal was then added to the pooled fractions in order to remove substances that could

interfere with the carbohydrate measurements and the samples were centrifuged again at 3500 rpm. The supernatant was immediately stored at -80^{o}C for further determination of sucrose and hexose (fructose and glucose) with a MultiScan Ascent Microplate Reader (Thermo Fisher Scientific Inc., Waltham, MA). The glucose (HK) assay kit from Sigma (Sigma Chemical Company, St Louis, MO) was used. A 20 µl aliquot of each extract was pipetted into a well of a microtitration plate and the plate was incubated at 50^{o}C for 40 min to evaporate ethanol. 10 µl of water were then added to each well along with 100 µl of glucose assay reagent and the plate was incubated again for 15 min at 30^{o}C. The absorbance was measured three times at 340 nm using a microplate reader. 0.25 EU of phosphoglucose isomerase was added to the extracts in each well of the plate and the absorbance was again measured at 340 nm. Subsequently, 83 units of invertase were added to the extracts and the microtitration plate was incubated at 30^{o}C for 60 min. The absorbance was measured three times at 340 nm and the results were expressed in mg carbohydrate/mg dry weight with the help of a standard curve made of known glucose concentrations.

Glutathione reductase measurements

Glutathione reductase activity was measured in pistils from sampled white flowers according to Anderson et al. (1992), with modification. Pistil tissue was homogenized using a mortar and pestle in an ice-cold extraction solution (10x pistil fresh weight) comprised of 50 mM PIPES (1,4-Piperazinediethanesulfonic acid) buffer (pH 6.8), 6 mM cysteine hydrochloride, 10 mM D-isoascorbate , 1 mM ethylenediaminetetraacetic acid, 0.3% Triton X-100 and 1% (w/v) soluble Polyvinylpyrrolidone (PVP). Solutions were further blended for 1 min in a tube

containing 0.25 g insoluble PVP and 1 drop of antifoam A emulsion using a homogenizer (Model Polytron; Brinkman Instruments Inc., Palo Alto, CA). Subsequently, samples were centrifuged at 21 000 g for 20 min (4°C) and the supernatants were stored at −80°C for further determination of glutathione reductase content according to Shaedle and Bassham (1977), with modification. To each well of a 96-well microtitration plate, a 15.7 µl aliquot of enzyme extract from each sample was added to a 300 µl reaction solution containing 50 mM Tris-HCl buffer (pH = 7.5), 0.15 mM reduced nicotinamide adenine dinucleotide phosphate (NADPH), 0.5 mM oxidized glutathione, and 3 mM $MgCl_2$. Oxidation of NADPH was determined as the decrease in absorbance at 340 nm during a 1 min reaction time was measured during a 1 min reaction time using an Ascent Multiscan microplate reader (Molecular Devices Corporation, Sunnyvale, CA), and glutathione reductase activity was expressed as GR units/g fresh weight.

Statistical analysis

The experimental design was a completely randomized design with twenty replications in each treatment. The trends of the data of the two growth chamber studies were similar so the results were pooled and the means were taken. Analysis of variance was performed using the Student's t-test (JMP8 software, SAS Institute, Cary, NC) for the comparison of data. Means were considered significantly different at $\alpha \leq 0.05$.

RESULTS

Leaf stomatal conductance

Stomatal conductance rates of the fourth main-stem leaf from the terminal was significantly decreased under conditions of water-deficit stress (Fig. 1). Day 1 of the experiment indicates the day after plants had reached visual wilting point and had been watered with 50% of the daily use of water quantity. Time was not considered as a factor and the results of each day were analyzed using a Student's t-test at $\alpha \leq 0.05$. Stomatal conductance rates of water-stressed plants were significantly lower compared to the control ($P \leq 0.001$) for day one, five and ten of the experiment. Rates of stomatal conductance of plants receiving 50% quantity of daily use water quantity were at 50.1, 38.7 and 36 mmol H_2O/m^2s the first, fifth and tenth day, respectively, while for plants receiving optimum quantities of water stomatal conductance rates were 220.9, 227.3 and 291.2 mmol H_2O $/m^2s$.

Photosynthesis and respiration

Leaf photosynthetic and respiratory rates of water-stressed plants were significantly decreased compared to control. Photosynthesis and respiration rates were determined from fifteen replications from each treatment and a two factor factorial design (main factors being water regime and days). The trends for the fifth and tenth day were similar and the results were pooled across the dates and analyzed using a Student's t-test at $\alpha \leq 0.05$. Leaf plotosynthesis of water-stressed plants was significantly lower compared to the control (Fig.2), with leaf photosynthetic rates of water-stressed plants being nearly 40% lower compared to

the control. A similar pattern was observed in the respiration activity of leaves (Fig. 3), with leaf respiratory rates being nearly 39% decreased compared to the control.

Water potential

Tissue water potential was determined form leaves and first position white flowers collected the ninth and the tenth day of the experiments. Eight flowers and their respective fourth-main stem leaves from each treatment were randomly chosen and the effect of water regime on leaf and pistil water potential was determined by using a Student's t-test at $\alpha \leq 0.05$. The trends for both days were similar, the results were pooled and the means were taken. Analysis of variance was performed using a Student's t-test at $\alpha \leq 0.05$. Limited water supply resulted in a nearly 50% decrease of leaf water potential (Fig. 4), whereas pistil water potential remained unaffected. Additionally, pistil water potential (Fig. 5) was at similar levels as leaf water potential of the control plants.

Total soluble carbohydrate content

Carbohydrate content of the pistils was determined from 25 replications collected from each treatment whenever they were available and the effects of water-deficit stress on carbohydrate concentrations were estimated using a Student'-t test at $\alpha \leq 0.05$. Water-stressed pistils contained significantly higher concentrations of hexose (Fig.6) ($P=0.0075$) as well as sucrose (Fig. 7) ($P=0.0033$) compared to pistils from plants receiving optimum quantity of water. Specifically, water-deficit stress resulted in a 14% and 64% higher accumulation of hexose and sucrose, respectively, in pistils.

Glutathione reductase levels

Glutathione reductase activity of the pistils was measured from 15 replications collected from each treatment whenever they were available and the differences between treatments were analyzed using a Student's-t test at $\alpha \leq 0.05$. Similarly to carbohydrate results glutathione reductase activity (Fig.8) of water stressed pistils was significantly higher ($P=0.0011$) compared to the control. However, the magnitude of the increase was higher with water-stressed pistils containing 94% more glutathione reductase compared to the control.

DISCUSSION

Stomatal conductance of water-stressed plants was significantly lower compared to the control in our experiment which is in accordance with Harris (1973) as well as Bielorai et al. (1975) who in experiments with potted plants reported that water-stressed cotton plants had significantly lower stomatal conductance rates compared to the control. Similar results were also reported by Wullschleger and Oosterhuis (1990), Kawakami et al. (2010) and da Costa and Cothren (2011) again with potted plants grown under controlled environmental conditions, however, Ackerson et al. (1977a) observed that stomata of field- grown cotton plants did not completely close even under very low water potential values and stomatal conductance was only slightly affected. We speculate that the contrasting to our results, the response reported by Ackerson et al. (1977a) is due to differences in light intensity between the growth chambers and field, as well as a slower imposition of water stress under field conditions allowing acclimation.

Similarly to stomatal conductance rates, leaf photosynthetic rates of water-stressed plants in our study were significantly lower compared to the control. Previous research in cotton has indicated that water-deficit stress, imposed at any developmental stage and at various intensities, results in great depression of leaf photosynthetic rate (Pallas et al., 1967; McMichael and Hesketh, 1982; Turner et al., 1986; Sung and Krieg, 1986; Genty et al., 1987; Ephrath et al., 1990; Wullschleger and Oosterhuis, 1990; Faver et al., 1996; Leidi et al., 1999; da Costa and Cothren, 2011) due to a combination of stomatal and non-stomatal limitations. Pettigrew (2004) described a biphasial response of leaf photosynthetic rates in field-grown cotton where leaf photosynthesis was initially increased in the morning and decreased in the afternoon. He speculated that this biphasial response could be attributed to the hydraulic conductivity of the soils in the Mississippi Delta that allowed the plants to rehydrate during the night and recover their photosynthetic activity in the morning. Our results are in agreement with Leidi et al. (1999) and da Costa and Cothren (2011) who subjected plants to water stress at flowering under field and greenhouse conditions, respectively, as well as with the majority of the previous conducted research that show leaf photosynthesis to be greatly reduced under conditions of water-deficit stress, however whether these decreases were solely due to stomatal limitations or due to a combination of stomatal and metabolic inhibitions is not known.

Photosynthesis is unavoidably connected with respiration since their balance ultimately determines plant growth and productivity (Lambers et al., 1998). In our study leaf respiration rates decreased similarly to photosynthesis, however the observed decreases were smaller than those reported in photosynthetic rates. This is in accordance with the observation by

Boyer (1970) that photosynthesis is more sensitive than respiration under conditions of water stress. In addition, even though rates of photosynthesis determine respiration rates under well-watered conditions (Noguchi, 2005), it has been suggested that this is not the case under conditions of water stress, where respiration seems to be dependent more on plant growth rate rather on photosynthetic rate and availability of assimilates (Wilson et al., 1980).

Decreased leaf respiration rates under conditions of limited water supply has been reported by a number of researchers (Palta and Nobel, 1989; Gonzalez-Meler et al., 1997; Escalona et al., 1999; Haupt-Herting et al., 2001), while others have described it to remain unaffected (Lawlor, 1976; Loboda et al., 1993) or even increase (Shearman et al., 1972; Zagdanska, 1995). Furthermore, Flexas et al. (2005) suggested that respiration's response is largely determined by the intensity and the duration of stress, decreasing until a certain stress threshold has been reached and increasing after that. Pallas et al. (1967) reported a biphasial response for cotton where leaf respiration of water-stressed plants initially decreased and then increased under conditions of more severe stress. On the contrary, Wullschleger and Oosterhuis (1990) reported that boll respiration remained unaffected under conditions of mild water stress and significantly decreased once the stress became more severe, however this differential response could be attributed to the different type of tissues investigated. Our results are in agreement with Palta and Nobel (1989), Escalona et al. (1999), and Haupt-Herting et al. (2001), while we assume that the differential responses reported by Pallas et al. (1967) are due to either the different growth stages that plants were subjected to stress in their study or the different duration and severity of stress in their experiments.

Despite cotton's ability to maintain leaf turgor under conditions of water stress through osmotic adjustment (Turner et al. 1986; Nepomuceno et al., 1998), leaf water potential of water-stressed plants was significantly lower compared to the control in accordance with Ackerson et al. (1977), Trolinder et al. (1993), Kawakami et al. (2010) and da Costa and Cothren (2011). On the contrary, ovary water potential of water-stressed plants remained unaffected and significantly higher compared to the water potential of the leaves showing cotton flowers to be relatively insensitive to the water status of the plant. In support of our observations Wullschleger and Oosterhuis (1990) reported that capsule wall water potentials of bolls varying in age and subjected to water stress were similar to the control and significantly higher than leaf water potential values. In addition, Trolinder et al. (1993) reported that even though water potential of petals of water-stressed field-grown cotton plants fluctuated similarly with plant water status, their water potential values remained unaffected compared to the control and higher than the leaves. They speculated that metabolic reasons, such as rapid breakdown of solutes, could be the reason for this inverted gradient, however further research by Van Iersel and Oosterhuis (1995, 1996) concluded that cotton reproductive units are apoplastically isolated due to fruit xylem connections being immature until three weeks past anthesis, and water potential gradient is directed from the fruits to the leaves since the main entrance of water in cotton reproductive units is through the phloem. Insensitivity of reproductive units to water stress has been reported for wheat as well as rice and maize (Tsuda and Takami, 1993; Dorion et al., 1996; Sheoran and Saini, 1996; Westgate et al., 1996; Saini, 1997). However, unlike cotton, immunity of floral organs of wheat, rice and maize was only evident if water-stress conditions were imposed on the plants prior to meiosis and this was attributed either to

the limited transpiration rates of the floral organs or to the hydraulic isolation between the floral stalk and the pericarp. Water stress at anthesis resulted in significant decreases in panicle and silk water potential in rice and maize, respectively (Tsuda and Takami, 1993; Westgate and Boyer, 1985b). It was suggested that increased transpiration rates from the exposed tissues should be the reason for those lower water potential values. Further research with covered rice panicles however, revealed that transpiration was not the driving force for those lowered water potentials (Garrity et al., 1986) and flower sterility still occurred indicating that other functions were affected.

One of the metabolic functions that have been reported to be greatly affected under conditions of water-deficit stress is carbohydrate metabolism of plant reproductive structures. Increased sucrose concentrations due to down-regulation of sucrose cleaving enzymes, such as invertase and sucrose synthase, under low water potentials have been described in a number of crops (Zinselmeier et al., 1995, 1999; Sheoran and Saini, 1996; Setter et al., 2001a,b; Nguyen et al., 2010), while variable results have been reported for hexose response. Higher than control concentrations of hexoses have been linked to decreased activity of enzymes that participate in the glycolysis cycle, a major provider of energy in the cells (Nguyen et al., 2010), while lower than control hexoses concentrations have been considered responsible for failed kernel set in maize (Andersen et al., 2002) and abortion of soybean pods (Liu et al., 2003) due to the inhibitory effect on cell division of a low hexose to sucrose ratio (Weber et al., 1998). In our study, both hexose and sucrose concentrations of water-stressed pistils were significantly higher compared to the control and our results are in accordance with Lalonde et al. (1997) in wheat, Setter et al. (2001a,b) in maize, Sheoran and Saini (1996), Nguyen et al. (2010), Fu et al.

(2010) in rice as well as Liu et al. (2003, 2004) in soybean. However, unlike the results in other crops, increased sugar concentrations in our study occurred at pistil water potential levels similar to the control leading us to assume that accumulation of sugars might be the physiological response to maintain water potential levels through osmotic adjustment. Nevertheless, an impairment of carbohydrate enzyme activity should not be excluded since Chang and Ryan (1987) reported significant increases in sucrose concentrations of water-stressed leaves, indicating in addition that sucrose synthase activity remained unaffected from water stress whereas sucrose-phosphate synthase activity was significantly increased. Enzymes involved in carbohydrate metabolism have been reported to have diverse responses depending on the type of tissue (Roitsch, 1999; Roitsch et al., 2000; Rolland et al., 2002; Koch et al., 2004) leading us to speculate that the increased sucrose concentrations observed under water deficit stress could be the result of significant reductions in sucrose cleaving enzymes activity in the cotton pistil. Similarly, increased hexose concentrations could be attributed to lack of utilization through glycolysis, which is further supported by the findings of Wullschleger and Oosterhuis (1990) that capsule wall respiration rates were decreased under conditions of water stress. Further research is needed in order to elucidate whether impairment of carbohydrate enzyme function is also responsible for accumulation of carbohydrates in cotton's reproductive units.

A similar pattern to the increasing carbohydrate concentrations was observed in pistil glutathione reductase, with its activity being significantly higher in the water-stressed pistils compared to the control. Water-deficit stress is intimately connected with increased oxidative stress as a result of higher production of reactive oxygen species (ROS) (Gill and Tuteja, 2010; Miller et al., 2010) and plants are able to prevent damage to cellular components due to a

highly regulated and rapidly responsive antioxidant system, comprising of enzymatic and non-enzymatic mechanisms, that balances production and scavenging of ROS (Miller et al., 2010). Similar increases in glutathione reductase activity in reproductive structures under conditions of water-deficit stress have been observed in rice anthers by Selote and Khanna-Chopra (2004) and Fu et al. (2010) and those increases were associated with enhanced drought tolerance, since only the drought-tolerant varieties in their studies exhibited increased glutathione reductase levels while the opposite was observed in the drought-sensitive varieties. In agreement with our results, Burke et al. (1985b) observed that glutathione reductase activity was consistently higher in leaves of cotton plants grown under dryland conditions compared to the control, however, Mahan and Wanjura (2005) and Kawakami et al. (2010) reported that glutathione reductase activity of water-stressed leaves was unaffected, while ascorbate peroxidase and superoxide dismutase activities were increased and decreased, respectively. Their results on glutathione reductase activity are in contrast with the results of our study, and we speculate that this is either due to the different growth stages that stress was applied in their studies since glutathione reductase activity has been shown to fluctuate during the season (Burke et al., 1985b) or the ways the stress was imposed.

In conclusion, water-deficit during flowering in cotton resulted in compromises in leaf physiology with stomatal conductance, photosynthesis as well as respiration rates being significantly decreased compared to the control. Similarly, leaf water potential was lower compared to the control showing that the osmotic adjustment mechanism of cotton was unable to maintain the water potential gradients under conditions of stress. On the contrary, pistil water potential of water-stressed plants was similar to those of the control and

significantly higher compared to the leaves. Higher concentrations of sucrose and hexose were found in water-stressed pistils compared to the control which could justify the ability of reproductive units to maintain a high water potential under conditions of stress. However, increases in glutathione reductase activity of water-stressed indicate metabolical implications in cotton flower's response to limited water supply. Evidence of cross-talking between carbohydrate and antioxidative metabolism has been reported for other crops (Koch 2004; Couee et al., 2006, den Ende and Valuru, 2009) suggesting for further investigation on the activity of sucrose cleaving enzymes as well as glycolysis enzymes in order to elucidate whether metabolic inhibitions are implicated in the sugar and antioxidant accumulations observed under water deficit stress.

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FIGURES

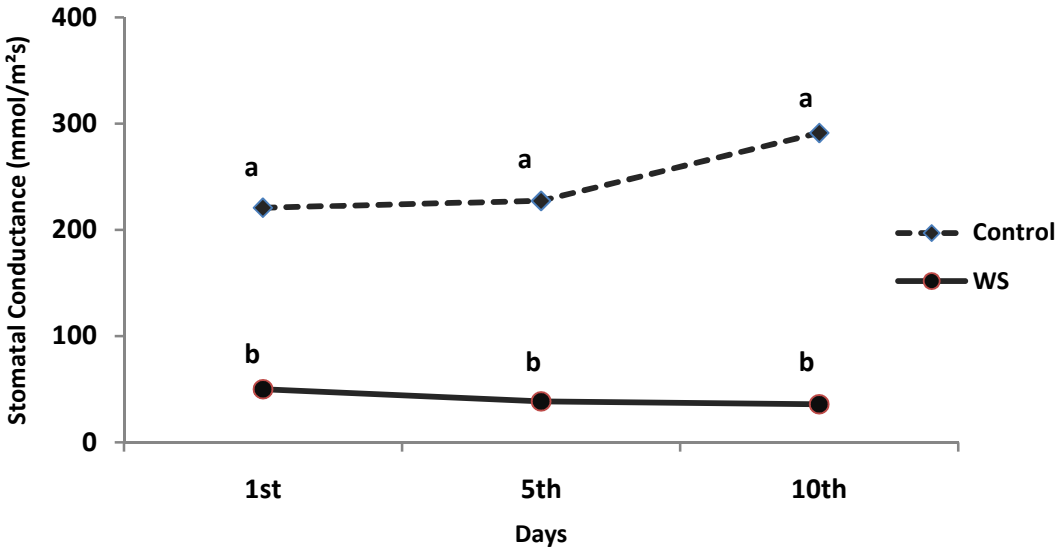

Figure 1: Effect of water-deficit stress on leaf stomatal conductance. Points with the same letter are not significantly different (P=0.05).

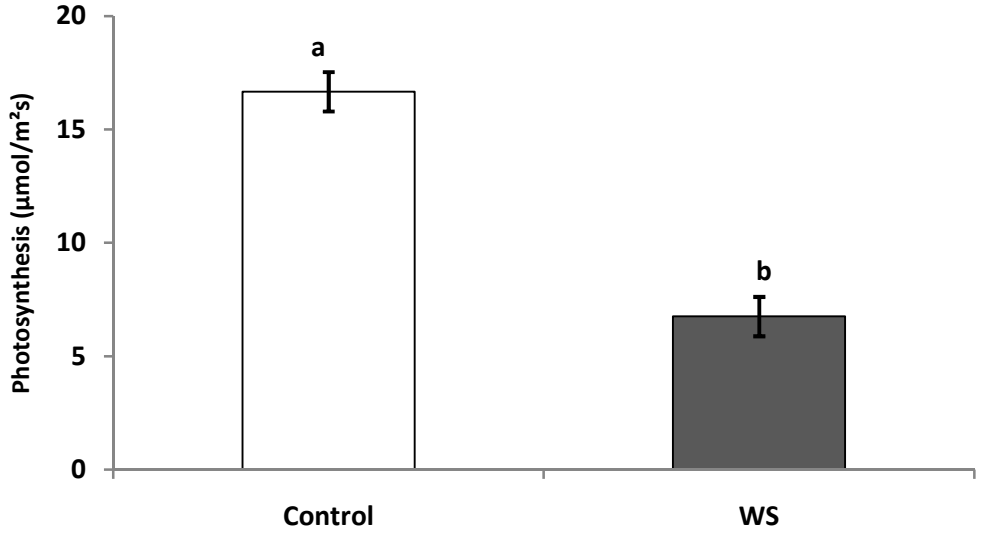

Figure 2: Effect of water-deficit stress on leaf photosynthetic rates. Columns with the same letter are not significantly different (P=0.05). Error bars represent ±1standard error.

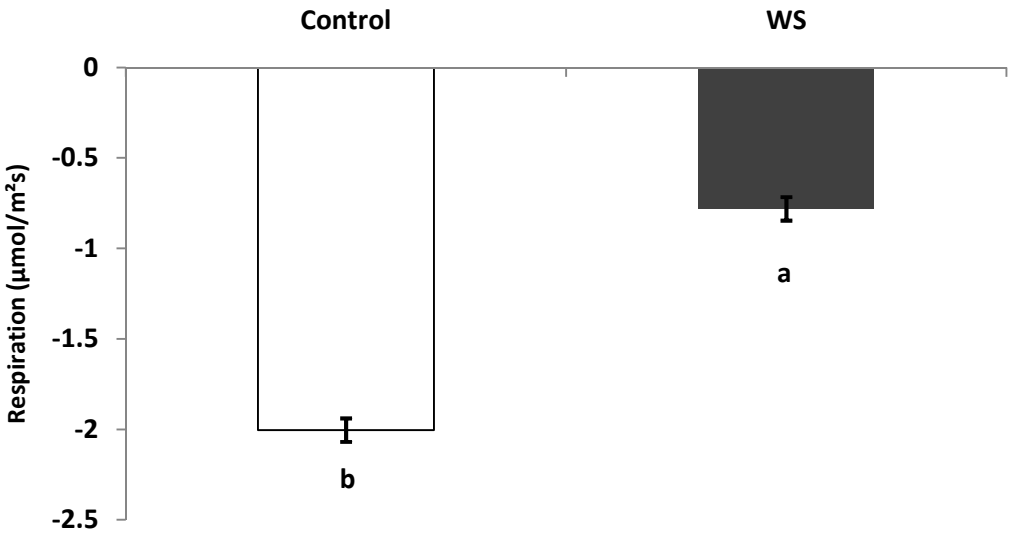

Figure 3: Effect of water-deficit stress on leaf respiration rates. Columns with the same letter are not significantly different (P=0.05). Error bars represent ±1 standard error.

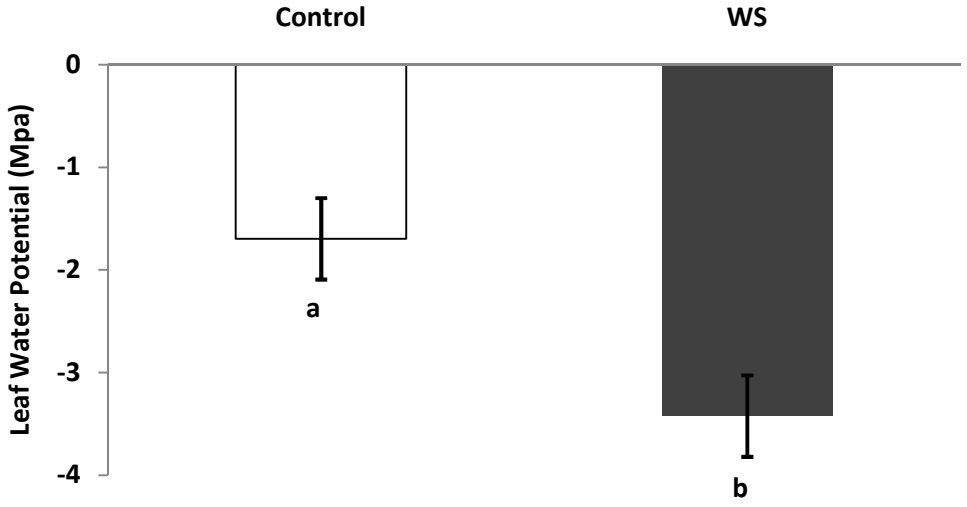

Figure 4: Effect of water-deficit stress on leaf water potential. Columns with the same letter are not significantly different (P=0.05). Error bars indicate ±1 standard error.

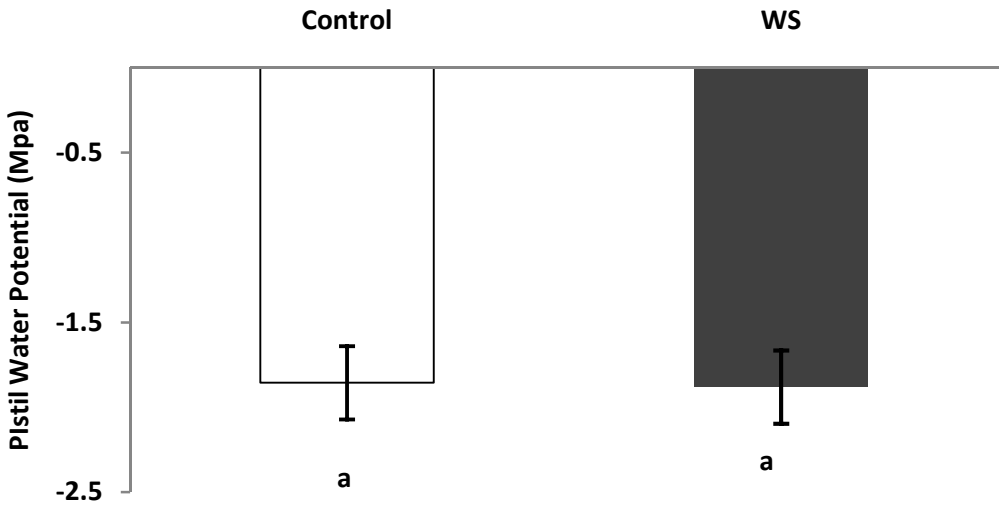

Figure 5: Effect of water-deficit stress on pistil water potential. Columns with the same letter are not significantly different (P=0.05). Error bars indicate ±1 standard error.

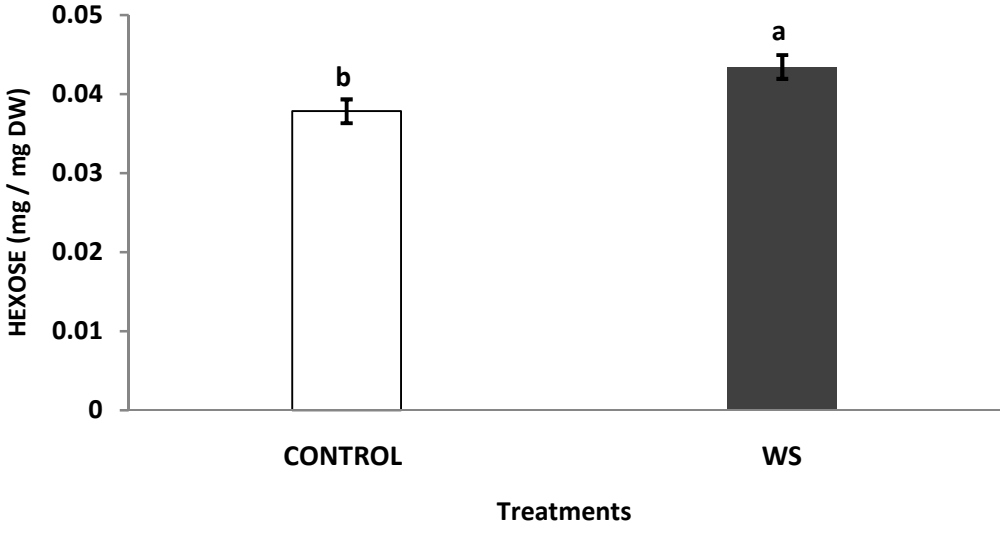

Figure 6: Effect of water-deficit stress on pistil hexose concentrations. Columns with the same letter are not significantly different (P=0.05). Error bars indicate ±1standard error.

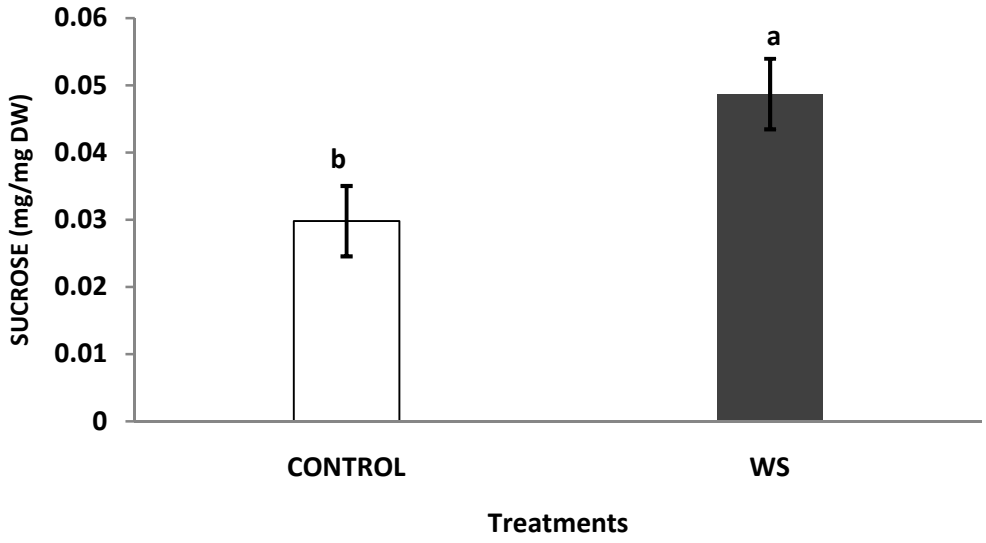

Figure 7: Effect of water-deficit stress on pistil sucrose concentration. Columns with the same letter are not significantly different (P=0.05). Error bars indicate ±1 standard error.

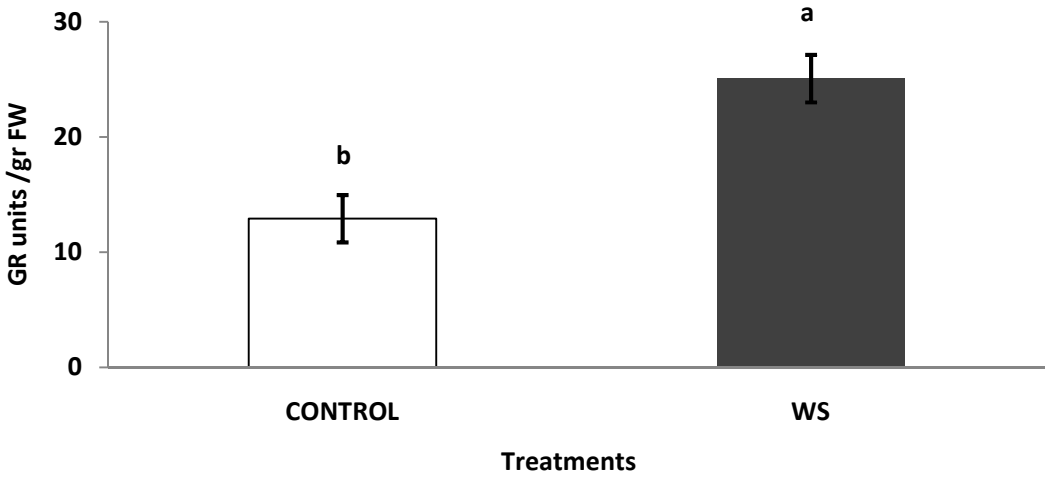

Figure 8: Effect of water-deficit stress on pistil glutathione reductase levels. Columns with the same letter are not significantly different (P=0.05).

CHAPTER II

EFFECT OF WATER-DEFICIT STRESS ON THE BIOCHEMISTRY OF THE COTTON FLOWER AND ITS SUBTENDING LEAF DURING REPRODUCTIVE DEVELOPMENT

ABSTRACT

Cotton (*Gossypium hirsutum* L.) growth and yield are greatly compromised under conditions of water-deficit stress. Although debate still exists on the most drought-sensitive developmental stage of the plant, it is generally accepted that limited supply of water during flowering results in significant yield reductions. However, the metabolism of reproductive units under conditions of water stress is largely unknown. A field study was conducted in 2011 in two locations (Fayetteville, AR, and Lubbock, TX) in order to investigate the effect of water-deficit stress during flowering on carbohydrate, glutathione reductase and polyamine metabolism of the cotton flower and its subtending leaf. Treatments consisted of control (well watered) and water-stress (irrigation was withheld for two weeks at the onset of flowering) in a split plot design. First position white flowers and their subtending leaves were collected at the end of each week of the stress period and used to determine carbohydrate levels, glutathione reductase activity and polyamine concentrations. Water-deficit stress resulted in significant increases in pistil and leaf sucrose concentrations, and a similar pattern was observed in leaf glucose and fructose levels, while pistil glucose and fructose concentrations remained similar to the control. Glutathione reductase activity of both pistils and leaves remained unaffected by the limited water supply. Conversely, putrescine and spermidine levels of water-stressed pistils and leaves were significantly higher compared to the control. Pistil and leaf spermine content significantly increased under drought conditions in one location, remaining unaltered in the other one. Leaf and pistil polyamine metabolism appeared to be more responsive under conditions of water-deficit stress compared to glutathione reductase. Nevertheless, the significant increases in polyamine levels, the observed increases in water-stressed pistil and leaf

carbohydrate concentrations indicated an inhibition of carbohydrate utilization that could potentially result in yield decreases.

INTRODUCTION

Cotton (*Gossypium hirsutum* L.) is a perennial with an indeterminate complex growth habit and is very sensitive to adverse environmental conditions (Oosterhuis, 1994). The U.S cotton crop in recent years has shown extreme and unpredictable year-to-year variability in yield, which has been attributed to genetics, management practices and unfavorable weather conditions (Lewis et al., 2000; Robertson, 2001) with water-deficit considered to be the main environmental factor contributing to variable yields.

Cotton is well equipped with stress mitigation mechanisms such as leaf and root osmotic adjustment (Oosterhuis and Wullschleger, 1987; Nepomuceno et al., 1998), production and accumulation of compatible osmolytes and heat shock proteins (Burke et al., 1985; Kuznetsov et al., 1999) as well as better water use efficiency compare to other crops such as corn and sorghum (Ackerson et al., 1977) since it originates from hot and arid areas (Lee, 1984). However, as a result of its domestication and cultivation as an annual crop, modern cultivars are characterized by drought-intolerance since the effectiveness of stress alleviation mechanisms is closely related to its indeterminate growth habit, as Quisenberry and Roark (1976) pointed out. Consequently, cotton's morphology, physiology and metabolism are significantly impaired under conditions of water-deficit stress with the extent of impairment depending on the developmental stage water stress occurs (Grimes et al., 1969; Gerik et al., 1996; Pace et al., 1999; Pettigrew, 2004). Even though considerable debate still exists on which

stage of development cotton is most susceptible to water stress, limited supply of water during flowering and boll development has been reported to be detrimental for yield (Constable and Hearn, 1981; Cull et al., 1981a,b; Turner et al., 1986).

Extensive research conducted to elucidate water-deficit stress effects on cotton, has focused mainly on leaf physiology and metabolism, with limited attention to cotton's reproductive units despite their great importance as yield determinants (Grimes et al., 1969). Apart from Trolinder et al. (1993), who indicated that cotton flowers and bolls are less sensitive to drought compared to the leaves due to their ability to maintain high water potentials under conditions of stress, Wullschleger and Oosterhuis (1990) who reported that water- deficit stress had no effect on cotton boll respiration, Van Iersel and Oosterhuis (1995, 1996), who attributed boll's insensitivity to plant water status to immature xylem connections and water supply of the boll through the phloem, and Guinn et al. (1976, 1988, 1990) who conducted extensive research on the hormonal balance of cotton bolls under conditions of limited water supply, no other information exist on the biochemistry and metabolism of cotton reproductive units, especially under conditions of water-deficit stress.

Carbohydrate metabolism is of fundamental importance to the plants since it provides the building blocks and the energy for plants (Smith and Stitt, 2007). Research in other crops has indicated that metabolism of reproductive units, and especially carbohydrate biochemistry, is greatly affected when drought stress coincides with flowering and the reproductive period. Zinselmeier et al. (1995, 1999) reported that an accumulation of sucrose in young water-stressed maize (*Zea* mays L.) ovaries occurs simultaneously with the cessation of ovary growth and an additional decrease in hexose concentration, and they speculated that the ratio of

hexose to sucrose could play an important role in ovary development. An inhibition of invertase activity due to drought stress could also result in an increase in ovary sucrose content (Schussler and Westgate, 1991; Liu et al., 2004a). This was also noted by Weber et al. (1998) for legume (*Vicia faba* L.) seed development. However, in cotton, Guinn et al. (1976), did not notice any difference in carbohydrate accumulation in 4 day old water-stressed cotton bolls, while Sung and Krieg (1979) reported that translocation of assimilates was less sensitive compared to photosynthesis, which has been reported to be greatly reduced under conditions of water stress (Faver et al., 1996, Lacape et al., 1998; Leidi et al., 1999). Despite the significant reductions in photosynthetic rates, water-stressed cotton plants have been reported to accumulate significant amounts of carbohydrates (structural and non-structural) in their leaves (Eaton and Ergle, 1948; Ackerson, 1981; Timpa et al., 1986; Parida et al., 2007) as an adaptive to stress mechanism, and similar results have been reported in cereals (Johnson and Moss, 1976; Boyer and McPherson, 1977) as well as in soybean (Liu et al., 2004a). Considering that growth of the reproductive units depends on the leaf for supply of nutrients, water and carbohydrates, it should be expected that perturbations in leaf carbohydrate metabolism would result in similar results in the carbohydrate metabolism of the reproductive units, however, this has not yet been elucidated in cotton.

Apart from osmotic adjustment through accumulation of carbohydrates, production of antioxidant enzymes is another defense mechanism plants possess to prevent lipid peroxidation of membranes from reactive oxygen species that are produced due to inhibition of photosynthesis (Noctor and Foyer, 1998; Mittler, 2002). Glutathione reductase, a non-enzymatic antioxidant that maintains the glutathione pool for constant functioning of the

ascorbate-glutathione cycle participating in scavenging of reactive oxygen species (Asada, 2006) was the first antioxidant whose activity was reported to increase in water-stressed wheat leaves (Gamble and Burke, 1984). Similar responses were observed in other crops such as clover (*Trifolium subterraneum* L.), rice (*Oryza sativa* L.), wheat (*Triticum aestivum* L.), corn (Van Rensburg and Kruger, 1994; Sairam et al., 1997; Jiang and Zhang, 2002; Srivalli et al., 2003; Selote and Khanna Cropra, 2006), whereas increasing evidence in the literature indicate that increased glutathione reductase activity is associated with increased drought tolerance (Smith et al., 1989; Broadbent et al., 1995; Sairam et al., 1998; Kocsy et al., 2004; Selote and Khanna-Chopra, 2004; Deng et al., 2012). However, diverse opinions exist about glutathione reductase activity in cotton. Burke et al. (1985) in field experiments reported that water-stressed cotton plants had increased levels of glutathione reductase compared to control plants, while Mahan and Wanjura (2005) again with field experiments observed that glutathione reductase activity remained unaffected under conditions of limited water supply. Similar results were reported by Kawakami et al., (2010) in growth chamber experiments, while Meloni et al. (2003) in experiments with salinity stress observed no significant effect of increasing salt concentrations in leaf glutathione reductase activity. However, as with carbohydrate metabolism, research on glutathione reductase activity, in cotton and in other crops, has been limited to leaf tissues despite the critical role that glutathione reductase has been observed to have in embryo and meristem development (Cairns et al., 2006; Reicheld et al., 2007; Frottin et al., 2009). In addition, studies in other crops have associated high levels of reactive oxygen species with ovary or ovule abortion (Sun et al., 2004; Hauser et al., 2005), whereas higher concentrations of glutathione reductase have been reported to decrease male sterility in water-stressed rice

anthers (Selote and Khanna-Chopra, 2004). In addition, associations between abscisic acid (ABA) and glutathione reductase have been reported with increasing ABA concentrations enhancing glutathione reductase activity both in vegetative and reproductive tissues in corn (Guan and Scandalios, 2000; Jiang and Zhang, 2002; Hu et al., 2005). Guinn et al., (1990) reported increases in concentrations of ABA in water-stressed white flowers however, glutathione reductase levels were not.

In addition to glutathione reductase, polyamine metabolism has been reported to be affected by conditions of water-deficit stress (Bouchereau et al., 1999). Polyamines are low molecular weight cationic polycations, with the main forms occurring in plants being the diamine, putrescine (PUT) and its derivatives, the triamine spermidine (SPD) and the tetramine spermine (SPM) (Kao, 1997). They have been observed to participate in a variety of physiological and metabolic functions (Kaur-Shawnhey, 2003) while their presence is indispensable during flowering (Evans and Malmberg, 1989; Kakkar and Rai, 1993). Furthermore, changes in polyamine concentrations have been reported to be a common plant response to a variety of abiotic stresses such as salinity and high or low temperatures, as well as drought (Bouchereau et al., 1999; Groppa and Benavides, 2008). Under conditions of water stress, polyamines function either as protective agents, similarly to antioxidants (Smirnoff, 1993; Capell et al., 1998) or as signaling molecules due to their connection to ABA metabolism (Alcazar et al., 2006a). Variable responses have been observed in different crops under conditions of water-deficit stress, however, increased polyamine concentrations have been associated with enhanced drought-tolerance (Capell et al., 1998; Liu et al., 2004b; Alcazar et al., 2005, 2006b, 2010a; Nayyar et al., 2005; Yamaguchi et al., 2007; Yang et al., 2007). Exogenous

application of polyamines or over-expression of polyamines in transgenic plants has been observed to result in enhanced drought tolerance (Kasukabe et al., 2004; Farooq et al., 2008). Despite the extensive research in other crops, little information exists for cotton. Davidonis et al. (1995) reported on the polyamine metabolism of the fiber, while Kuehn et al. (1990) accounted for the presence of uncommon polyamines, such as nonspermidine, nonspermine, pentamine and hexamine. Regarding polyamine metabolism in cotton under abiotic stresses Bibi et al., (2010) reported that application of PUT on cotton flowers resulted in increases in PUT levels of the ovary and seed set efficiency under conditions of heat stress, while SPD and SPM concentrations were significantly decreased. Considering that water-deficit stress is the most important abiotic factor affecting crop production in almost 40% of cultivated areas around the world (Kramer, 1983; Massacci et al., 2008) and that drought events are projected to intensify in the future (IPCC, 2007), it is imperative that research efforts should be focused on finding ways to ameliorate the consequences of water-deficit stress. Therefore, the objectives of our study were to monitor and evaluate the alterations caused by water-deficit stress on the carbohydrate, polyamine and glutathione reductase metabolism of the cotton pistil and its subtending leaf.

MATERIALS AND METHODS

Plant material

Cotton cultivar ST5288B2F seeds were sown at a density of ten plant per meter in a Captina silt loam (Typic Fragidult) soil on June 6th at the Arkansas Agricultural Research in Fayetteville, AR and in a sandy loam (Amarillo) soil on May 30th at the experimental station in

Lubbock, TX. Plots were 4m x 7m with 1m borders between each plot. To maintain well-watered conditions until stress was imposed, plants in Fayetteville, AR were irrigated to soil saturation every six days in the absence of saturating rainfall, while in Lubbock, TX drip irrigation was provided daily. Fertilizer application, weed control, and insecticide applications were performed according to extension center recommendations and practices.

Irrigation was withheld when plants reached the flowering stage (approximately 50% of the plants in the field had white flowers) which was July 20th in Fayetteville, AR and July 13th in Lubbock, TX. First position white flowers and their subtending leaves were sampled at noon at the end of the first week and second week after irrigation was withheld. Tissues were immediately placed in an ice chest filled with dry ice and transferred to the laboratory, and stored at -80^{o}C in Fayetteville, while tissues from Lubbock were shipped overnight to Fayetteville for further determination of carbohydrate, polyamine and glutathione reductase levels.

Soil moisture content

Six soil samples were collected from each treatment plot in both locations at the end of the first and the second week after irrigation was withheld with the help of a soil auger. Fresh weight of the samples was determined after sampling, after which the samples were placed in a dryer (50^{o}C) until they were completely dry. Samples were weighed and their dry weight was determined. Soil moisture content was expressed as the ratio of dry weight/fresh weight.

Stomatal conductance measurements

Stomatal conductance rates were taken at the end of the first and second week only at Fayetteville. Measurements were taken from the subtending leaf (n=20) from 11 :00 a.m. until 1:00 p.m. using a Decagon SC-1 Porometer (Decagon Inc. , Pullman, WA). Due to the small surface area of the cuvette(6.25cm^2), three measurements on various areas of the leaf were taken and then averaged. The results were expressed as mmol H_2O/m^2s.

Carbohydrate measurements

Soluble carbohydrate content was measured according to a modification of the Hendrix (1993) protocol. White flowers were sampled at 12:00pm from control and water-stressed plants at the end of each week. Twenty flowers and their subtending leaves were chosen randomly from each treatment and the pistils were excised and oven dried for 3 days at 50^oC. The dried tissues were subsequently ground with a mortar and pestle. Forty mg of the ground tissue were extracted 3 times with 80^oC aqueous ethanol (800 ml ethanol /L) and the samples were centrifuged after each extraction at 5000 rpm and finally the fractions were pooled. Active charcoal was then added to the pooled fractions in order to remove substances that could interfere with the carbohydrate measurements and the samples were centrifuged again at 3500 rpm. The supernatant was immediately stored at -80^oC for further determination of sucrose, fructose and glucose with a MultiScan Ascent Microplate Reader (Thermo Fisher Scientific Inc., Waltham, MA). The glucose (HK) assay kit from Sigma (Sigma Chemical Company, St Louis, MO) was used. A 20 µl aliquot of each extract was pipetted into a well of a microtitration plate and the plate was incubated at 50^oC for 40 min to evaporate ethanol. 10 µl

of water were then added to each well along with 100µl of glucose assay reagent and the plate was incubated again for 15 min at 30°C. The absorbance was measured three times at 340 nm using a microplate reader. 0.25 EU of phosphoglucose isomerase was added to the extracts in each well of the plate and the absorbance was again measured at 340 nm. Subsequently, 83 units of invertase were added to the extracts and the microtitration plate was incubated at 30°C for 60min. The absorbance was measured three times at 340 nm and the results were expressed in mg carbohydrate/mg dry weight with the help of a standard curve made of known glucose concentrations.

Glutathione reductase measurements

Glutathione reductase activity was measured in pistils from sampled white flowers and in their subtending leaves (n=20) according to Anderson et al. (1992), with modifications. Pistil tissue was homogenized using a mortar and pestle in an ice-cold extraction solution (10x pistil fresh weight) comprised of 50 m*M* PIPES (1,4-Piperazinediethanesulfonic acid) buffer (pH 6.8), 6 m*M* cysteine hydrochloride, 10 m*M* D-isoascorbate , 1 m*M* ethylenediaminetetraacetic acid, 0.3% Triton X-100 and 1% (w/v) soluble Polyvinylpyrrolidone (PVP). Solutions were further blended for 1 min in a tube containing 0.25 g insoluble PVP and 1 drop of antifoam A emulsion using a homogenizer (Model Polytron; Brinkman Instruments Inc., Palo Alto, CA). Subsequently, samples were centrifuged at 21 000 *g* for 20 min (4°C) and the supernatants were stored at −80°C for further determination of glutathione reductase content according to Shaedle and Bassham (1977), with modification. To each well of a 96-well microtitration plate, a 15.7 µl aliquot of enzyme extract from each sample was added to a 300 µl reaction solution containing

50 mM Tris-HCl buffer (pH = 7.5), 0.15 mM reduced nicotinamide adenine dinucleotide phosphate (NADPH), 0.5 mM oxidized glutathione, and 3 mM $MgCl_2$. Oxidation of NADPH was determined as the decrease in absorbance at 340 nm during a 1 min reaction time was measured during a 1 min reaction time using an Ascent Multiscan microplate reader (Molecular Devices Corporation, Sunnyvale, CA), and glutathione reductase activity was expressed as GR units/g fresh weight.

Polyamine extraction and analysis

Polyamines were extracted according to Smith and Davies (1985) with modifications. Cotton ovary and leaf tissue, 0.1 and 0.2 g, respectively, were excised and homogenized in mortars with pestles in 0.2 N $HCLO_4$. To monitor the extraction and quantification procedure unfortified and fortified samples were prepared. For unfortified samples 100 µl of 1 mM hexamethylenediamine in 0.2 N $HCLO_4$ was added to the tissue prior to homogenization as an internal standard. The final volume of 2ml was obtained by adding 1900 µl 0.2 N $HCLO_4$. For the fortified samples that contained a certain amount of the three polyamines, 100 µl hexamethylenediamine 1mM in 0.2 N $HCLO_4$ was added plus the desired volume of fortification solution in 0.2N $HCLO_4$ which was 120 µl 1mM putrescine in 0.2 N $HCLO_4$, 120 µl 1 mM spermidine in 0.2 N $HCLO_4$, and 120 µl 1 mM spermine of 0.2 N $HCLO_4$. The final volume of 2 ml was obtained by adding 1540 µl of 0.2 N $HCLO_4$. An aliquot of 1.5 ml of the homogenate was transferred to 2 ml micro centrifuge tubes and the samples were centrifuged at 4^oC for 20min at 14000 rpm. The supernatant was collected and used for dansylation of polyamines.

The polyamines were derivatized by adding 100 µl aliquots of the supernatant to 1000 µl 21.2 mM of aqueous Na_2CO_3, 400 µl of 99.9% acetone and 50 µl of 12.5 mM and 100 µl of 87.5 mM of dansyl chloride in acetone. The mixture was incubated in a thermal reaction block at 60^{o}C for 1h in the dark. After 1h in the thermal block, the samples were removed and cooled to near room temperature, and 100 µl 1N $HCLO_4$ were added to the mixture and mixed. The samples were then centrifuges at 4^{o}C for 20 min at 14000 rpm, after which 500 µl of centrifugate were transferred into 2 ml sample vial and 500 µl of 0.02 N $HCLO_4$ were added. The samples were capped and mixed before injection into the High Performance Liquid Chromatography (HPLC). Derivatization needs to be in a basic solution, whereas the final solution for HPLC needs to be acidic.

A total of 5 standards were used for the preparation of the standard curves. The standards included putrescine, spermidine, spermine and the internal standard hexamethylenediamine. The concentrations of putrescine and spermidine in the five standards ranged from 5 to 30 nmoles/ml, whereas the concentrations of spermine ranged from 10 to 60 nmoles/ml. A 500 µl aliquot of hexanemethylenediamine was added to the standards. All standards were brought to a final volume of 10 ml with 0.2 N $HCLO_4$.

HPLC analysis was performed using a Hitachi HPLC (Hitachi High Technologies America, Inc., Canada) system that included a model L-7100 pump, and L-7200 autosampler, a D-7000 interface, and an ERC-3415a degasser and an L-7480 fluorescence detector. The column used in this analysis was a 25 cm x 2 mm, i.d. 0.5 micron Phenomenex Gemini C18. Injection volume was 50µl. Polyamines were eluted from the column at 0.3 ml/min with methanol:water (v/v) gradient from 70% methanol to 95% methanol over 6 min and then remaining at 95% methanol

for 16.4 min. The system was re-equilibrated with 70% methanol for 15 min before the next injection. For dansyl polyamines, an excitation wavelength of 510 nm. Data collection and processing were with Hitachi System Manager (HSM) software on the internal standard concentration.

Statistical analysis

The experimental design used was a split plot and treatments consisted of control, receiving optimum quantity of water all through the season, and water stress, where irrigation was withheld for two weeks during flowering. 100 first position white flowers and their subtending leaves were randomly selected from each treatment plot at the end of each week from both locations and each sample was considered as an experimental unit. Analysis of carbohydrate, polyamine and antioxidant results was done utilizing a three factor factorial analysis with factors being water-regime, time (week) and location, with twenty replications. JMP 8 software (SAS institute, Cary, NC) was used to evaluate the results while interactions and main effects were tested with Analysis of Variance (ANOVA) at $\alpha \leq 0.05$.

RESULTS

Soil moisture content and environmental data

Significant differences were observed between the water-stressed and control plots in Fayetteville, AR at the end of the first and second week (Fig.1A), however the opposite was observed in Lubbock, TX, where moisture content of both water-stressed and control plots were similar at the end of the first and second week of the stress (Fig. 1B). Additionally, similar

mean temperatures were recorded in both locations (Fig.2) all through the stress period, while the contrary was recorded for relative humidity (Fig.2). Values of relative humidity were consistently higher in Fayetteville, AR compared to Lubbock, TX resulting in substantial differences in vapor pressure difference (VPD) with average VPD during the stress period in Fayetteville, AR being 28 KPa and in Lubbock, TX being 39.5 KPa.

Stomatal conductance

Water-deficit stress resulted in significant decreases in leaf stomatal conductance rates. Water-stressed plants had significantly lower stomatal conductance rates compared to the control for the first week ($P \leq 0.0001$) as well as the second week ($P \leq 0.0001$) (Fig.3). Stomatal conductance rates were 38% and 42% lower compared to the control at the end of the first and second week of stress, respectively.

Carbohydrate concentrations

White flowers and their subtending leaves were collected randomly from control and water-stressed plots in the end of each week and were analyzed for their carbohydrate content. No interaction was observed in carbohydrate values of the pistils between water regime, location and time (weeks) ($P_{GLU} = 0.1090$, $P_{FRU} = 0.0687$, $P_{SUC} = 0.3555$), and the results from both locations were pooled and analyzed using a two factor factorial with the factors being water-regime and time (weeks). No interaction was observed between the two factors for all the carbohydrates analyzed ($P_{GLU} = 0.2292$, $P_{FRU} = 0.0520$, $P_{SUC} = 0.9823$) and the results from both weeks were pooled again and the effects of water-deficit stress were determined using a

Student's t-test at a≤0.05. No interaction was also observed in carbohydrate values of the leaves between treatments, location and weeks (P_{GLU}= 0.5552, P_{FRU}= 0.2918, P_{SUC}= 0.3733) and the results from both locations were pooled and analyzed using a two factor factorial with the factors being water-regime and time (weeks). Similarly to the pistils, no interaction was observed between the two factors for all the carbohydrates analyzed (P_{GLU}= 0.0510, P_{FRU}= 0.5293, P_{SUC} =0.1827). The results from both weeks were pooled and the effects of water-deficit stress on leaf carbohydrate concentrations were determined using Student's t-test at α≤0.05.

Pistil glucose and fructose concentrations of water-stressed flowers were not significantly altered. Pistil glucose increased by 16% compared to the control and the same was observed in fructose levels of water-stressed pistils which were increased only by 8% compared to the control (Fig. 4). Conversely, pistil sucrose concentrations under conditions of limited water supply were nearly 1.5 fold higher compared to the control (Fig. 4). Water-deficit stress resulted in significant increases in leaf glucose, fructose and sucrose concentrations (Fig. 5) with their levels being 47%, 24% and 55% higher compared to the control, respectively.

Glutathione reductase activity

Glutathione reductase was analyzed from twenty white flowers and their subtending leaves that were collected only the second week from both locations. No interaction was observed between the two locations in leaf reductase values (P=0.4369) or pistil reductase values (P=0.2996) and the results from both locations were pooled for each tissue and water-deficit's effects on glutathione reductase were estimated using a Student's t-test (α≤0.05). No

significant differences were observed between water-stressed and control leaf glutathione reductase (Fig.6), and a similar pattern was observed for pistil glutathione reductase activity, where water-stressed pistils contained similar quantities with control pistils (Fig. 7).

Polyamine concentrations

Polyamine content of twenty white flowers (ovaries) and their subtending leaves sampled randomly from each treatment plot at the end of each week from both locations. No interaction was observed in ovary PUT or SPD concentrations between water-regime, time (weeks) and location (P_{PUT} =0.9112, P_{SPD} =0.3674), while a significant interaction was observed in levels of SPM (P_{SPM} = 0.0198). However, results were analyzed separately for each location for all three polyamines using a two factor factorial with the factors being water-regime and time (weeks). No interaction was observed between water-regime and time (weeks) in Lubbock, for ovary PUT, SPD and SPM (P_{PUT}=0.4297, P_{SPD}= 0.3873, P_{SPM}=0.0926), while a significant interaction was observed for ovary PUT and SPD but not for SPM in Fayetteville (P_{PUT}=0.0079, P_{SPD} =0.0011, P_{SPM} =0.0896), and the effects of water-deficit stress were analyzed separately for each week using Student's t-test at $\alpha \leq 0.05$.

A significant interaction was observed in leaf PUT concentrations between water-regime, time (weeks) and location (P_{PUT} =0.00029) whereas no interaction was observed in leaf SPD and SPM concentrations (P_{SPD} = 0.3584, P_{SPM} = 0.3127), however again all results were analyzed separately for each location using a two factor factorial with the factors being water-regime and time (weeks). In Lubbock, a significant interaction was found between the two factors in leaf PUT levels (P_{PUT}= 0.0001) while not interaction was observed in leaf SPD and SPM

levels (P_{SPD}= 0.2559, P_{SPM} =0.3387) and a similar pattern was notices in Fayetteville where a significant interaction between water-regime and time (weeks) was found only in leaf PUT levels (P_{PUT}= 0.0092) while the opposite was observed in leaf SPD and SPM (P_{SPD} =0.8589, P_{SPM}= 0.6117), and the effects of water-deficit stress were analyzed separately for each week using Student's t-test at $\alpha \leq 0.05$.

Water-deficit stress in Fayetteville had no effect on ovary PUT levels at the end of the first week, however leaf PUT levels were nearly 3-fold higher compared to the control (Fig. 8). A similar pattern was observed at the end of the second week with leaf PUT concentrations increasing by 256% compared to the control while ovary PUT concentrations significantly increased as well however, only by 54% compared to the control (Fig.8). Conversely, both ovary SPD (Fig.9) and SPM (Fig.10) concentrations significantly decreased at the end of the first week, by 30 and 31%, respectively whereas leaf SPD significantly increased by 27% (Fig. 9), while leaf SPM levels remained similar to the control (Fig.10). Nevertheless, ovary and leaf SPD levels were significantly higher compared to the control at the end of the second week (Fig.9), while both ovary and leaf SPM levels remained unaffected by water stress (Fig. 10).

In Lubbock, PUT concentrations of water-stressed ovaries were almost 2.5-fold higher compared to the control (Fig. 11) and the same was observed in PUT levels of water-stressed leaves with them being though almost 9-fold higher compared to the control (Fig. 11). Similarly, at the end of the second week, both ovary and leaf PUT concentrations were significantly higher compared to the control with the increases in the leaf being more pronounced (Fig.11).In accordance to PUT levels, both SPD (Fig.12) and SPM (Fig. 13) concentrations of water-stressed ovaries were higher compared to the control by 46% and 47%,

respectively, while leaf SPD (Fig. 12) and SPM (Fig.13) levels were also significantly enhanced compared to the control at the end of the first week by 90% and 40%, respectively. Unlike ovary PUT levels, however, water-deficit stress had no effect on either ovary SPD (Fig. 12) or ovary SPM (Fig. 13) levels at the end of the second week, while the opposite was observed in leaf SPD and SPM concentrations where water-stressed leaves contained 67% and 30% more SPD(Fig. 12) and SPM (Fig. 13) respectively, compared to the control.

DISCUSSION

Water supply is a key factor regulating cellular growth and development (Hsiao, 1973) and ultimately crop yield since it determines the function of several physiological and metabolic processes. Stomatal conductance has been reported to greatly decrease under limited water supply conditions (Socias et al., 1997; Chaves and Medrano, 2002), however, diverse opinions have been expressed for cotton. Experiments with potted plants have reported that water-deficit stress decreased cotton leaf stomatal conductance (Wullschleger and Oosterhuis, 1990; Kawakami et al., 2010; da Costa and Cothren, 2011) while conversely, Ackerson et al. (1977a) in studies with field-grown cotton reported that stomatal conductance remained essentially unaltered even under very low water potentials. In our study, in accordance with the majority of the previous research, significant decreases in leaf stomatal conductance rates of water-stressed plants were observed compared to the control. We speculate that the different results reported by Ackerson et al. (1977a) are due to the different way of imposition of stress and acclimation since they subjected the plants in a series of drying-rewatering cycles.

Despite the significant reductions in leaf stomatal conductance rates and the consequent decrease in CO_2 fixation rates, significant increases in glucose, fructose and sucrose levels were observed in water-stressed leaves in our study. Increased leaf hexose and sucrose concentrations in cotton under conditions of limited water supply have also been reported by Timpa et al. (1986), Chang and Ryan (1987), and Parida et al. (2007). Accumulation of carbohydrates in water-stressed leaves is considered to be an adaptive response of plants to drought stress for maintenance of leaf cell turgor through osmotic adjustment (Bray, 1997) and has been observed also in a variety of other crops such as maize (Kim et al., 2000), barley (*Hordeum vulgare* L.) (Teulat et al., 2001), sorghum (*Sorghum bicolor* L.) (Turner et al., 1978), and grapevine (*Vitis vinifera* L.) (Cramer et al., 2007). Increased leaf hexose concentrations under conditions of water stress were attributed to up-regulated activity of sucrose cleaving enzymes under conditions of water-stress (Kim et al., 2000) while higher sucrose levels has been suggested to be the result of higher activity of sucrose synthesizing enzymes and a decreased activity of starch synthesizing enzymes (Quick et al., 1989). Chang and Ryan (1987) reported that sucrose synthase activity of cotton leaves remained unaffected by limited water supply however, sucrose phosphate synthase and α-amylase were significantly increased and decreased, respectively, resulting in increased sucrose concentrations and diminished starch levels. Similar to their results, leaf starch concentrations were significantly decreased under conditions of water stress in our study (data not shown) while the opposite was observed for sucrose levels, leading us to assume that the increased carbohydrate levels observed in water-stressed leaves were the result of the effect water-deficit stress had on the activity of carbohydrate enzymes.

Growth and development of flowers and fruits have been observed to be strongly dependent on leaf supply of carbohydrates (Ho et al., 1988) while in cotton the leaf subtending to the flower has been reported to provide the majority of carbohydrates (Ashley, 1972). In addition, water-deficit stress has been reported to alter partitioning of photoassimilates due to changes in sink strength in plant tissues (Ho et al., 1988, Muller et al., 2011). Sung and Krieg (1979) observed that water stress diverted more carbohydrates to the upper canopy leaves however, translocation rates of carbohydrate export from leaves were less sensitive compared to photosynthesis under water stress (Sung and Krieg, 1979) suggesting that carbohydrate metabolism of the flower should proceed unaffected. Nevertheless, that was not what was observed in our study, where sucrose concentrations of water-stressed pistils were significantly higher compared to the control, while glucose and fructose levels remained unaffected. Higher than control sucrose levels, with unaltered, decreased or increased hexose levels under conditions of limited water supply have also been reported to occur in the reproductive units of other crops such as maize, rice, wheat and soybean (*Glycine max* L.) (Zinselmeier et al., 1995; 1999; Sheoran and Saini, 1996; Lalonde et al., 1997; Setter et al., 2001; Andersen et al., 2002; Liu et al., 2003). Similarly to the leaves, sucrose increases were attributed to alterations in sucrose cleaving enzymes activity, and specifically to down regulation of acid invertase activity (Zinselmeier et al., 1995; Lalonde et al., 1997, Liu et al., 2003), whereas higher hexose levels were suggested to be the result of decreased utilization in the respiratory pathway (Couee et al., 2006; Rolland et al., 2006; Nguyen et al., 2010). However, the increases in sucrose concentrations in the aforementioned studies were observed to occur at pistil water potentials lower than control, while in previous studies cotton pistils and bolls were reported to maintain

high water potential under water stress (Trolinder et al., 1993) leading us to speculate that the increased sucrose concentrations in our study was the plant's response to maintain high water potential. However inhibitions in the functions of sucrose cleaving enzymes should not be out-ruled especially, since hexose levels remained similar to the control. In view of Weber et al. (1998) and Liu et al. (2003) reports that hexose to sucrose ratio controls fruit growth with low values of this ratio resulting in abortion and abscission, it is evident that further research in the pistil's carbohydrate metabolism of cotton is needed.

Soluble carbohydrates have also been reported to act as signaling agents interacting with a variety of metabolical functions including antioxidant metabolism. Sucrose has been observed to have antioxidant function while glucose is also the main precursor for ascorbate (Smirnoff et al., 2001) as well as for carbon skeletons used for synthesis of amino acids such as cysteine, glutamine and glycine which are used in glutathione synthesis (Noctor and Foyer, 1998). However, despite the significant differences in leaf carbohydrate concentrations no significant differences were observed in the antioxidant, glutathione reductase, activity in either of the tissues analyzed in our study. Under conditions of water stress, glutathione reductase activity has been reported to increase in other crops, such as clover, rice, wheat, corn (Van Resburg and Kruger, 1994; Sairam et al., 1997, 1998; Jiang and Zhang, 2002; Srivalli et al., 2003; Selote and Khanna-Cropra, 2006) resulting in increased drought tolerance. Burke et al. (1985) reported similar results with water-stressed cotton leaves containing higher levels of glutathione reductase. Conversely, and in accordance with our results, leaf glutathione reductase activity has been reported to remain unaffected under limited supply of water under both field (Mahan and Wanjura, 2005) and controlled environment conditions (Kawakami et al.,

2010). Mahan and Wanjura (2005) additionally, speculated that the lack of differences in glutathione reductase activity was due to the increases that were observed in ascorbate peroxidase activity that maintained cellular integrity and no further increase in other antioxidants was needed. In support of this observation, Loggini et al. (1999) in experiments with wheat cultivars differing in drought tolerance, reported that levels of glutathione reductase activity in the drought-tolerant cultivar remained unaffected under conditions of water stress but was significantly increased in the drought-sensitive one. Therefore, we assume that the stress imposed to the plants in our study was not enough to increase the activity of glutathione reductase, while the differential responses between our study and Burke et al. (1985) are due to the different cotton strains (photoperiodic) they used compared to ours (non-photoperiodic).

Similarly to leaf glutathione reductase, pistil glutathione reductase levels remained unaffected by limited water supply. In one other study focusing on glutathione reductase metabolism in reproductive units, Selote and Khanna-Chopra (2004), conversely to our results, in experiments with rice cultivars differing in drought tolerance reported that panicle glutathione reductase levels in the drought-tolerant cultivar significantly increased under conditions of water stress, while the opposite was observed in the drought-sensitive cultivar. Increased pistil glutathione reductase levels in water-stressed pistils were also observed in our growth chamber studies (Chapter 1), however, the stress in that study was less severe in duration and extent compared to the present one. Since antioxidant metabolism is reported to be more active at the onset of the stress (Smirnoff, 1993; Sgherri et al., 1996) we speculate that the lack of differences in glutathione reductase is due to the later time into the stress that the

samples analyzed were attained. However, similarly to the leaves, an increase in other antioxidant activities could also be the reason for water-stressed pistils containing similar quantities of glutathione reductase to the control.

Nevertheless, contrary to the insignificant responses in glutathione reductase activity in both tissues analyzed, pistil and leaf polyamine metabolism was significantly affected by water-deficit stress in both locations. PUT concentrations of water-stressed leaves were significantly increased in both locations, compared to the control, at the end of the first and the second week and a similar pattern was observed in leaf SPD levels. Regarding leaf SPM concentrations, water stress resulted in a significant increase only in Lubbock, whereas in Fayetteville its levels remained similar to the control. Increased polyamine concentrations have been reported as a response to water-deficit stress in a variety of other crops (Nayyar et al., 2005; Liu et al., 2006; Yang et al., 2007). Furthermore, Alcazar et al. (2010b) reported that transgenic *Arabidopsis* plants transformed to over-produce PUT were more drought-tolerant compared to the wild types due to their ability to control and induce stomatal closure more effectively, while Liu et al. (2000) made similar observations for SPD and SPM. Stomatal conductance rates of water-stressed leaves in Fayetteville, were significantly decreased at the end of the first and the second week, similar to the increases observed in PUT and SPD concentrations, while SPM remained unaffected leading us to assume that in cotton, PUT and SPD are more efficient in inducing stomatal closure and protecting plants from water loss compared to SPM. Additionally, we speculate that the lack of response in leaf SPM concentrations in Fayetteville compared to Lubbock was due to the environmental differences with the stress being more severe in Lubbock.

Regarding ovary polyamine concentrations, a differential response was observed between the two locations at the end of first week as well as at the end of the second week. Water-stressed PUT concentrations were significantly higher compared to the control in Lubbock at the end of the first and second week, while ovary SPD and SPM levels were significantly increased only at the end of the first week, but they remained unaffected at the end of the second. Conversely, in Fayetteville ovary PUT concentrations remained unaffected at the end of the first week, whereas SPD and SPM levels significantly decreased, while at the end of the second week ovary PUT, SPD and SPM concentrations were significantly higher in water-stressed ovaries compared to the control. Similar to leaves, increases in polyamine concentrations in reproductive units have been observed (Nayyar et al., 2005). In addition, Capell et al. (2004) observed that PUT concentrations in rice have to surpass a certain threshold in order for SPD and SPM biosynthesis to be triggered. Similar results were reported also by Alcazar et al. (2010b) in experiments with *Arabidopsis*. We speculate that the decreases in SPD and SPM concentrations in Fayetteville in the first week were due to the insignificant increases in PUT levels that failed to initiate SPD and SPM biosynthesis, while the stress resulted in activation of SPD and SPM catabolic pathway, which has also been observed by Capell et al. (1993). Conversely, in Lubbock at the end of first week, the stress was enough to enhance PUT levels past the threshold, resulting in significant increases in both SPD and SPM concentrations. A similar pattern was observed in Fayetteville at the end of the second week, with both SPD and SPM levels of water-stressed being significantly higher compared to control after PUT concentrations were apparently past the critical level to induce their biosynthesis. However, the over-production of PUT observed at the end of the second week in Lubbock resulted in SPD

and SPM levels of water-stressed ovaries remaining similar to the control, leading us to speculate either that nitrogen pools of the plants were depleted or plants were unable to utilize them, as has also been reported by Lazcano-Ferrat and Lovett (1999). Hence, polyamine metabolism in cotton appears to be greatly affected by water stress, with leaf polyamine metabolism being more sensitive compared to ovary polyamine metabolism, which is in accordance with the observed enhanced drought tolerance of cotton reproductive units compared to leaves.

The results of our study indicated that water-deficit stress affected carbohydrate metabolism of both leaf and pistil, resulting in significant increases in total carbohydrates and in sucrose, respectively. Similarly, leaf and ovary polyamine metabolism were significantly affected by limited water supply, suggesting that polyamines have a critical role in cotton protection under adverse environmental conditions, especially PUT and SPD. However, more research needs to be conducted in order to elucidate the exact function of each polyamine. Despite our expectations, glutathione reductase activity was not affected by water stress in either of the tissues, leading us to assume that other antioxidant systems are more responsive in cotton under conditions of water stress.

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FIGURES

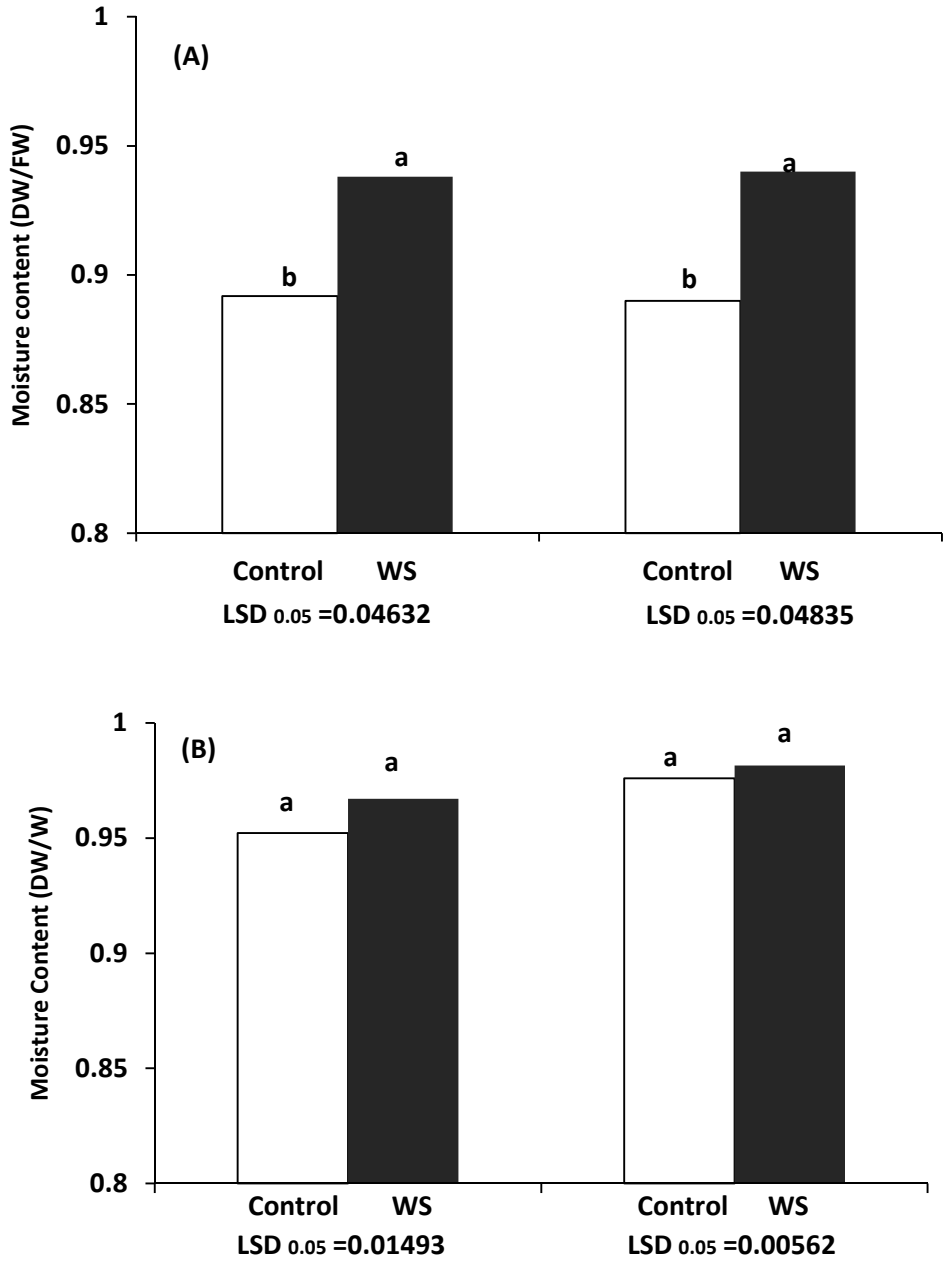

Figure 1. Effect of water-deficit stress on soil moisture content in Fayetteville, AR (A) and Lubbock, TX (B) at the end of the first and second week. Columns connected with the same letter are not significantly different (P≤0.05).

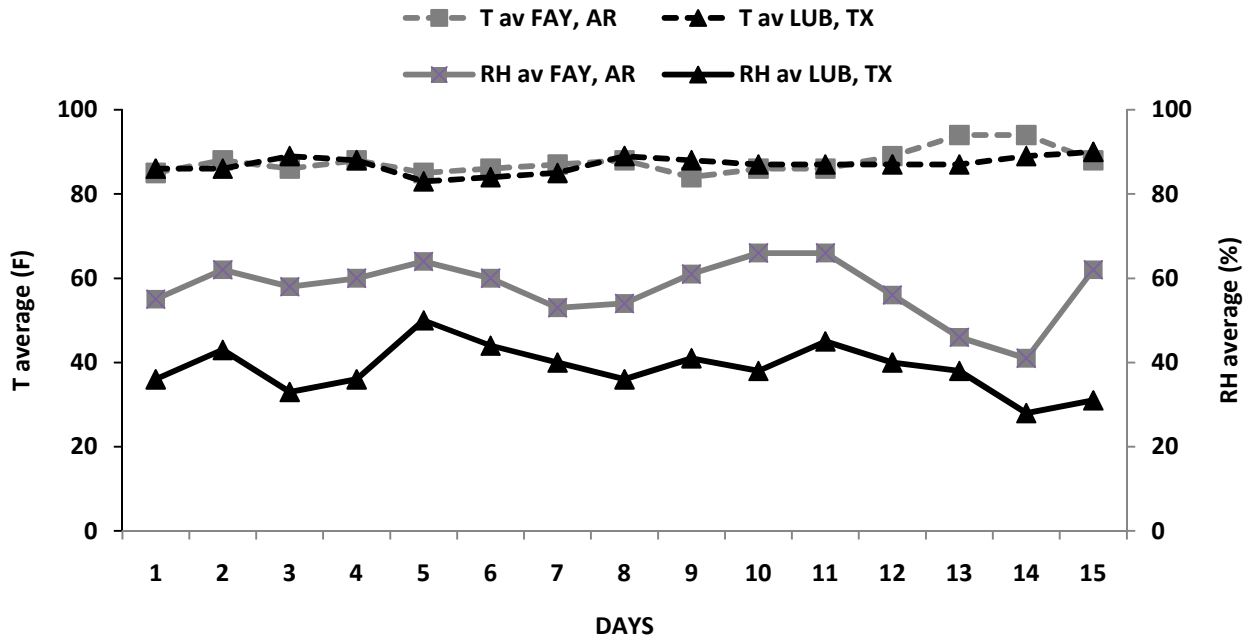

Figure 2: Mean day temperatures and relative humidity during the two weeks of the water-deficit stress period for Fayetteville, AR and Lubboc, TX. Samples were taken on days 7 and 14.

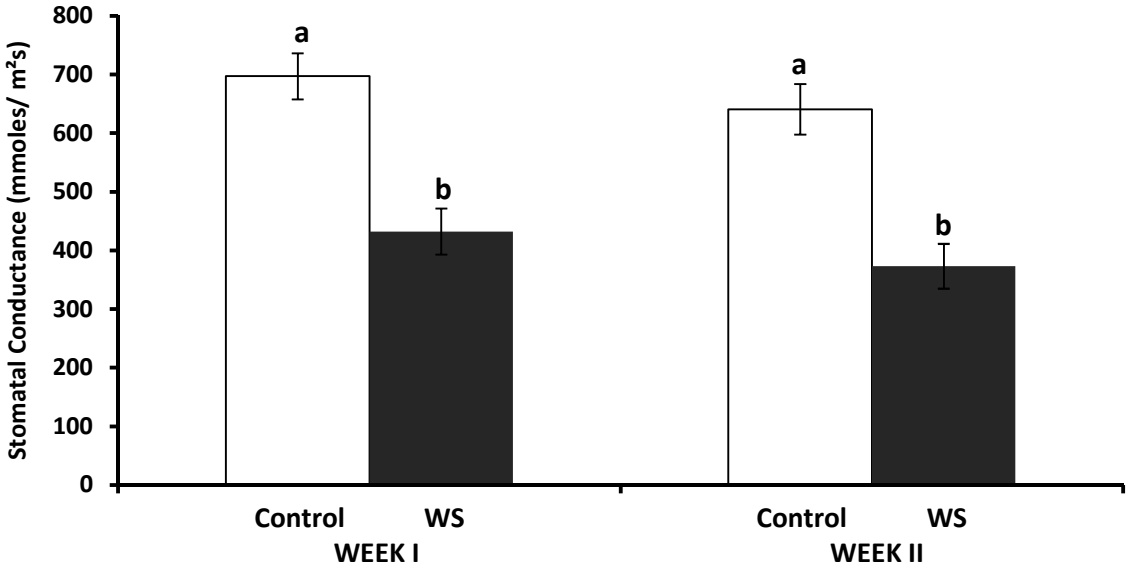

Figure 3 : Effect of water-deficit stress on leaf stomatal conductance rates at the end of the first and the second week in Fayetteville. Pairs of columns in the same time interval (week) connected with different letters are significantly different (P=0.05). Error bars indicate ±1 standard error.

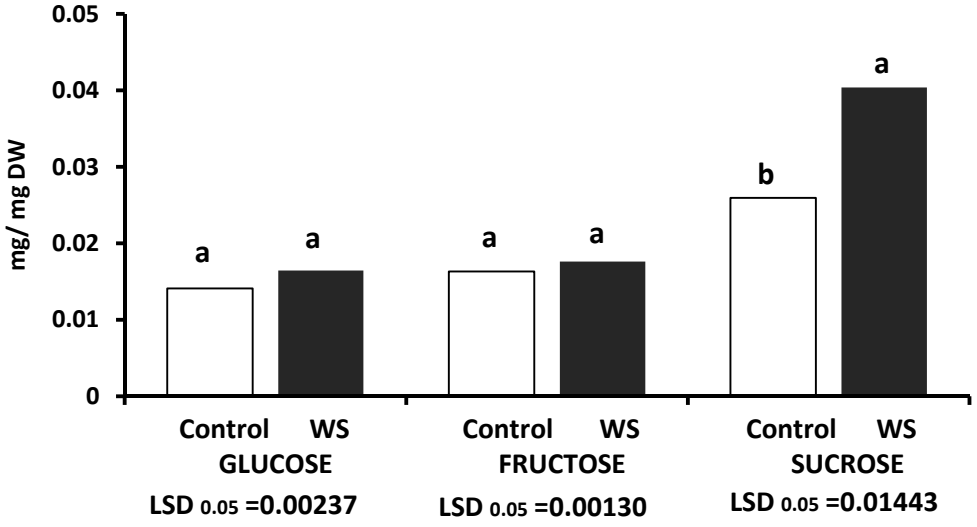

Figure 4: Effect of water-deficit stress on pistil glucose, fructose and sucrose concentrations. Columns connected with different letters are significantly different (P=0.05).

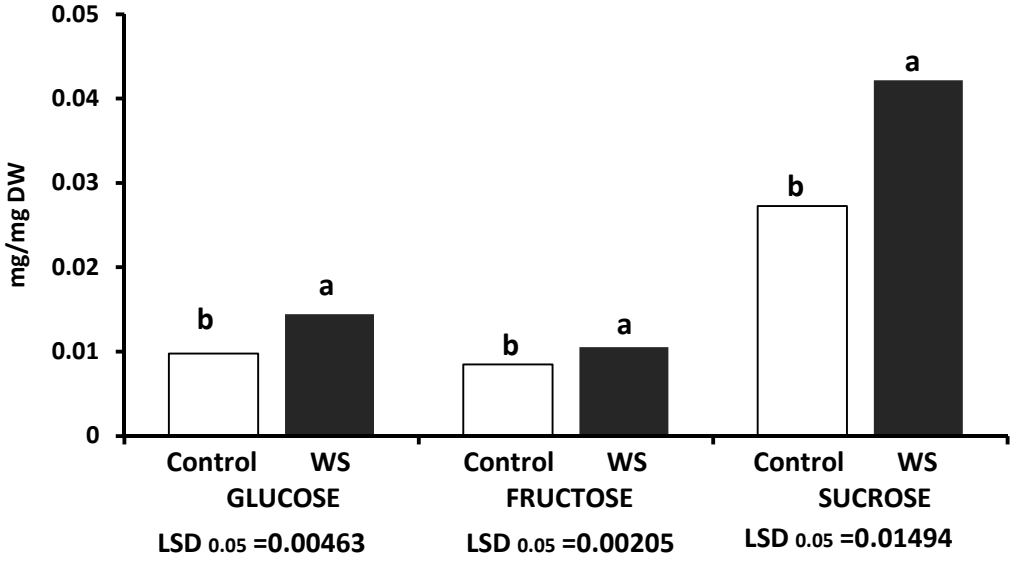

Figure 5: Effect of water-deficit stress on leaf glucose, fructose and sucrose concentrations. Columns connected with different letters are significantly different (P=0.05).

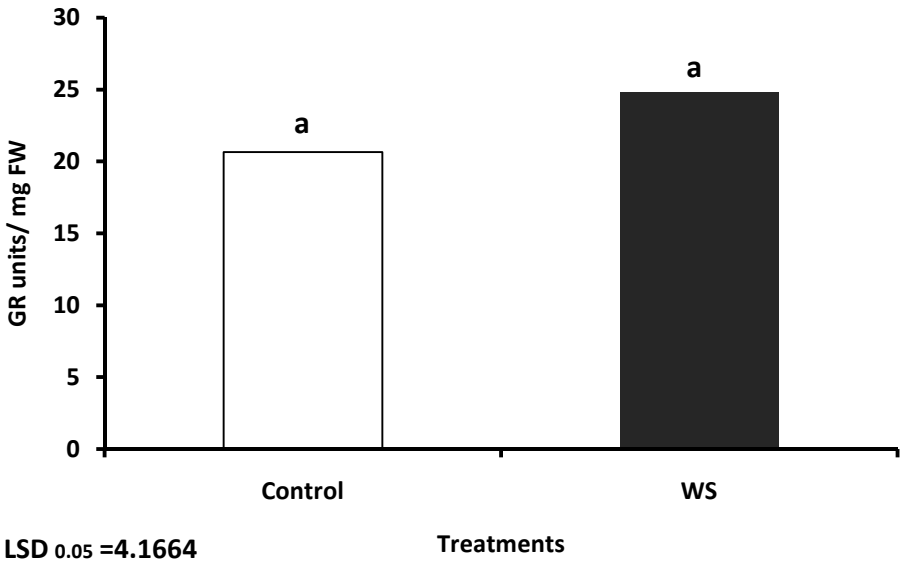

Figure 6: Effect of water-deficit stress on leaf glutathione reductase activity. Columns connected with the same letter are not significantly (P=0.05).

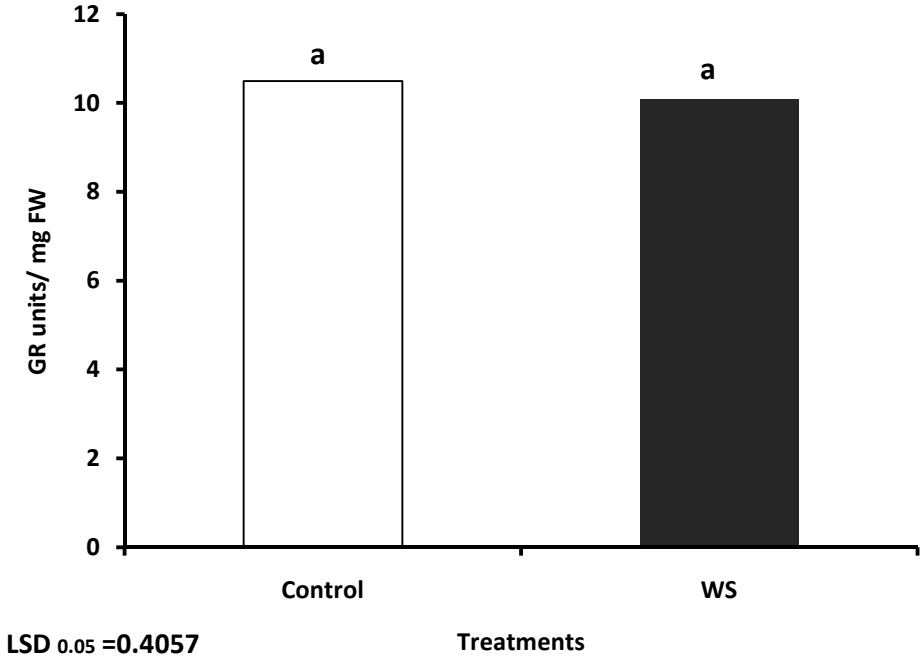

Figure 7: Effect of water deficit stress on pistil glutathione reductase activity. Columns connected with the same letter are not significantly different (P=0.05). $LSD_{0.05}$=0.4057.

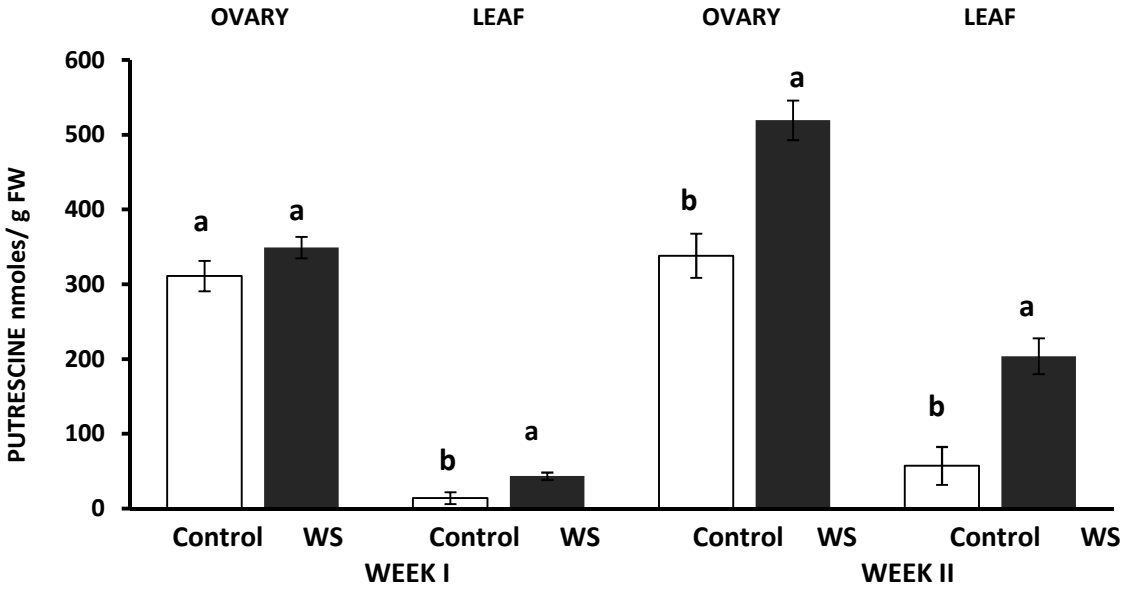

Figure 8: Effect of water deficit stress on ovary and leaf putrescine (PUT) concentrations in Fayetteville at the end of the first and second week. Pairs of columns within each type of tissue connected with the same letter are not significantly different (P= 0.05). Error bars represent ±1 standard error

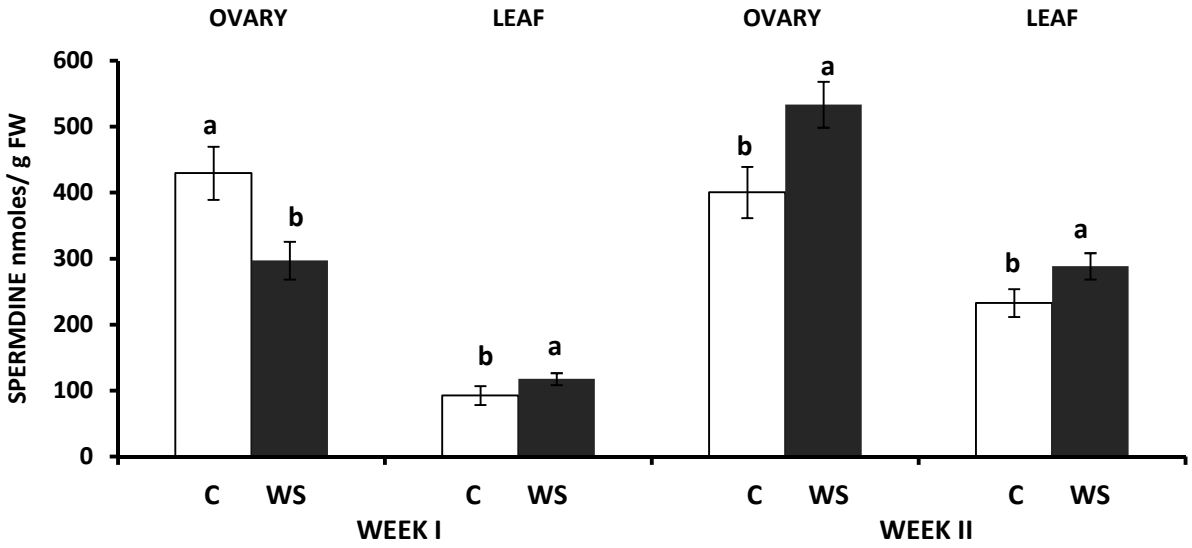

Figure 9: Effect of water deficit stress on ovary and leaf spermidine (SPD) concentrations in Fayetteville at the end of the first and second week. Pairs of columns within each type of tissue connected with the same letter are not significantly different (P= 0.05). Error bars represent ±1 standard error.

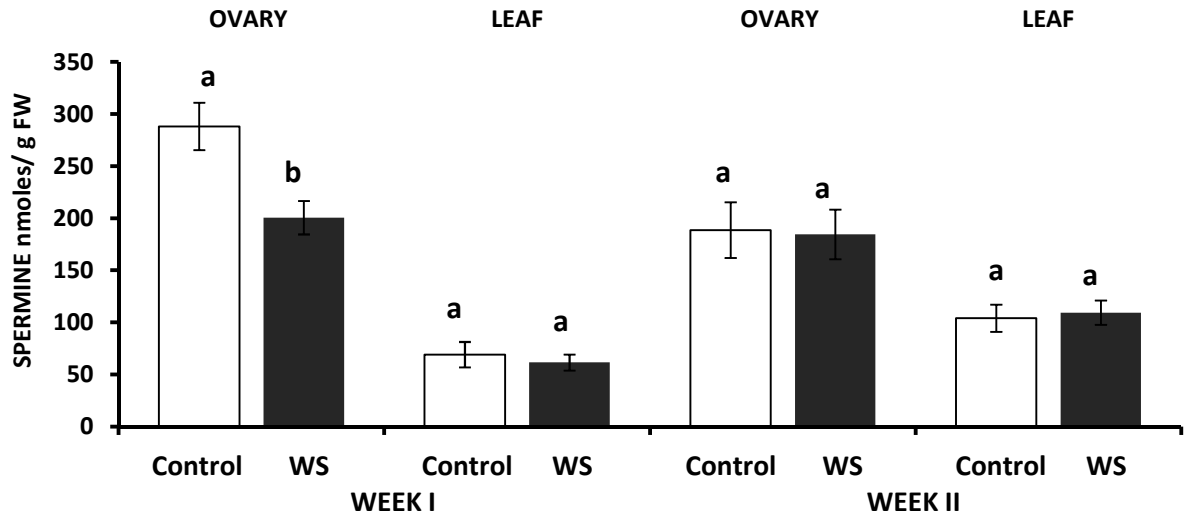

Figure 10: Effect of water deficit stress on ovary and leaf spermine (SPM) concentrations in Fayetteville at the end of the first and second week. Pairs of columns within each type of tissue connected with the same letter are not significantly different (P= 0.05). Error bars represent ±1 standard error.

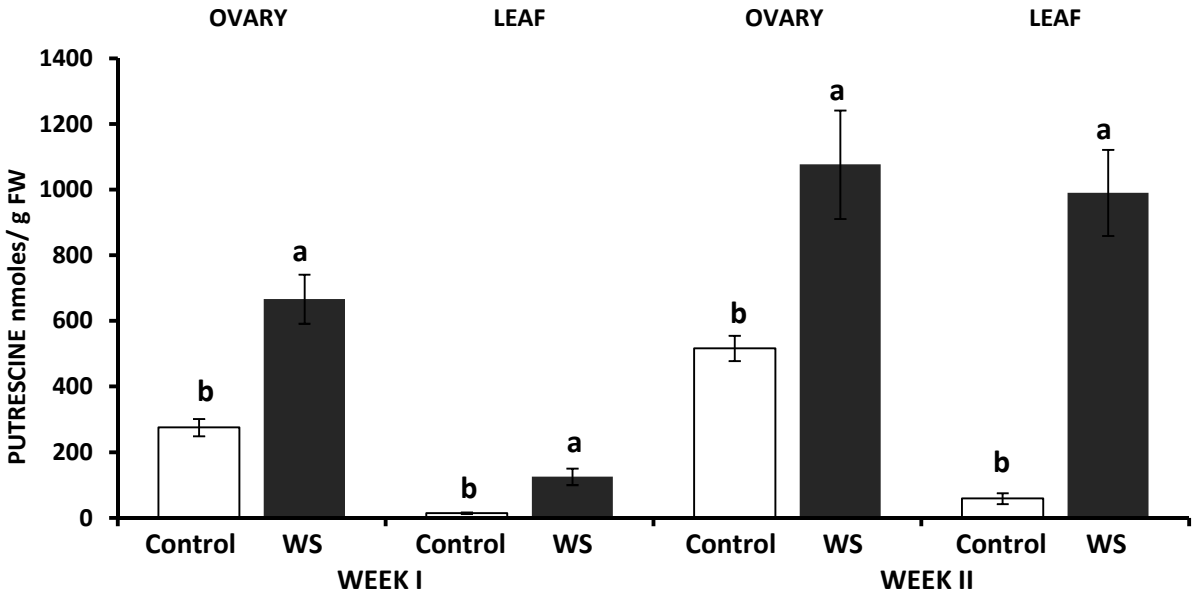

Figure 11: Effect of water deficit stress on ovary and leaf putrescine (PUT) concentrations in Lubbock at the end of the first and second week. Pairs of columns within each type of tissue connected with the same letter are not significantly different (P= 0.05). Error bars represent ±1 standard error.

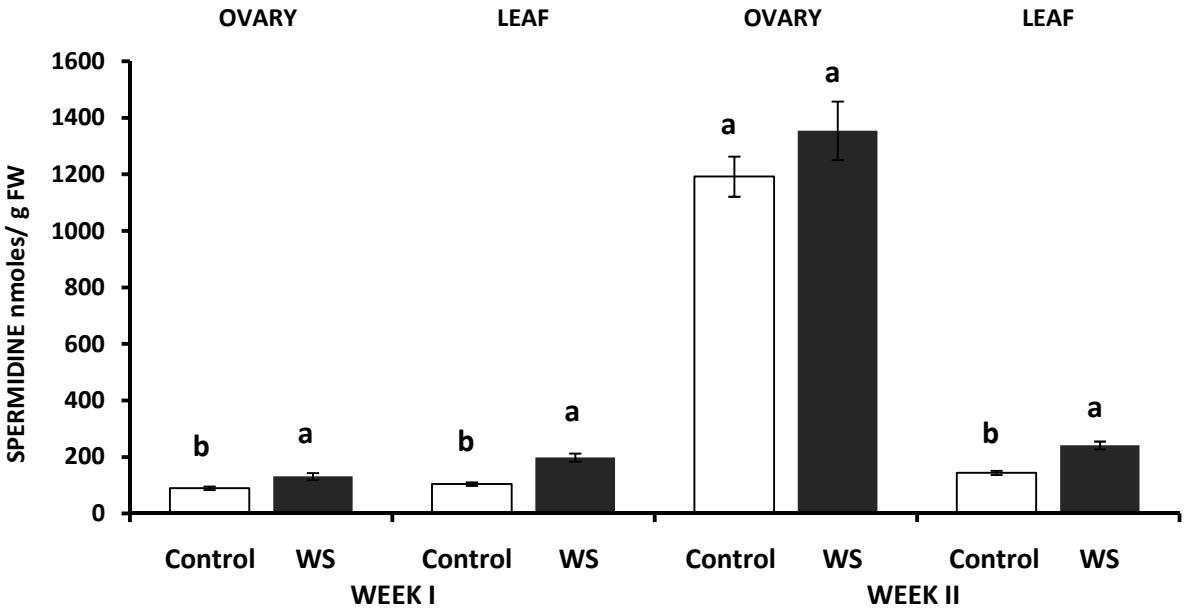

Figure 12: Effect of water deficit stress on ovary and leaf spermidine (SPD) concentrations in Lubbock at the end of the first and second week. Pairs of columns within each type of tissue connected with the same letter are not significantly different (P= 0.05). Error bars represent ±1 standard error.

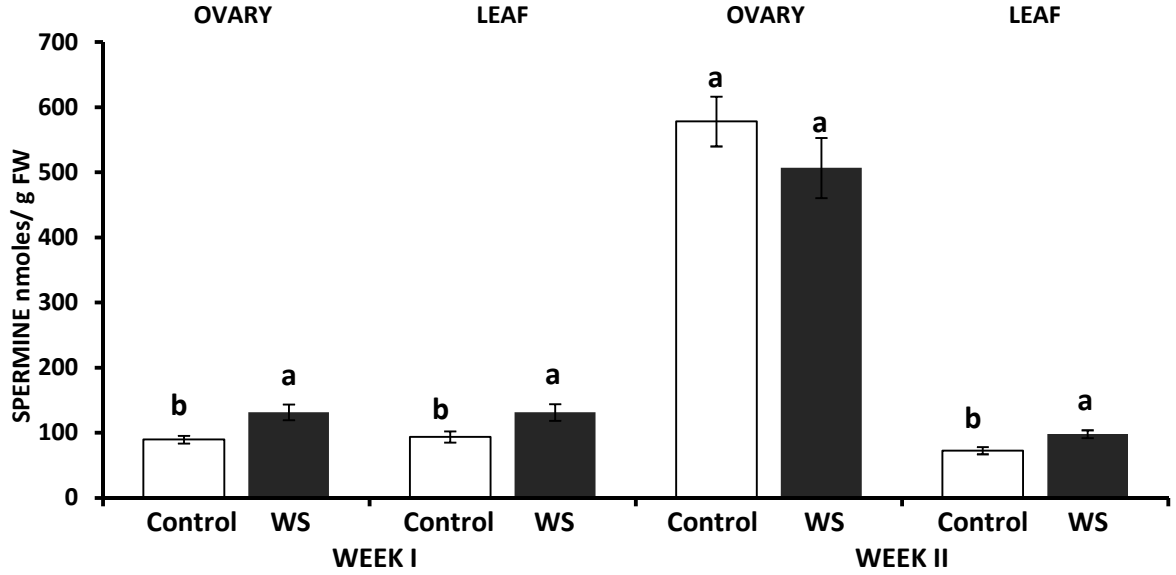

Figure 13: Effect of water deficit stress on ovary and leaf spermine (SPM) concentrations in Lubbock at the end of the first and second week. Pairs of columns within each type of tissue connected with the same letter are not significantly different (P= 0.05). Error bars represent ±1 standard error.

CHAPTER III

EFFECT OF 1-MCP ON GAS EXCHANGE AND CARBOHYDRATE METABOLISM OF THE COTTON FLOWER AND SUBTENDING LEAF UNDER WATER-DEFICIT STRESS

ABSTRACT

Ethylene, an endogenous plant hormone, has often been observed to increase under environmental stress conditions, resulting in abscission of leaves and fruiting forms and ultimately in yield reduction. In cotton, however, the effects of water-deficit stress on ethylene production have been uncertain. In this study it was hypothesized that application of an ethylene inhibitor 1-Methylcyclopropene (1-MCP) would prevent ethylene production and result in alleviation of water-deficit stress consequences on the physiology and metabolism of the cotton flower and subtending leaf. To test this, growth chamber experiments were conducted in 2009-2010 with treatments consisting of (C) untreated, well-watered control, (C +1MCP) well-watered plus 1-MCP, (WS) untreated, water-stressed control, and (WS+1MCP) water-stressed plus 1-MCP. The plants were subjected to two consecutive drying cycles during flowering, 8 weeks after planting, and 1-MCP was foliar applied at a rate of 10g. ai/ha at the beginning of each drying cycle. The results showed that 1-MCP application had no significant effect on gas exchange functions and did not prevent reductions in leaf photosynthesis, respiration and stomatal conductance. However, application of 1-MCP resulted in a decrease in sucrose concentrations of water-stressed pistils leading us to speculate that 1-MCP has the potential to interfere in carbohydrate metabolism resulting in a more efficient utilization of carbohydrates.

INTRODUCTION

Plant growth and crop yields are greatly affected in many areas of the world due to limited water supply (Kramer, 1983). Approximately one third of cultivated areas around the world are subjected to inadequate supplies of water (Massaci, et al., 2008), and the severity of the problem is expected to increase due to the changing climatic trends (Le Houerou, 1996). Advances in irrigation technology aid crops in achieving their potential yields, however, already low ground water supplies and increasing energy costs show that irrigation cannot be a sustainable solution.

Cotton (*Gossypium hirsutum* L.) is a perennial with a complex and indeterminate growth habit that originated in hot and arid areas (Lee, 1984), and is considered to be relatively drought tolerant due to defense mechanisms such as leaf and root osmotic adjustment (Oosterhuis and Wullschleger, 1987; Nepomuceno et al., 1998), accumulation of compatible osmolytes (Kuznetsov et al., 1999), production heat shock proteins (Burke et al., 1985) as well as greater water use efficiency compared to other crops (Ackerson et al., 1977b). However, drought tolerance traits are associated with cotton's indeterminate growth habit (Quisenberry and Roark, 1976), and due to its domestication and cultivation as an annual crop, modern cotton cultivars suffer significant yield decreases under conditions of limited water supply (Basal et al., 2005).

Ethylene is a plant growth hormone occurring abundantly in plants during each stage of growth and involved in numerous physiological function (Abeles, 1973). Even though ethylene synthesis is continually occurring at low rates under normal conditions, its production significantly increases under conditions of biotic or abiotic stresses such as high temperatures,

light quantity and quality, and water-deficit stress (Morgan et al., 1990; Morgan and Drew, 1997; Narayana et al., 1991). However, significant increases in ethylene evolution rates have also been reported to occur after flower pollination and fertilization (O'Neill, 1997) in several plant species creating confusion about the role of ethylene as a senescence inducing or growth promoting hormone. Recent findings that ethylene has a biphasial response, either as a growth inhibitor or a growth promoter, that is concentration dependent as well as tissue and species specific (Pierik, 2006) provides insight on the mode of action of ethylene, however considerable debate still exists concerning ethylene's response under adverse environmental conditions.

Water-deficit stress and ethylene interaction has always been the subject of much debate. Significant increases in ethylene quantities under conditions of water stress have been reported for a number of crops (Abeles, 1973; Stumpff and Johnson, 1987; Irigoyen et al., 1992) while on the contrary, many researchers have reported that water-deficit stress does not induce changes in ethylene evolution rates (Morgan and Drew, 1997, Klassen and Bugbee, 2003, Bugbee, 2011). These contradicting opinions are attributed to the lack of comparable results due to the variety of methods used to evaluate the ethylene response under water stress conditions (Morgan and Drew, 1997).

In cotton, early studies have shown water- deficit stress to cause abscission of fruiting forms and leaves after relief of the stress (Stockton et al., 1961). Beyer and Morgan (1971) reported that increases in ethylene concentrations of cotton cotyledons resulted in a decrease of auxin transport and subsequent abscission, while Lipe and Morgan (1972a) subjected plants to water-stress and reduced atmospheric pressure and they observed a decrease in the rate of abscission presumably due to the removal of some of the ethylene. McMichael et al. (1972) in

studies with intact petioles observed that water-deficit stress resulted in a significant increase in ethylene concentrations and reported a linear relationship between water deficit and leaf abscission, even though a relationship between water deficit and ethylene production rates was not established. Additionally, Jordan et al. (1972) suggested that water-deficit resulted in ethylene mediated abscission either due to increased ethylene production or due to increased ethylene sensitivity. Further research by McMichael et al. (1973) showing a linear relationship between water deficit and fruit abscission concluded that increased ethylene production under conditions of water deficit were responsible for impairing auxin transport and ultimately resulting in abscission. This was confirmed by Guinn (1976) who in studies with detached and intact 4-day old cotton bolls, reported that ethylene rates of dehydrated detached and intact cotton fruits were higher compared to the control, however detached bolls produced more ethylene than intact bolls. Morgan et al. (1990) in experiments with intact plants concluded that ethylene rates were decreased under conditions of water stress, and suggested that ethylene production depends on the rate that the stress is imposed, with quick osmotic shocks causing ethylene production, while slow occurring water-deficits did not. Those findings were confirmed by Bugbee et al. (2011) and Klassen and Bugbee (2003) although, no distinction was made for ethylene production between vegetative and reproductive units or at different growth stages. Limited water conditions affect vegetative and reproductive tissues differently (Van Iersel and Oosterhuis, 1996). Additionally, Lipe and Morgan (1972b) reported that ethylene production by cotton plants showed two peaks during growth, one at the day of anthesis and one prior to fruit dehiscence. Observations in other crops, such as carnations (*Dianthus caryopohyllus* L.) (Nichols, 1976), wheat (*Triticum aestivum* L.) (Yang et al., 2004) and

maize (*Zea mays* L.)(Andersen et al., 2002), where high ethylene concentrations appear to increase sucrose accumulation and consequently impede grain filling rate or stimulate ovary abortion only emphasize the need for more information about the role of ethylene in cotton reproductive units the day of anthesis.

Ethylene biosynthesis inhibitors such as silver, silver thiosulfate (STS), and aminoethylvinylglycine (AVG) as well as blockers of ethylene receptors have provided valuable help in ethylene research. 1-Methylcyclopropene (1-MCP), an ethylene inhibitor that acts by binding on ethylene receptors (Sisler and Serek, 1997) and reducing plant sensitivity to ethylene has been shown to result in a decrease or a delay of the ethylene activity and symptoms (Blankenship and Dole, 2003). Kawakami et al. (2010) observed that application of 1-MCP on 4 week-old water-stressed plants resulted in an increase in stomatal resistance, water potential and activity of antioxidant enzymes superoxide dismutase and glutathione reductase. Additionally, water-stressed plants treated with 1-MCP had decreased membrane leakage compared to the control (Kawakami et al., 2010). On the contrary, da Costa and Cothren (2011) reported that 1-MCP had no effect on gas exchange, chlorophyll content and dry matter partitioning of water-stressed cotton plants. Accordingly, the increase in the number of reproductive nodes that was observed in 1-MCP treated water-stressed plants did not result in higher yield since 1-MCP caused higher fruit abscission (da Costa and Cothren, 2011), however, no data exist on the effect of 1-MCP on the biochemistry of the cotton flower under water-deficit stress conditions. The objective of these studies was to evaluate the possible ameliorating effect of the anti-ethylene plant regulator, 1-MCP on cotton's floral buds and subtending leaves under conditions of limited water supply during reproductive development.

It was hypothesized that application of 1-MCP would prevent ethylene production and result in alleviation of water-deficit stress effects on the cotton flower and consequently prevent yield loss.

MATERIALS AND METHODS

Growth chamber studies were conducted and repeated at the Altheimer Laboratory, University of Arkansas, during 2009-2010. Cotton (*Gossypium hirsutum* L.,) cultivar ST 5288B2F was planted in 2L pots containing Sunshine potting media mix#1 (SunGro Distribution Inc., Bellevue, WA). Pots were arranged in a growth chamber (Conviron PGW36, Conviron Inc., Winnipeg, Canada) that was equipped with incandescent and fluorescent lamps and set for a 12h photoperiod with a photosynthetic flux density (PPFD) of 800-850 µmol/m²s and a relative humidity of 60%. Normal day/night temperatures of 32/24ºC (maximum during the day and minimum during the night) were imposed throughout the duration of the experiments simulating a normal diurnal variation. All pots received half-strength Hoagland's nutrient solution daily to maintain adequate nutrients and water until flowering, approximately eight weeks after planting and induction of water-deficit stress and 1-MCP application treatments, after which plants were watered only with deionized water. The experiments were arranged in a completely randomized design with two factors that consisted of water-deficit stress and 1-MCP application. The experimental unit was a single plant and fifteen replications were used. Application of 1-MCP started approximately eight weeks after planting and the treatments consisted of: Untreated well-watered control (C), Control +1-MCP (C+1MCP), Untreated water stressed (WS), and Water Stress +1-MCP (WS+1MCP).The rate of 1-MCP was 10g a.i./ha.

Control plants received optimum quantities of water during the experiment. Optimum quantity was determined by weighing the plants the day before and after watering to saturation and allowing for excess drainage. A water-stress cycle consisted of withholding water from the pots until visual wilting point, after which the stress was relieved by re-watering with optimum quantity. This process was repeated twice. 1-MCP was applied with a CO_2 backpack sprayer calibrated to deliver 187 l/ha two days after water supply had been discontinued (day 2 and day 8). The adjuvant AF-400 (Rohm Hass, Philadelphia, PA) was used for all 1-MCP applications at 0.375%v/v.

Stomatal conductance measurements

Stomatal conductance measurements were taken daily from the fourth uppermost main-stem leaf (n=10) from 11 :00 a.m. (five hours into the photoperiod) until 1:00 p.m. using a Decagon SC-1 Porometer (Decagon Inc. , Pullman, WA). Due to the small surface area of the cuvette (6.25cm^2), three measurements on various areas of the leaf were taken and then averaged. The results were expressed as $mmol/m^2s$.

Photosynthesis and respiration measurements

A Li-Cor Model 6200 portable photosynthesis system (LICOR Inc., Lincoln, NE) was used to determine photosynthetic and respiratory rates for the attached, fourth main-stem leaf from the terminal of the plant (n=10). Photosynthesis measurements were taken at 1:00 p.m. one and four days after spraying. Respiratory rates were taken at 2:00 p.m. one and four days after spraying after turning off the lights in the growth chambers for 15 minutes and additionally covering the plant with a black cloth during the measurement.

Carbohydrate Content Measurements

White flowers and their subtending leaves were collected the last two days of each drying cycle at noon. Soluble carbohydrate content was measured according to a modification of the Hendrix (1993) protocol. Ten white flowers and their subtending leaves were sampled from each treatment and they were oven dried for 3 days at 50°C and then ground with a mortar and pestle. The ground tissue was extracted 3 times with 80% aqueous ethanol (800ml ethanol /L) and the samples were centrifuged after each extraction at 5000 rpm and finally the fractions were pooled, while the remaining pellet was used for the determination of starch content. Active charcoal was then added to the pooled fractions to remove substances that could interfere with the carbohydrate measurements and the samples were centrifuged again at 3500 rpm. The supernatant was immediately stored at -80ºC for later determination of sucrose and hexose (fructose and glucose) with a MultiScan Ascent Microplate Reader (Thermo Fisher Scientific Inc., Waltham, MA). A glucose HK-assay kit (Sigma Chemical Company, St Louis, MO) was used. A 10µl aliquot of each extract was pipetted into a well of a microtitration plate and the plate was incubated at 50°C for 40 min to evaporate ethanol. Ten microliters of water were then added to each well along with 100 µl of glucose assay reagent and the plate was incubated again for 15min at 30°C. The absorbance was measured three times at 340 nm using a microplate reader. Subsequently, 0.25 enzyme units of phosphoglucose isomerase was added to the extracts in each well of the plate and the absorbance was again measured at 340nm after which, 83 enzyme units of invertase were added to the extracts and the microtitration plate was incubated at 30°C for 60 min. The absorbance was measured three times at 340nm.

Statistical analysis

A two factor factorial statistical analysis with fifteen replications was used to evaluate the results using JMP8 software (SAS Institute, Cary, NC). The factors consisted of experiment, water regime, and 1-MCP application. No interaction was observed between the two separate experiments, so the results were pooled and the means were taken. Analysis of variance and student's t-test were used to analyze statistical significance. The days of the experiment were not considered a factor and a single ANOVA was done for each day to compare differences among treatment combinations.

RESULTS

Stomatal conductance

Leaf stomatal conductance was significantly lower in water-stressed plants compared to the control (Fig. 1) in both watering cycles of the study. Stomatal conductance began to decrease 3 days after water supply was stopped, reaching its lowest (visual wilting point) at 48mmol/m²s for untreated water-stressed plants and 42 mmol/m²s for 1-MCP treated water-stressed plants in 6 days. However, upon re-watering, stomatal conductance measurements returned to same levels as the control. No significant interaction ($P=0.1043$) was observed between 1-MCP application and water-deficit stress regime in any day of the experiment while a significant effect of the water regime ($P<0.001$) was observed on days 2, 3, 4, 5, 7, 8, 9, 10 and 11 with water-deficit stress significantly lowering stomatal conductance rates compared to the

control. On the other hand, stomatal conductance rates of 1-MCP treated water-stressed plants were similar to the rates of untreated water-stressed leaves and significantly lower compared to the control.

Photosynthesis and respiration measurements

Photosynthetic rates for the fourth main-stem leaf from the terminal were significantly decreased under conditions of water-deficit stress compared to the control (Fig.2). 1-MCP appeared to have no significant effect on preventing reductions in photosynthetic rates under water-limited supply, since photosynthetic rates showed 43% decrease without 1-MCP application, and 41% decrease after 1-MCP application, compared to the control, respectively.

A similar trend was observed for the respiration rates of the fourth main-stem leaf from the terminal (Fig. 3). Again water-limited supply caused a significant reduction in the respiratory rates in the water-stressed leaves compared to the control. Similarly to stomatal conductance and photosynthesis, 1-MCP had no significant ameliorating effect on the respiration rates under water stress, with respiration rates decreasing 35% compared to the control without application of 1-MCP, and 42% compared to the control after 1-MCP application.

Carbohydrate content measurements

Water-deficit stress caused a significant increase in sucrose concentrations of the pistil (Fig.4) however, sucrose concentrations of the subtending leaf remained unaffected (Fig. 5). On the other hand, glucose levels of the pistil remained at the same levels as the control (Fig.6), whereas a significant increase was observed in the glucose concentrations of the water-

stressed subtending leaves compared to the control (Fig. 7). Both fructose levels of both the pistil (Fig. 8) and the subtending leaf (Fig.9) remained unaffected under conditions of water-deficit stress and their levels were similar to the control.

Application of 1-MCP resulted in a significant decrease in sucrose concentration of the pistils under conditions of water-deficit stress as well as a decrease in glucose levels of water-stressed subtending leaves, however non-significant. No effect of 1-MCP application was observed in pistil glucose concentrations and leaf sucrose levels, while both leaf and pistil fructose levels were unaffected by 1-MCP application.

DISCUSSION

Significant reductions of stomatal conductance were observed under conditions of water-deficit stress in our study. Water-deficit stress has long been known to decrease stomatal conductance by inducing abscisic acid (ABA) accumulation in the guard cells of stomata (Hsiao, 1973) in order to prevent excess water loss from the plants. Apart from ABA however, ethylene has also been reported to decrease stomatal conductance (Pallas and Kays, 1982; Vitogliano and Hoad, 1978). In cotton, Bielorai et al. (1975) reported that in potted experiments stomatal conductance was significantly decreased under water-limited conditions, while Ackerson et al. (1977a) observed that leaf stomatal conductance of field-grown cotton was slightly affected and leaf stomata did not completely close even under very low water potentials and those differential responses were attributed to the differences in light intensity as well as on the more gradual imposition of water-deficit stress in the field. However, Radin and Ackerson (1981) reported that in potted experiments water-deficit stress resulted in significant increases

in ABA leaf concentrations and concluded that ABA was a key factor controlling stomatal function under conditions of water-deficit stress. Further research by Ackerson (1982) revealed that ABA continued to accumulate in the guard cells of stomata in cotton leaves long after stomatal closure had occurred, indicating that there is another possible role for ABA apart from stomatal closure. In support of this observation, Sharp (2002) suggested that increases in ABA concentrations have as a focus not only to protect the plant from excessive water loss, but also to prevent increases in ethylene evolution rates.

In our experiments application of 1-MCP resulted in no significant effect on the stomatal conductance of water-stressed plants. Similar results were reported by Klassen and Bugbee (2003), Bugbee (2011) as well as da Costa and Cothren (2011) while Kawakami et al. (2010) observed that 1-MCP treated water-stressed plants exhibited higher stomatal resistance. Even though leaf ABA concentrations were not determined in our study, we speculate that the lack of 1-MCP effect on leaf stomatal conductance rates in our study was either due to the lack of water-deficit stress on ethylene evolution rates (Klassen and Bugbee, 2003) or a result of increased ABA concentrations in the leaf that prevented ethylene evolution rates from increasing, while the opposite results reported by Kawakami et al. (2010) are attributed to the different plant growth stage and consequently the difference in ABA and ethylene evolution rates (Verelst et al., 2010).

Similarly to stomatal conductance rates, water-deficit stress resulted in significant decreases of leaf photosynthetic and respiratory rates in our study. Leaf photosynthetic rates have been reported to decrease as relative water content or water potential decrease (Lawlor and Cornic, 2002).Unlike photosynthesis, diverse opinions have been expressed on the effect of

water-deficit stress on respiration. According to Atkin et al. (2009) water-deficit stress effects on respiration are variable depending on the type and the age of tissue, the duration and severity of stress, changes in activity of respiratory enzymes, substrate availability as well as ATP demand. Flexas et al. (2006) pointed out that the percentage of daily fixed carbon that is respired is expected to be higher in water-stressed plants mainly because of the inhibitory effect that water deficit has on photosynthesis. De Vries et al. (1979) observed that while respiration rates remained unaffected at low or moderate water stress, they decreased at severe water stress. A similar pattern was also reported in cotton by McCree et al. (1984) and in soybean (*Glycine max* L.) Ribas-Carbo et al. (2005). On the contrary, Ghashgaie et al., (2001) noticed an increase in respiration rates under severe water stress, while Lawlor and Fock (1977) reported no change.

In cotton, decreases of photosynthetic rates due to water-deficit stress have been reported by a number of researchers (e.g. Lacape et al., 1998; Pettigrew, 2004, da Costa and Cothren, 2011), while regarding respiration Pallas et al. (1967) reported a biphasic response of cotton leaf respiration rates where rates initially decreased with increasing severity of the water stress and eventually increased at more severe stress, while Wullschleger and Oosterhuis (1987) reported that boll respiration significantly decreased under conditions of severe water stress. Our results indicated significant decreases in photosynthetic rates of water-stressed leaves compared to the control in accordance with the aforementioned reports. Respiration's similar decreasing rates are in agreement with the findings of DeVries et al. (1979), McCree et al. (1984), Ribas-Carbo et al. (2005) and Wullschleger and Oosterhuis (1987). The opposite results reported by Pallas et al. (1967) we speculate was due to the different growth stage of

the plants in their experiment. Application of 1-MCP failed to prevent reduction of both photosynthetic and respiratory rates in our study similar to the findings of Bugbee (2011) and da Costa and Cothren (2011). Even though ethylene has been reported to be implicated in photosynthesis and respiration in other crops (Adato and Gazit, 1974; Hanson and Kende,1975; Pallas and Kays, 1982; Zhou et al., 1998) we speculate either that water-deficit stress had no effect on ethylene concentrations (Bugbee, 2011) or that ABA concentrations increased sufficiently to prevent ethylene increases (Sharp, 2002).

Our results indicated that leaf glucose concentrations were significantly increased under conditions of water-deficit stress, whereas fructose and sucrose concentrations remained at similar to the control levels. According to previous research the effect of water-deficit stress on the carbohydrate metabolism of cotton appears to be variable. Eaton and Ergle (1948) reported that water-stressed cotton leaves exhibited large reductions in starch concentrations compared to the control, variable sucrose levels and significant increases in hexose concentrations. Similar results were observed by Parida et al. (2007), while Ackerson (1981) in field experiments reported that higher quantities of starch were accumulated in water-stressed plants compared to the control. He also noted that sucrose export rate from non-acclimated plants was severely inhibited under conditions of water stress and speculated that water-deficit stress results in significant impairment of photosynthate translocation. In support of this observation, Timpa et al. (1986) recorded significant increases in leaf glucose levels of water-stressed plants indicating that the source-sink relationship is affected by drought, even though no significant differences were observed in leaf sucrose levels. Significant increases in leaf hexose concentrations under conditions of water-deficit stress have also been reported in other

crops such as soybean (*Glycine max* L.) (Liu et al., 2004), and barley (*Hordeum vulgare*, L.) (Teulat et al., 2001). Additionally, inhibition of photosynthate translocation under water limited conditions has been observed in sugarcane (*Beta vulgaris* L.) (Hartt, 1967), maize (Boyer and McPherson, 1975), and wheat (*Triticum aestivum*, L.) (Johnson and Moss, 1976). Liu et al. (2004) made a similar observation for soybean source-sink relationships and reported that sucrose and starch leaf concentrations decreased significantly under water stress which resulted in a decrease in the rate of sucrose export from the leaves. In our study water-deficit stress resulted in significant increases in leaf glucose concentrations, whereas leaf fructose and sucrose concentrations remained unaffected, which is in accordance with the majority of the aforementioned studies indicating that water-deficit stress results in increases in leaf carbohydrate concentrations.

A pattern similar to the leaf carbohydrate concentrations was observed in pistil carbohydrate content with the only difference being that pistil sucrose concentrations were significantly increased while pistil glucose and fructose remained at the same levels as the control. However, Guinn (1976) reported that no significant differences were observed in carbohydrate accumulation of 4-day old bolls that had been subjected to limited water supply. On the contrary, Zinselmeier et al. (1999) observed that an increase in sucrose concentrations in young water-stressed maize ovaries occurs simultaneously with the cessation of ovary growth and they reported that water-deficit stress had an inhibiting effect on sucrose cleaving enzymes such as invertase and sucrose synthase. Analogous results were reported by Saini and Westgate (2000), Schussler and Westgate (1995) as well as Liu et al. (2004) and Yang et al. (2004). Even though no significant change in carbohydrate concentrations of water-stressed 4-

day old cotton bolls were reported by Guinn (1976) our results are in accordance with Zinselmeier (1999) in maize and Liu et al. (2004) in soybean. It is suggested that the significant increases in pistil sucrose concentrations observed in the water-stressed plants in our study are due to down regulation of sucrose cleaving enzymes due to limited water supply, whereas the differential responses between leaf and pistil carbohydrate concentrations are attributed to tissue specific regulation of sucrose cleaving enzymes, with invertase being up-regulated in the leaves and down-regulated in the fruiting forms under conditions of water-deficit stress (Yang et al., 2004; Koch, 2004).

Another explanation for these differential responses, between the leaf and the pistil, is the interaction of ethylene with photosynthesis and photosynthate translocation under conditions of water-deficit stress (Zhou et al., 1998). Ethylene has also been observed to modulate carbohydrate metabolism not only through regulation of enzymes such as invertase or sucrose synthase (Rolland et al., 2006) but also through carbohydrate concentrations (Koch, 2004; Leon and Sheen, 2003). Furthermore, Rolland et al. (2006) reported that hexokinase was found to interact with ABA and ethylene signaling pathways while ethylene was observed to modulate sucrose concentrations of reproductive units in a number of crops (Saftner, 1986; Ishizawa and Esashi, 1988; Chervin et al., 2006). In support of these observations, reductions in grain filling rate and weight due to increases in ethylene under conditions of water stress have been reported in wheat (Xu et al., 1995) and rice (Yang et al., 2004), while Mohapatra et al. (2000) and Naik and Mohapatra (2000) observed that application of ethylene inhibitors on rice improved grain filling and enhanced sucrose synthase activity under conditions of water stress.

In cotton, Guinn (1976) reported no significant change in carbohydrate concentrations of 4-day old bolls under water-deficit stress, even though ethylene concentrations increased. In our study, application of 1-MCP resulted in a reduction of glucose concentration compared to untreated water-stressed glucose levels, however, not as low as the control. On the contrary, application of 1-MCP had a pronounced effect on pistil sucrose concentration, compared to the leaves, significantly inhibiting sucrose accumulation under water-stress compared to the untreated water-stressed plants. However, reductions in sucrose concentrations, even though not significant, were observed between the treated well-watered and the untreated well-watered. We speculate that the differential response of 1-MCP observed in cotton leaves and pistils is attributed either to the lower number of receptors existing in the leaves compared to the reproductive units (Burns, 2008) or to the differential regulation of sucrose cleaving enzymes due to tissue specific response of ethylene under conditions of water-deficit stress (Verelst et al., 2010).

In summary, the results of our study indicated that water-deficit stress during reproductive development resulted in significant decreases in cotton leaf stomatal conductance, photosynthesis and respiration. Application of 1-MCP failed to ameliorate the negative consequences of water-deficit stress on cotton gas exchange functions indicating that either ethylene evolution from the leaves is minimal under conditions of water stress or ethylene evolution is uncoupled from cotton leaf's gas exchange functions. However, the significant decrease in pistil sucrose concentrations and the non-significant decrease in leaf glucose content indicate that ethylene plays an important role in the regulation of carbohydrate metabolism in both vegetative and reproductive tissues. Further research is

needed to elucidate the exact site of modulation and factors controlling the interaction between ethylene production under water-deficit stress and cotton carbohydrate accumulation.

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FIGURES

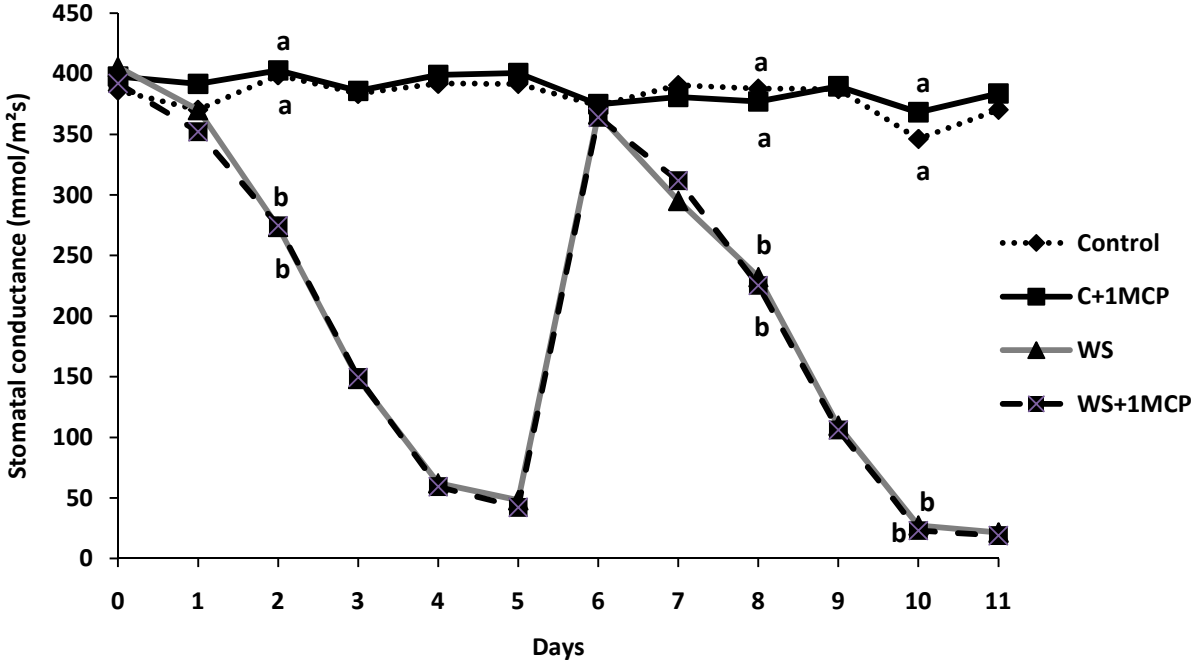

Figure 1: Effect of water-deficit stress and 1-MCP application on leaf stomatal conductance. Plants were imposed on two 5-day drying cycle. Points connected with the same letter are not significantly different (P=0.05). Water was withheld on day 0 and reapplied at the end of day 5. 1-MCP was applied on days 2 and 8.

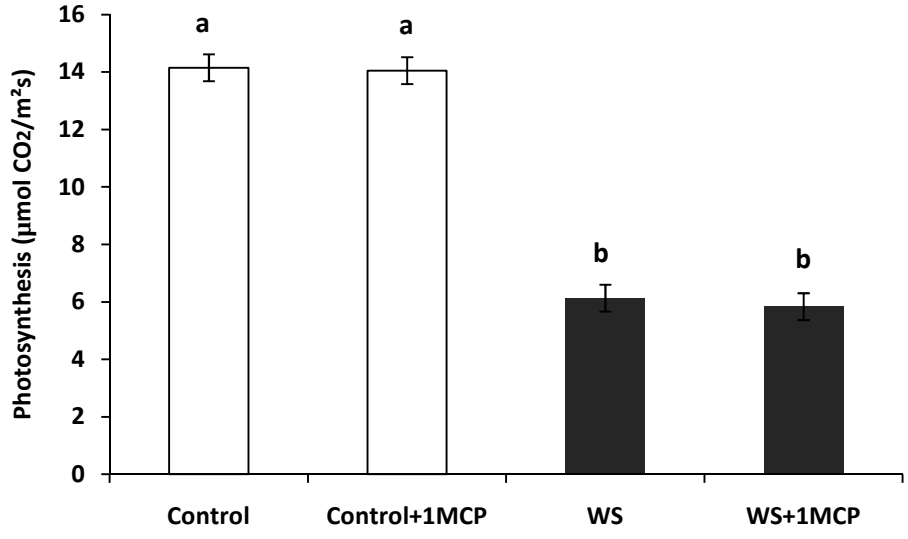

Figure 2: Effect of water-deficit stress and 1-MCP application on leaf photosynthesis 4 days after induction of stress. Bars with the same letter are not significantly different (P=0.05). Error bars represent ±1 standard error**.**

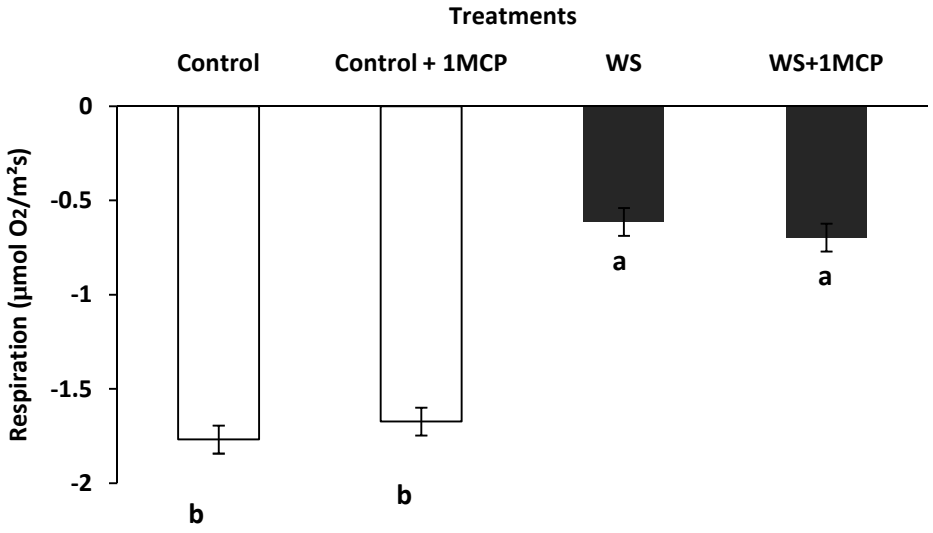

Figure 3: Effect of water-deficit stress and 1-MCP application on leaf respiration 4 days after induction of stress. Bars with the same letter are not significantly different (P=0.05). Error bars represent ±1 standard error.

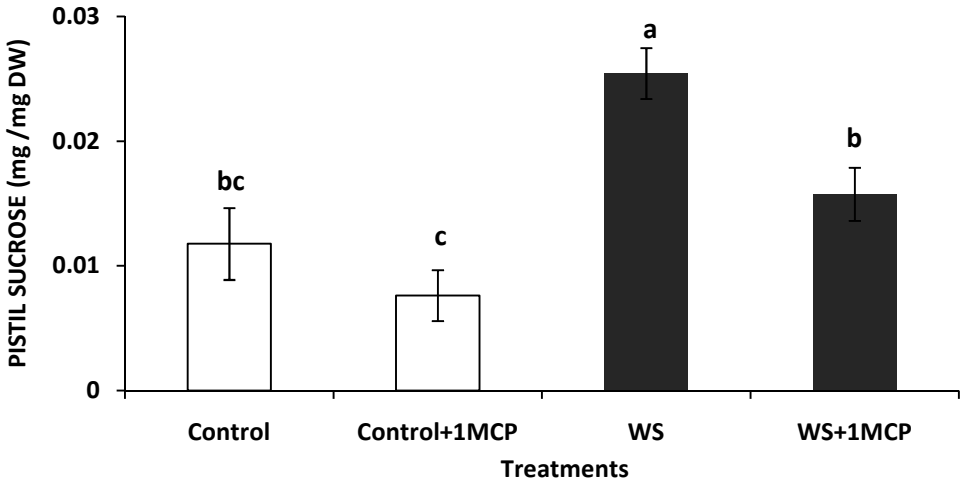

Figure 4: Effect of water-deficit stress and 1-MCP application on pistil sucrose content. Columns with the same letter are not significantly different (P=0.05). Error bars represent ±1 standard error.

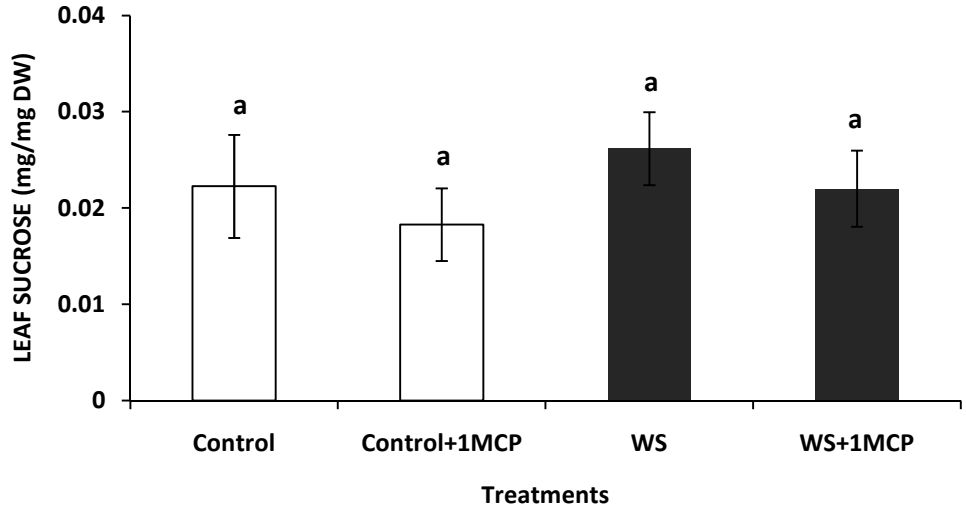

Figure 5: Effect of water-deficit stress and 1-MCP application on leaf sucrose content. Columns with the same letter are not significantly different (P=0.05). Error bars represent ±1 standard error.

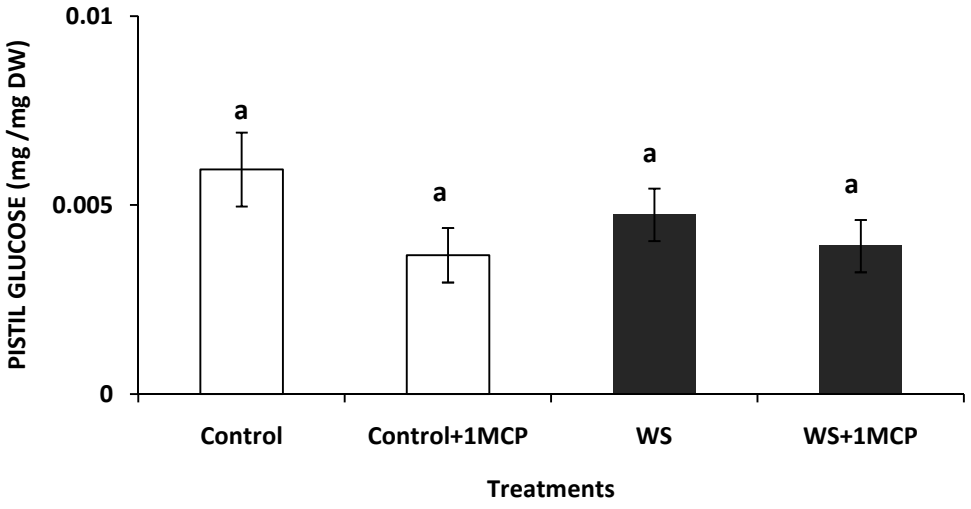

Figure 6: Effect of water-deficit stress and 1-MCP application on pistil glucose content. Columns with the same letter are not significantly different (P=0.05). Error bars represent ±1 standard error.

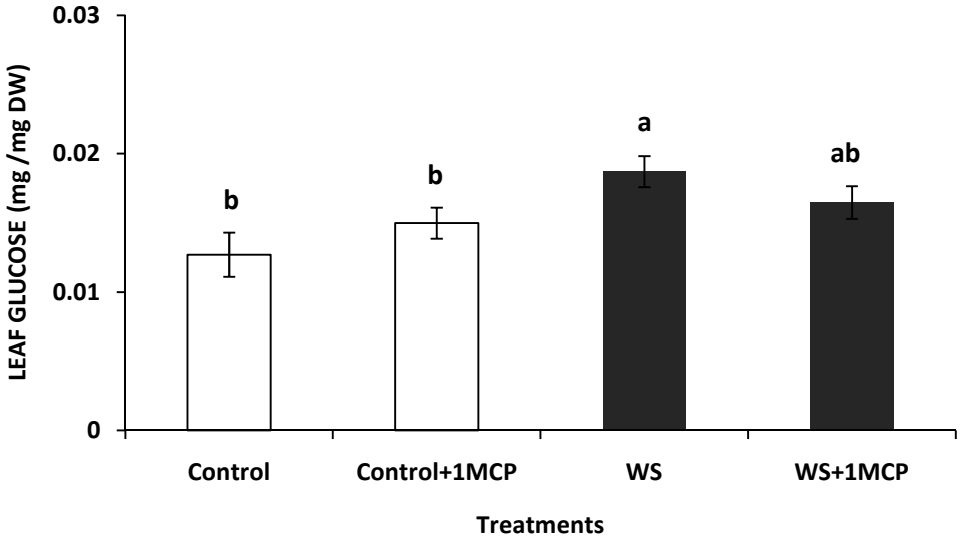

Figure 7: Effect of water-deficit stress and 1-MCP application on leaf glucose content. Columns with the same letter are not significantly different (P=0.05). Error bars represent ±1 standard error.

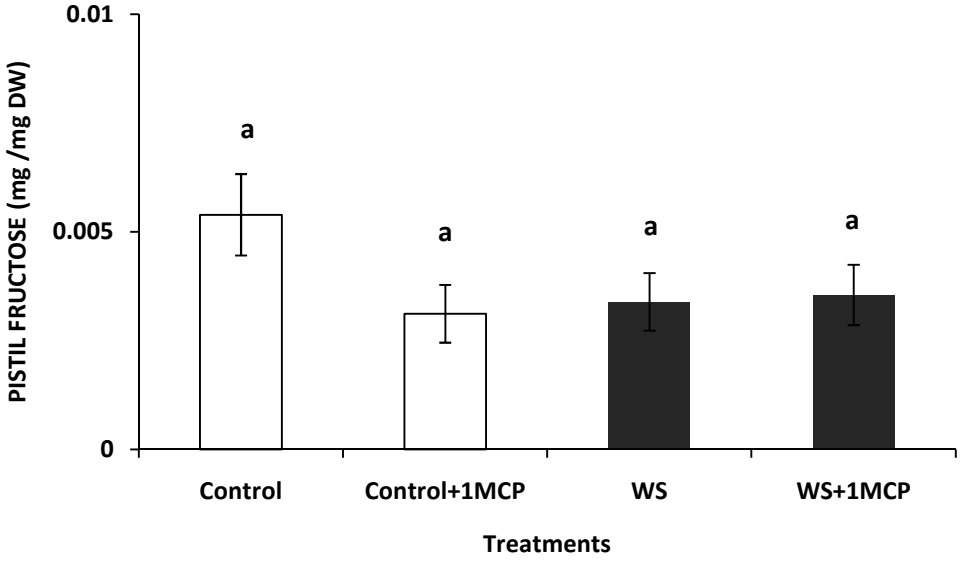

Figure 8: Effect of water-deficit stress and 1-MCP application on pistil fructose content. Columns with the same letter are not significantly different (P=0.05). Error bars represent ±1 standard error.

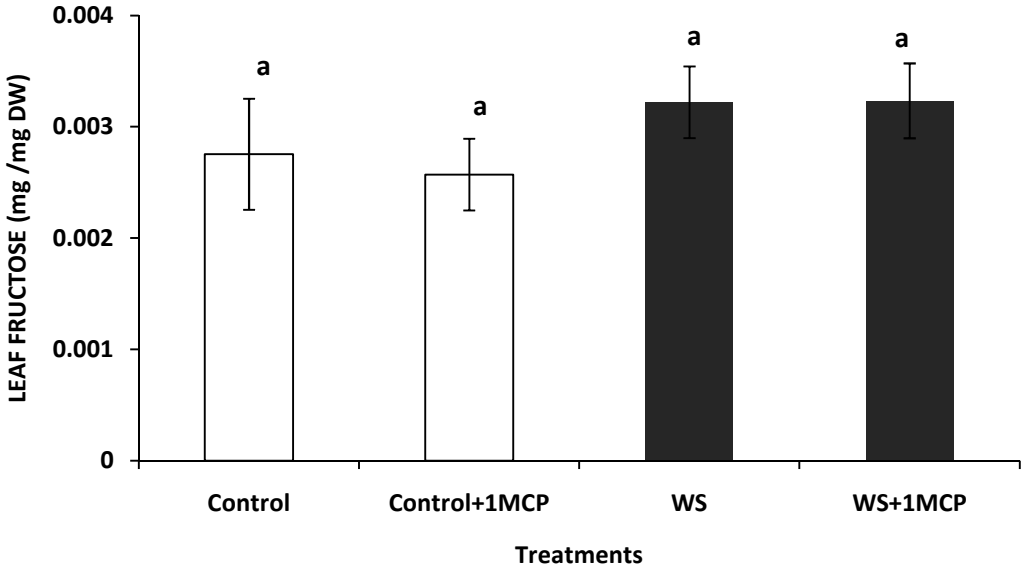

Figure 9: Effect of water-deficit stress and 1-MCP application on leaf frustose content. Colunns with the same letter are not significantly different (P=0.05). Error bars represent ±1 standard error.

CHAPTER IV

EFFECT OF WATER-DEFICIT STRESS ON POLYAMINE METABOLISM OF COTTON FLOWER AND THEIR SUBTENDING LEAF

ABSTRACT

Polyamines, putrescine (PUT), spermidine (SPD) and spermine (SPM) are ubiquitous components of all living cells. Apart from their participation in numerous physiological and metabolical functions of the plant, they are also implicated in plants' responses under conditions of abiotic stress. Previous research in other crops has indicated that polyamines and changes in their concentrations are associated with drought tolerance under conditions of water deficit stress, however no information exist on cotton (*Gossypium hirsutum* L.). Growth chamber experiments were conducted in 2011-2012, with two cotton cultivars differing in drought tolerance, ST 5288B2F (average drought-tolerant) and Siokra L23 (drought-tolerant) were planted in order to investigate the distribution of polyamines, the effect of water-deficit stress on the polyamine metabolism of cotton reproductive units and their subtending leaves as well as the possible relationship between polyamines and drought tolerance in cotton. According to our results cotton ovaries contained significantly higher levels of total polyamines compared to their subtending leaves under both control and water stress conditions. Water-deficit stress significantly increased PUT concentrations in ST5288, while SPM levels significantly decreased in Siokra L23. The results indicated that water-deficit stress significantly affected cotton polyamine metabolism in reproductive structures and their subtending leaves, however no clear relationship between drought-tolerance and changes in polyamine accumulation was established. Further research is needed in order to elucidate the mechanism according to which water-deficit stress affects polyamine metabolism.

INTRODUCTION

Polyamines (PA) are low-molecular-weight organic polycations with two or more primary amino groups -NH_2 and they are present in bacteria, plants and animals. They were discovered as early as 1678 (van Leeuwenhoek, 1678), however, their correct chemical composition and structure were only determined in the 1920's (Galston and Kaur-Sawhney, 1990).

In plants, the diamine putrescine (PUT) and its derivatives, the triamine spermidine (SPD) and the tetramine spermine (SPM) are the most common polyamines. PUT is produced either directly from ornithine or indirectly from arginine through ornithine decarboxylase (ODC) or arginine decarboxylase (ADC), respectively (Kao, 1997; Adiga and Prasad, 1985). The activities of ADC and ODC enzymes appear to regulate overall PUT biosynthesis in plants (Bagni and Tassoni, 2001) with ADC being associated with tissues undergoing cell expansion and ODC with tissues growing by cell division. The triamine SPD and tetramine SPM are synthesized from PUT and SPD, respectively, with addition of one aminopropyl moiety (Palavan-Unsal, 1995). The aminopropyl group, decarboxylated S-adenosylmethionine (dcSAM), is produced from S-adenosylmethionine (SAM) mediated by S-adenosylmethionine decarboxylase (SAMDC) (Bagni and Tassoni, 2001). Polyamines are degraded by diamine oxidases (DAO), most commonly found in dicots (Cona et al., 2006), or polyamine oxidases (PAO) that occur in high levels in monocots (Sebela et al., 2001). PAOs have been also reported to participate in back-conversion of SPM to SPD with simultaneous production of H_2O_2 (Moschou et al., 2008).

Due to their positive charge, depending on the ionic and pH conditions of the cells, polyamines occur in free forms, conjugated with phenolic acids or bound to macromolecules

such as proteins (Galston and Kaur-Sawhney, 1990; Kasukabe et al., 2004). Only free PAs have been observed to be translocated throughout the plant in the phloem and the xylem sap mainly as PUT (Antognoni et al., 1998) and their concentrations are dependent not only on their synthesis but also on their translocation, conjugation and degradation (Groppa and Benavides, 2008).

Polyamines' versatility has them implicated in a variety of plant functions, either physiological or metabolic. These functions include photosynthesis and stomatal conductance, enzyme activation and maintenance, cell division and differentiation, morphogenesis and embryogenesis as well as organogenesis (Evans and Malmberg, 1989; Galston et al. 1997; Kakkar and et al., 2000; Konigshofer and Lechner, 2002; Ioannidis and Kotzabasis, 2007). Correct conformation of nucleic acids, membrane stabilization and ion channel regulation, gene expression and translation, hormone action mediation, as well as heat shock proteins and macromolecule synthesis are also characterized by PA participation (Kumar et al., 1997; Malmberg et al., 1998; Bouchereau et al., 1999; Liu et al., 2000; Alcazar et al., 2006b, 2010b; Kusano et al., 2008; Groppa and Benavides, 2007). However, PA role in reproductive development appears to be more than mere participation due to the significant increase in their concentrations as plants transition from their vegetative to reproductive stage of growth (Kakkar and Rai, 1993). Kloareg et al. (1986) indicated that PAs are indispensable to plants at the time of flowering and early fruit development. In support of this observation, experiments with PA-deficient mutants or mutants with unbalanced PA metabolism resulted in abnormal growth and flowering patterns as well as delayed flowering (Galston et al., 1997; Kakkar and Sawhney, 2002; Alcazar et al., 2005). Flower induction also includes PA function (Evans and

Malmberg, 1989; Faust and Wang, 1992; Bagni et al., 1993; Bouchereau et al., 1999; Kakkar and Sawhney, 2002) along with flower initiation (Kaur-Sawhney et al., 1988), pollination (Falasca et al., 2010), fruit growth and ripening (Kakkar and Rai, 1993) while sexual differentiation of tissues is reported to be strongly dependent on PA biosynthesis and catabolism (Martin-Tanguy et al., 1985; Martin-Tanguy, 1997).

Apart from flowering, PAs have been reported to play a fundamental role in regulation of post-fertilization development (Galston, 1983; Slocum and Galston, 1985; Lin, 1984; Evans and Malmberg, 1989). Numerous reports on PA and their role in stimulating fruit set and fruit development exist, i.e., in apple (*Malus domestica* L.) (Costa and Bagni, 1983; Biasi et al., 1991), pear (*Pyrus communis* L.) (Crisosto et al., 1986; Crisosto et al. 1988), pepper (*Capsicum annuum* L.) (Serrano et al., 1995), olive (*Olea europaea* L.) (Rugini and Mencuccini, 1985), mango (*Mangifera indica* L.) (Singh and Singh, 1995), tomato (*Lycopersicon esculentum*) (Antognoni et al. 2002), and strawberry (*Fragaria amanassa* Dutch.) (Tarenghi and Martin-Tanguy, 1995).

After the first observation of PUT accumulation in barley leaves under potassium deficiency (Richards and Coleman, 1952) investigations in a number of plant species has shown that changes in PA concentrations is a common plant response to a variety of abiotic stresses, including salinity (Maiale et al., 2004; Roy et al., 2005; Liu et al., 2006), high or low temperatures (Song et al., 2002; Hummel et al., 2004; Imai et al., 2004) and drought (Capell et al., 1998; Capell et al., 2004; Kasukabe et al., 2004; Ma et al., 2005) as well as biotic stresses (Walters, 2003). Experiments with cultivars differing in stress tolerance have shown that stress-tolerant plants have generally the ability to increase PA biosynthesis as a response to stress and enhance endogenous PA levels compared to the unstressed plants (Shen et al., 2000; Kasukabe

et al., 2004; Yang et al., 2007). The opposite or no changes in PA levels have also been observed (Zhang et al., 1996; Lazcano-Ferrat and Lovatt, 1999). Interestingly, apart from the endogenous PA, application of exogenous PA has been reported to increase stress tolerance in plants (Lutts et al., 1996; Liu et al., 2007; He et al., 2008), while application of PA biosynthesis inhibitors has the opposite effect (Lee et al., 1997; He et al., 2002; Liu et al., 2004). Furthermore, research with transgenic plants transformed to increase PA production has reported that transformed plants have enhanced stress tolerance compared to their respective wild types (Roy and Wu, 2001; Kumria and Rajam, 2002; Kasukabe et al., 2006; Alcazar et al., 2010b, Hussain et al., 2011). Regarding specifically water-stress, PA have been observed to enhance drought tolerance, functioning either as protective agents, due to their ability to stabilize macromolecules and act as reactive oxygen species scavengers (Kuznetsov et al., 2007) or as signaling molecules through their degradation product H2O2 and its connection to ABA (Alcazar et al., 2006a). Changes in endogenous PA levels under conditions of water-deficit stress have been reported in a number of crops such as rice(*Oryza sativa* L.) (Capell et al., 2004, Yang et al., 2007), oat (*Avena sativa* L.) (Flores and Galston, 1984), wheat (*Triticum aestivum* L.) (Liu et al., 2006), Arabidopsis (*Arabidopsis thaliana* L.) (Alcazar et al., 2010a,b), soybean (*Glycine max* L.) (Nayyar and Chander, 2004) and jack pine (*Pinus banksiana* L.) (Rajasekaran and Blake, 1999). Exogenous application of PA has also been reported to be beneficial, as well as overexpression of PA biosynthesis genes (Kasukabe et al., 2004; Farooq et al., 2008).Thus, it is evident that PA could be used not only for selection but also for creation of drought-tolerant cultivars through manipulation of their metabolism.

Cotton (*Gossypium hirsutum* L.), is a relatively drought tolerant crop since its wild, perennial ancestors originated in hot and dry areas (Lee, 1984). However, cotton modern cultivars that are cultivated as annuals appear to be less drought- tolerant since water-deficit stress occurring at any stage of development, but especially during flowering, results in significant compromises in the morphology, physiology, metabolism and ultimately yield (Grimes et al., 1969; Gerik et al., 1996; Pettigrew, 2004). Since drought is the major abiotic factor, already affecting 30% of cultivated areas around the world and projections anticipate that water-stress episodes are going to intensify in the future due to increased greenhouse gas concentrations, tools to help with drought-tolerant selection genotypes are greatly needed. PA metabolism is an enticing target, however, despite the extensive research on other crops, limited information on PA metabolism exists for cotton with the only reports being on the distribution of polyamines in the cotton plant (Bibi et al., 2011) polyamine content just prior to rapid fiber elongation (Davidonis, 1995), the effect of heat stress on PAs (Bibi et al., 2010), and the occurrences of uncommon polyamines (norspermidine, norspermine, pentamine, and hexamine) (Kuehn, et al., 1990).

The purpose of this study was to investigate the changes in PA concentrations in first day flower ovaries and their subtending leaves under conditions of water deficit stress by using two cultivars differing in drought tolerance in order to determine whether PAs are involved in drought tolerance.

MATERIALS AND METHODS

Cotton (*Gossypium hirsutum* L.) cultivars ST5288B2F and Siokra L23 were planted in February 2011 at the Altheimer Laboratory, University of Arkansas into 1 L pots containing Sunshine potting media mix#1 (SunGro Distribution Inc., Bellevue, WA). The growth chamber (Conviron PGW36, Conviron Inc., Winnipeg, Canada) equipped with incandescent and fluorescent lamps were set for a 14-h photoperiod with a photosynthetic photon flux density (PPFD) of 800-850 μmol/m²s and a relative humidity of 60%. Cotton was grown under normal day/night temperatures of 32/24ºC and all pots received half-strength Hoagland's nutrient solution daily to maintain adequate nutrients and water. Irrigation was withheld at flowering (8 weeks after planting) until plants were visually wilted which after plants received 50% of their daily use of water for ten days.

Stomatal conductance measurements

Stomatal conductance measurements were taken daily from the fourth uppermost main-stem leaf (n=10) and the subtending to the flower leaf, whenever flowers were available, from 11 :00 a.m. (five hours into the photoperiod) until 1:00 p.m. using a Decagon SC-1 Porometer (Decagon Inc., Pullman, WA). Due to the small surface area of the cuvette (6.25 cm^2), three measurements on various areas of the leaf were taken and then averaged. The results were expressed as mmol H_2O/m^2s.

Photosynthesis measurements

A Li-Cor Model 6200 portable photosynthesis system (LICOR Inc., Lincoln, NE) was used to determine photosynthetic rates of the attached, fourth main-stem leaf from the terminal of the plant(n=10). Measurements of photosynthesis and respiration were taken at 12:00 pm the fifth and tenth day of the stress and the results were expressed as µmol CO_2/m^2s.

Sampling

First day cotton flowers and their subtending leaves were sampled whenever they were available through the duration of the experiment.

Polyamine extraction and analysis

Polyamines were extracted according to Smith and Davies (1985) with modifications. Cotton ovary and leaf tissue, 0.1 and 0.2 g respectively, were excised and homogenized in mortars with pestles in 0. 2N $HCLO_4$. To monitor the extraction and quantification procedure unfortified and fortified samples were prepared. For unfortified samples 100 µl of 1mM hexamethylenediamine in 0.2 N $HCLO_4$ was added to the tissue prior to homogenization as an internal standard. The final volume of 2ml was obtained by adding 1900 µl 0.2 N $HCLO_4$. For thefortified samples that contained a certain amount of the three polyamines, 100 µl hexamethylenediamine 1 mM in 0.2 N $HCLO_4$ was added plus the desired volume of fortification solution in 0.2 N HCLO4 which was 120 µl 1mM putrescine in 0.2 N $HCLO_4$, 120 µl 1mM spermidine in 0.2 N $HCLO_4$, and 120 µl 1mM spermine of 0.2N $HCLO_4$. The final volume of

2ml was obtained by adding 1540 µl of 0.2 N $HCLO_4$. An aliquot of 1.5 ml of the homogenate was transferred to 2 ml micro centrifuge tubes and the samples were centrifuged at 4°C for 20 min at 14000 rpm. The supernatant was collected and used for dansylation of polyamines.

The polyamines were derivatized by adding 100 µl aliquots of the supernatant to 1000 µl 21.2 mM of aqueous Na_2CO_3, 400 µl of 99.9% acetone and 50 µl of 12.5mM and 100 µl of 87.5 mM of dansyl chloride in acetone. The mixture was incubated in a thermal reaction block at 60°C for 1h in the dark. After 1h in the thermal block, the samples were removed and cooled to near room temperature, and 100 µl 1N $HCLO_4$ were added to the mixture and mixed. The samples were then centrifuges at 4°C for 20 min at 14000 rpm, after which 500 µl of centrifugate were transferred into 2ml sample vial and 500µl of 0.02N $HCLO_4$ were added. The samples were capped and mixed before injection into the High Performance Liquid Chromatography (HPLC). Derivatization needs to be in a basic solution, whereas the final solution for HPLC needs to be acidic.

A total of 5 standards were used for the preparation of the standard curves. The standards included putrescine, spermidine, spermine and the internal standard hecamethylenediamine. The concentrations of putrescine and spermidine in the five standards ranged from 5 to 30nmoles/ml, whereas the concentrations of spermine ranged from 10 to 60 nmoles/ml. A 500µl aliquot of hexanemethylenediamine was added to the standards. All standards were brought to a final volume of 10 ml with 0.2 N HCLO4.

HPLC analysis was performed using a Hitachi HPLC (Hitachi High Technologies America, Inc., Canada) system that included a model L-7100 pump, and L-7200 autosampler, a D-7000 interface, and an ERC-3415a degasser and an L-7480 fluorescence detector. The column used in

this analysis was a 25cmx2mm, i.d. 0.5 micron Phenomenex Gemini C18. Injection volume was 50 µl. Polyamines were eluted from the column at 0.3 ml/min with methanol :water (v/v) gradient from 70% methanol to 95% methanol over 6 min and then remaining at 95% methanol for 16.4 min. The system was re-equilibrated with 70% methanol for 15min before the next injection. For dansyl polyamines, an excitation wavelength of 510 nm. Data collection and processing were with Hitachi System Manager (HSM) software on the internal standard concentration.

Statistical analysis

A two factor factorial statistical analysis with the main factors being water regime and cultivar and fifteen replications, completely randomized, in each treatment was used to evaluate the results using JMP8 software (SAS Institute, Cary, NC). Similar trends were observed between the two growth chamber studies so the results were pooled and the means were taken. Interactions and main effects were tested with Analysis of Variance (ANOVA) at $\alpha \leq 0.05$. When significant effects were detected, means were separated with Student's t-test ($\alpha \leq 0.05$). For stomatal conductance results, the days of the experiment were not considered a factor and a single ANOVA was done for each day to compare differences among treatment combinations.

RESULTS

Leaf stomatal conductance

Stomatal conductance rates of the fourth main-stem leaf from the terminal were significantly decreased under condition of water-deficit stress for both cultivars (Fig.1). Day 1 of the experiment indicates the first day after plants were visually wilted (5 mmol H_2O/m^2s) and had been watered with 50% of daily use quantity. Stomatal conductance rates were significantly different ($P \leq 0.001$) for days 1-10 of the experiment. However significant differences were observed between the two cultivars in the well-watered group, with Siokra L23 having consistently significantly ($P \leq 0.001$) lower stomatal conductance rates than ST 5288 for all the days of the experiment. A similar pattern was observed in the stomatal conductance rates of the subtending leaf (data not shown) with control rates of both cultivars being significantly higher compared to the water-stressed. Subtending leaves of control plants of Siokra L23 additionally, had significantly lower stomatal conductance rates compared to control plants of ST5288.

Photosynthesis

Photosynthetic rates were determined from a total of 10 replications from each cultivar and treatment group and the results were analyzed separately for each date using a two factor factorial design with the main factors being water regime and cultivars. Results from both days were similar so the results were pooled. No significant interaction ($P=0.3039$) was observed between the two main factors. Significant decreases in leaf photosynthetic rates were observed in the water-stressed plants of both cultivars compared to the control. Water-stressed plants of

ST5288 had 45% lower photosynthetic rates compared to their control and a similar pattern, nonetheless with a higher decrease (59%) observed between control and water-stressed plants of Siokra L23.

Polyamine levels

Significant differences were observed in the distribution of total polyamines with the ovaries containing higher concentrations of total polyamines compared to the leaves (Fig.3) under both control and water-deficit stress conditions.

The total polyamine levels in both leaf and ovaries of Siokra L23 remained unaffected under conditions of limited water supply compared to the control (Fig.4). Conversely, water-deficit stress resulted in a marked increase in the total polyamine content of the ovaries in the average drought-tolerant ST 5288, however leaf total polyamine levels remained unaffected (Fig.5).

No significant interaction was observed between the main factors water regime and cultivar in leaf PUT, SPD and SPM concentrations either for the leaf or the ovary (PLEAF=0.2558, POVARY=0.3555/ PLEAF=0.8522, POVARY=0.2748/ PLEAF=0.1280, POVARY=0.0937, respectively)and the effects of water-deficit stress on polyamine concentrations were analyzed using Student's t ($P \leq 0.05$).

A significant increase in leaf PUT concentrations was observed for ST 5288 where water-stressed plants containing 61nmole/gr FW PUT compared to 24 nmoles/gr FW of the control (Fig.6) Leaf PUT concentrations of Siokra L23 also increased, however not significant. Siokra L23 water-stressed plants contained comparably similar levels of PUT (63 nmoles/gr FW) to water-

stressed ST5288 however, control plants contained similar concentrations (55 nmoles/gr FW). A similar pattern was observed in ovary PUT concentrations (Fig. 7). Water-stressed ovaries of ST 5288 contained significantly higher concentrations of PUT compared to the control, while PUT in water-stressed Siokra L23 was not significantly different compared to the control.

Water-deficit stress had no significant effect on the levels of leaf SPD for either cultivar (Fig. 8). Both cultivars contained similar concentrations of SPD with ST 5288 containing 146 nmoles/gr FW and 141 nmoles/gr FW in control and water-stress treatments, respectively, while Siokra L23 levels were 119 nmoles/gr FW and 125 nmoles/gr FW for control and water stress respectively. The SPD concentrations of the ovary, (Fig.9), in Siokra L23 SPD concentrations remained unaffected under conditions of water stress with control ovaries containing 336 nmoles/gr FW SPD and water-stressed ovaries containing 334 nmoles/gr FW. ST 5288.

Unlike to SPD, water-deficit stress resulted in a significant decrease in leaf SPM levels of water-stressed SIokra L23 compared to the control, whereas no effect was observed in ST 5288 (Fig.10). Concentrations of SPM in water-stressed Siokra L23 were nearly 40% lower compared to the control while leaf SPM levels of water-stressed ST 5288 were 4% higher compared to the control. Similarly to leaf SPM concentrations Siokra L23 ovary SPM concentrations were also decreased however, not significantly compared to the control (Fig.11). ST 5288 SPM levels of water-stressed ovaries were higher compared to the control but not statistically significant. However, water-stressed ST 5288 ovaries contained significantly higher concentrations of SPM compared to water-stressed Siokra L23 ovaries.

DISCUSSION

The results of our present study showed that cotton polyamine concentrations vary depending on the type of tissue. Specifically, leaf polyamine concentrations were significantly lower compared to the ovaries. Cotton ovaries from both cultivars contained nearly twice the total polyamines concentrations compared to the leaves indicating that polyamines are closely associated with cotton's flowering mechanism. Significant increases in polyamine concentrations in reproductive structures as plants transition from vegetative to reproductive stage have been reported (Kakkar and Rai, 1993) and the importance of polyamine participation in flowering function has been established through experiments with polyamine deficient mutants that resulted in abnormal flowering structures or growth or even delayed flowering (Hanzawa et al., 2002; Alcazar et al., 2005). Additionally, variations in polyamine concentrations have been reported depending on the species, the organ as well as the type of tissue analyzed (Bouchereau et al., 1999; Kaur-Sawhney et al., 2003), with the general trend being that Increased polyamine concentrations occur in reproductive units compared to vegetative tissues (Kakkar and Rai, 1993). However, Alabadi et al. (1998) in experiments with tomato noticed that up-regulation of polyamine biosynthetic enzymes resulting in higher polyamine concentrations occur in both mature leaf tissues and ovaries. Similar results were reported by Bae et al. (2008) in cacao (*Theobroma cacao*) young leaf and ovary concentrations. Hence, we assume that

cotton's polyamine metabolism is more active in the reproductive organs compared to the vegetative tissues.

Focusing specifically on each cultivar, both leaf and ovary total polyamine levels of drought- tolerant Siokra L23 remained unaltered under conditions of limited water supply compared to the control. On the other hand, average drought-tolerant cultivar ST 5288, had significantly higher concentrations of total PAs in the water stressed ovaries while leaf concentrations remained unaffected. Modulation of PA metabolism with concomitant changes in their concentrations have been observed in a variety of crops under conditions of water-deficit stress (Bouchereau et al., 1999) and PAs have been considered as protective agents due to their ability to function either as antioxidants (Smirnoff et al., 1993) or as signaling molecules for initiation of other protective mechanisms through their catabolism (Moschou et al., 2008). However, whether increases or decreases in their levels indicate enhanced drought-tolerance is still elusive. Lazcano-Ferrat and Lovatt (1999), in field experiments with bean varieties differing in drought tolerance observed that leaf total PA content of the drought-sensitive cultivar remained unaltered under conditions of water-deficit stress however, leaf total PA concentrations of drought-tolerant cultivar were decreased compared to the control. On the other hand, Yang et al. (2007) reported that both drought tolerant and sensitive rice cultivars increased total PA concentrations however, the drought tolerant ones were faster in their response. Similar results were reported by Yamaguchi et al. (2007) in *Arabidopsis*, while Nayyar et al. (2005) noticed that in drought-sensitive soybean total PA content was decreased. Considering that PA response to abiotic stresses varies between species, tissues, duration and intensity of stress as well as developmental stages, we speculate that the stress, intensity or

duration, imposed on the plants in our study was not enough to result in significant changes in total PA concentration of the drought-tolerant Siokra L23, while it opposite was observed in average tolerant ST5288. Nevertheless, bearing in mind that each polyamine (PUT, SPD and SPM) appears to control different as well as have diverse responses under conditions of stress depending on the species, the type of tissue and the developmental stage (Takahashi and Kakehi, 2011) we need to look at the effect of water-deficit stress in each polyamine individually.

Hence, in our study, PUT concentrations in both water-stressed leaf and ovary of ST 52288 were significantly increased compared to the control while PUT levels in both tissues remained unaltered under conditions of water-deficit stress for the drought-tolerant Siokra L23. Lefevre et al. (2001) reported that diverse patterns in PUT accumulation under conditions of stress between photosynthetic and non-photosynthetic tissues, however they investigated the effect of salt stress in rice shoots and roots. We assume that the incompatibility between the results is due to the difference of stress, species and tissues investigated. Increased leaf PUT concentrations have been associated with decreased drought tolerance in wheat (Liu et al., 2006) as well as decreased salt tolerance in rice (Yamaguchi et al., 2007) which is in partial agreement with the results of our study since higher levels than control were observed in both cultivars, however significant increases were only for ST 5288. Conversely, Nayyar et al. (2005) reported that PUT levels increased in drought tolerant chickpea, while they decreased in more drought sensitive soybean. Yang et al. (2007) reported that PUT levels in both drought tolerant and sensitive rice cultivars were increased however, a lag in time was observed in the drought sensitive ones. In addition, Alcazar et al. (2010b) reported that transgenic *Arabidopsis* plants

transformed to over-express PUT were more drought tolerant compared to wild type plants and this was attributed to the decrease in stomatal conductance, which was similar to our results. Water-stressed leaves of ST5288 had similar stomatal conductance rates with water-stressed leaves of Siokra L23 and a similar pattern was observed in photosynthetic rates. Interestingly, however control leaves of Siokra L23 had significantly lower stomatal conductance rates compared to control ST5288, while leaf PUT concentrations of control and water-stressed drought-tolerant Siokra L23 were similar to those of ST5288 under water-stress. Significantly lower stomatal conductance rates of okra isolines compared to normal under well-watered conditions has been reported by Pettigrew et al. (1993) and the difference was attributed to the lower abaxial stomatal density observed between the okra and the normal isolines. Additionally, Pettigrew et al. (2004) in field experiments as well as Nepomuceno et al. (1998) in experiments with PEG-induced water stress reported higher photosynthetic rates for okra isolines compared to normal isolines at similarly low water potentials, which was not observed in our study since Siokra L23 had significantly lower photosynthetic rates compared to ST5288 under water-deficit stress conditions. We speculate that the differential responses in photosynthetic rates are due to the differences in the stress conditions between the studies. Additionally, even though stomatal density was not measured in our experiments, we assume that in cotton PUT levels affect stomatal conductance under both normal and water-deficit conditions with high concentrations inducing stomatal closure, which is in accordance with Alcazar et al. (2010b). The insignificant increase in PUT levels of drought-tolerant Siokra L23 could be attributed to the already high levels of PUT and the stress duration or intensity not being enough to trigger PUT biosynthesis (Cappell et al. 2004).

Similarly to leaf PUT levels, ovary PUT levels of both ST5288 and Siokra L23 were increased under conditions of limited water supply, however only significantly for the ST 5288. Conversely, Bibi et al. (2010) reported that PUT concentrations of heat-stressed ovaries were similar to those of the control. Nayyar et al. (2005) observed increases in PUT levels of flowers and pods in both drought-tolerant and sensitive chickpea cultivars, however drought-sensitive cultivars had a faster response to the stress compared to the drought-tolerant ones. This differential response was attributed by the authors to the ability of drought-tolerant cultivars to preserve high water potentials under water-deficit stress. Even though water potentials were not monitored in our study Siokra L23 has been reported to have significantly higher leaf water potential under conditions of stress compared to other cultivars (Voloudakis et al., 2002). Hence, we assume that the insignificant increase in ovary PUT levels of water-stressed Siokra L23 in our study is a result either of the low intensity of stress imposed to the plants, which was not enough to trigger PUT biosynthesis, according to the threshold model suggested by Cappell et al. (2004) or/and Siokra L23 ability to maintain good water status under conditions of stress.

Triamine SPD derives directly from PUT by addition of one aminopropyl group. Associations between high SPD and enhanced drought-tolerance have been observed in a variety of crops (Nayyar et al., 2005; Liu et al., 2006; Yamaguchi et al., 2007; Yang et al., 2007), while exogenous application of SPD or over-expression of SPD biosynthesis genes in transformed plants have resulted in higher concentrations of SPD and enhancement of drought tolerance (Kubis et al., 2003; Kasukabe et al., 2004; Farooq et al., 2008) suggesting that SPD plays a major role in protecting plants from water-stress. However, leaf SPD concentrations in our study were observed to remain unaltered under conditions of water-deficit stress for both

cultivars. No change in SPD concentrations of water-stressed wild-type or transformed to over-produce PUT Arabidopsis plants, no matter the increases in PUT levels, was reported also by Alcazar et al., (2006a, 2010b). The authors suggested that SPD biosynthesis is under tighter control compared to PUT, whereas Capell et al. (2004) attributed the lack of changes in SPD concentrations in rice drought-tolerant cultivars to the fact that the level of stress applied was insufficient to initiate PUT biosynthesis and further synthesis of SPD. Contrary to Alcazar et al. (2010b), who suggested that PUT controls stomatal control more effectively than SPD or SPM, Liu et al. (2000) observed that SPD is more effective in inducing leaf stomatal closure on wheat. Nevertheless, this was not observed in our study. Leaf stomatal conductance rates were similar for both cultivars under conditions of water stress and significantly lower compared to the control while their SPD levels were similar under both control and water-stress conditions. Zhang et al. (2009) reported that exogenous application resulted in increased photosynthetic rates in both drought sensitive and tolerant cucumber cultivars. In our study, photosynthetic rates of water-stressed Siokra L23 were significantly lower not only compared to the control but also compared to water-stressed ST 5288. Hence, we assume that in cotton, SPD is not as effective as PUT in modulating stomatal function. We speculate that regarding average drought-tolerant ST 5288, either the increases in PUT concentrations did not surpass a certain threshold in order to trigger SPD biosynthesis or that the synthesis rates of SPD were very slow resulting in no significant change as it has been shown previously by Flores and Galston (1984), whereas the insignificant increase in PUT levels of drought tolerant Siokra L23 were definitely insufficient to result in changes in SPD levels.

Water-stressed ovary SPD concentrations remained similar to the control in Siokra L23, as well as the control in ST5288, whereas water-stressed ST 5288 ovaries contained significantly higher SPD levels. Increases in both flowers and early pods of chickpea in SPD concentrations of drought-tolerant as well as drought-sensitive cultivars were observed also by Nayyar et al. (2005). However, similarly to their PUT pattern drought-tolerant cultivars increased their SPD levels later into the stress, while drought-sensitive ones at the onset of the stress which leads us to assume that drought-tolerant Siokra L23 might needed a more extended period of stress or a more intensive stress in order to increase its SPD concentrations, while the opposite is assumed for average tolerant ST 5288. In accordance with our assumption is also the threshold model suggested by Capell et al. (2004) where contrary to the drought-tolerant, drought-sensitive rice cultivars initiated SPD synthesis at the imposition of the stress.

Similarly to SPD, SPM has also been attributed significant roles regarding plant defense under conditions of abiotic stress. Water-deficit stress has been observed to result in increased levels of SPM in drought-tolerant cultivars or species (Capell et al., 1998; Nayyar et al., 2005; Liu et al., 2006; Yamaguchi et al; 2007; Yang et al. 2007). In support of these observations, SPM-deficient mutants have been reported to be more susceptible to drought (Yamaguchi et al., 2007) while exogenous application of SPM has been observed to enhance drought tolerance (Pang et al., 2007; Farooq et al., 2008) due to its protective role in lipid peroxidation of membranes. However, in our study water-stressed leaf SPM levels of drought-tolerant Siokra were significantly decreased compared to the control, while no significant change was observed in leaf SPM levels of ST 5288. Similar decreases in leaf SPM concentrations under water-stress were also reported for the drought tolerant *Phaseolus acutifolius* while for drought-sensitive

Phaseolus vulgaris SPM levels remained unaffected (Lazcano-Ferrat and Lovatt, 1999). In their study, the observed decrease in SPM levels was attributed to the inability of *P. Acutifolius* to use the accumulated nitrogen pools of the plant, while Capell et al. (1993) in experiments with PEG-induced water-stress on oat protoplasts suggested that SPM decreased due to the activation of the catabolic pathway. Alcazar et al. (2010b) speculated that the reduced SPM in *Arabidopsis* were due to the PUT concentrations not increasing over a threshold level and hence not initiating SPM synthesis, which is in accordance with our results. In addition, the low leaf SPM levels of Siokra L23 observed in our study, resulted in a significant decrease in its photosynthetic rates not only compared to the control but also compared to water-stressed ST 5288, indicating that SPM levels significantly modulate photosynthetic function in cotton. In support of this observation, Besford et al. (1993) reported that exogenous application of SPM decreased chlorophyll degradation and protected Rubisco under conditions of water stress, while increased levels of SPM were associated with enhanced photosynthesis (Islam et al.,2003). Contrary to Liu et al. (2000) and Farooq et al. (2008) SPM did not appear to have an important role in stomatal function since water-stressed ST 5288 had similar stomatal conductance rates as water-stressed Siokra L23 even though it contained significantly higher levels of SPM.

Ovary SPM levels followed a similar to the leaf SPM content pattern. Under well watered conditions, SPM concentrations of the ovaries were similar for both cultivars however, water-stressed Siokra L23 contained significantly lower concentrations of SPM compared to ST 5288. Decreases in SPM content of cotton ovaries were also reported by Bibi et al. (2010) under conditions of heat stress, whereas Nayyar et al (2005) contrastingly reported increases in SPM

levels of both drought tolerant and sensitive cultivars of chickpea under conditions of water-deficit stress. In their experiment however, both SPD and PUT ovary concentrations were increased under stress. Since ovary levels of SPD and especially PUT of Siokra L23 remained fairly unaltered under conditions of water-deficit stress, while a significant increase was observed in PUT levels of water-stressed ST 5288, we speculate that the differential response in ovary SPM levels between the two cultivars is due to the PUT not reaching the levels necessary to activate SPD and SPM synthesis, a suggestion made also by Alcazar et al. (2010b).

In conclusion, the results of our study indicated that polyamines in cotton accumulate in higher concentrations in the reproductive structures compared to the vegetative tissues. Total polyamine concentrations were not shown to be affected significantly by water-deficit stress conditions, however the opposite was observed when each polyamine concentration was analyzed individually. PUT was shown to significantly affect stomatal function in cotton, with increasing concentrations inducing stomatal closure. SPD levels on the other hand remained unaffected, suggesting that SPD does not play a significant role in cotton defense mechanism under conditions of water-deficit stress. Conversely, SPM levels significantly affected photosynthetic rates since decreases in its concentration resulted in significantly lower photosynthetic rates. We speculate that polyamines play an important role in cotton protection under adverse environmental conditions and changes in their concentrations, especially PUT and SPM, could be used as potential markers for selection of drought tolerant cultivars. However, further research is needed in order for a clearer cause-effect relationship to be established.

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FIGURES

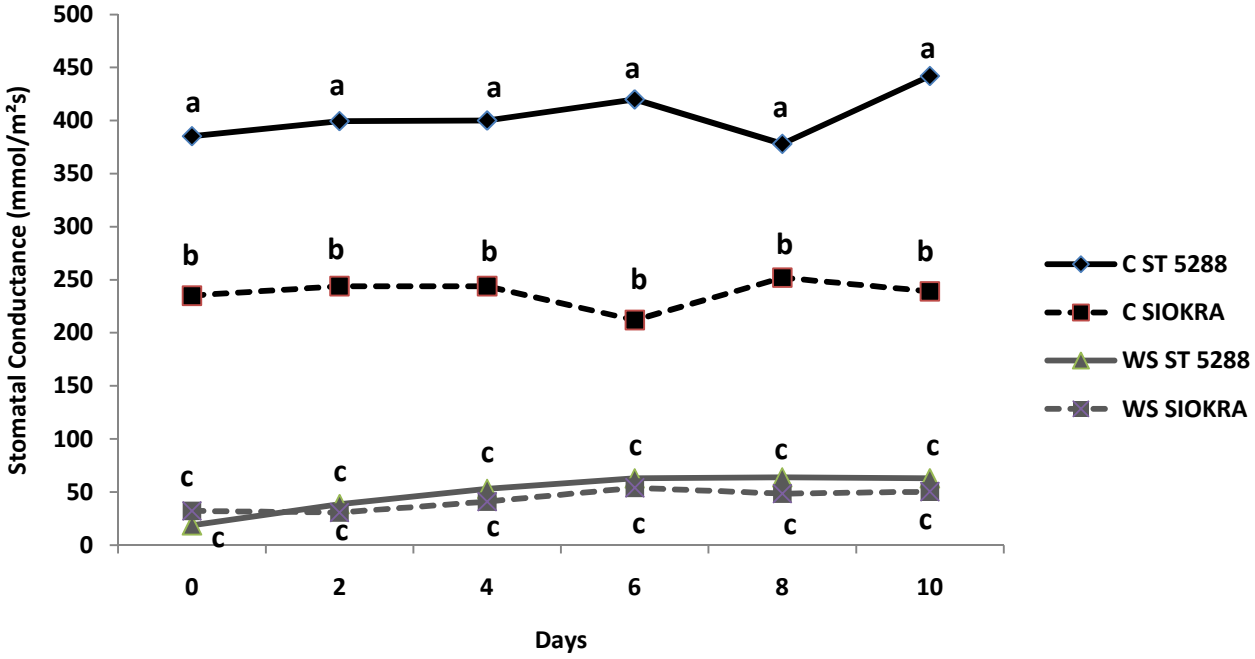

Figure 1: Effect of water-deficit stress on stomatal conductance rates of ST 5288 and Siokra L23. Day 0 represents the first day after plants had reached visual wilting point and had been rewatered with 50% of daily use water quantity and day 10 is the last day of the experiment. Points with the same letter were not significantly different (P=0.05).

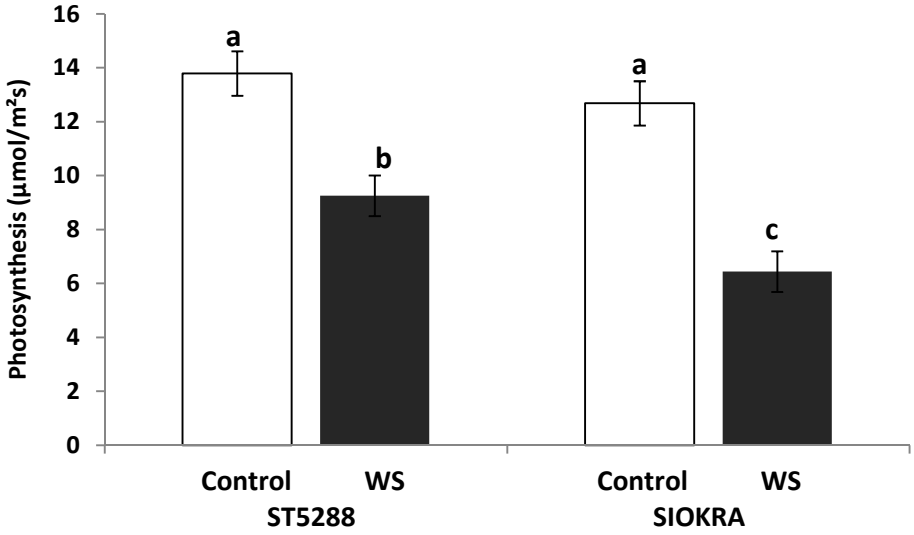

Figure 2: Effect of water-deficit stress on leaf photosynthetic rates of ST 5288 and Siokra L23. Columns connected with the same letter are not significantly different (P=0.05). Error bars represent ±1 standard error.

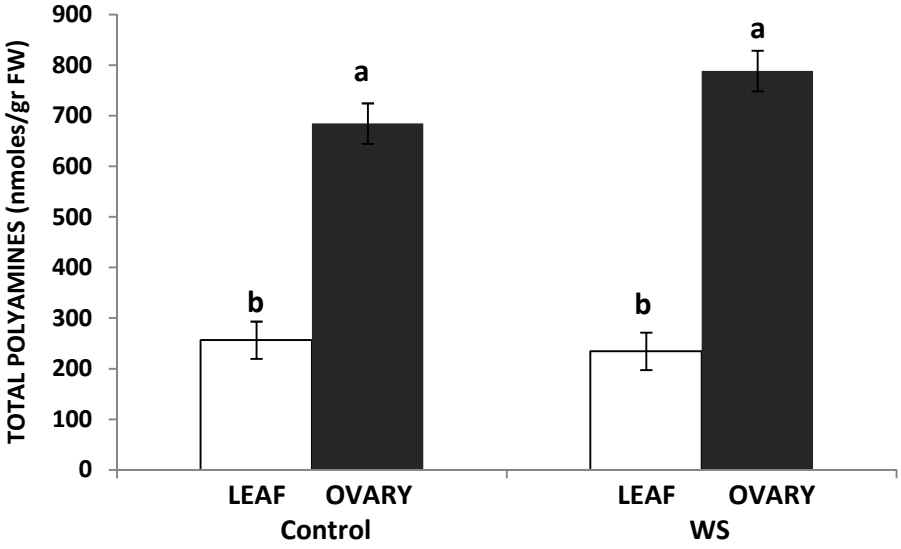

Figure 3: Effect of water-deficit stress on the distribution of total polyamine levels in the leaf and the ovary. Columns connected with the same letter are not significantly different. Error bars represent ±1 standard error.

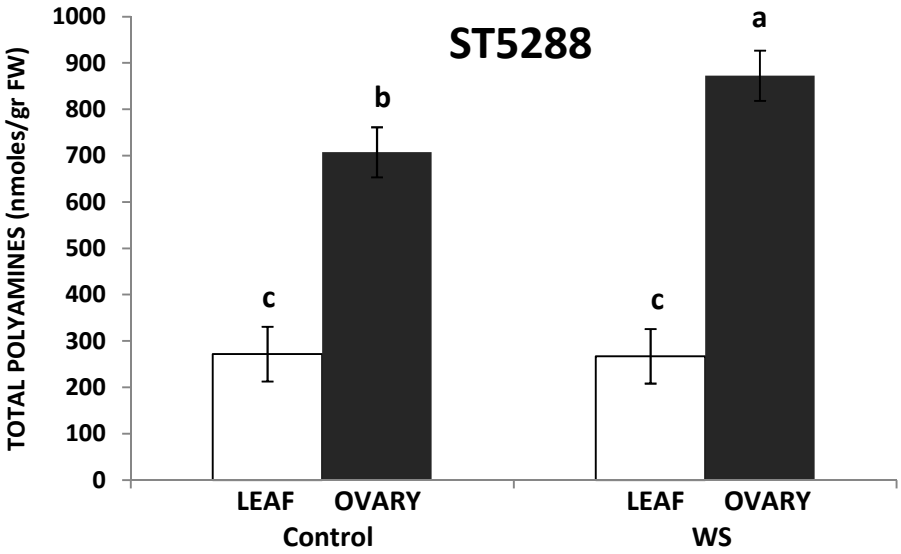

Figure 4: Effect of water-deficit stress on total polyamine content in leaf and ovary of ST 5288. Columns connected with the same letter are not significantly different (P=0.05). Error bars represent ±1 standard error.

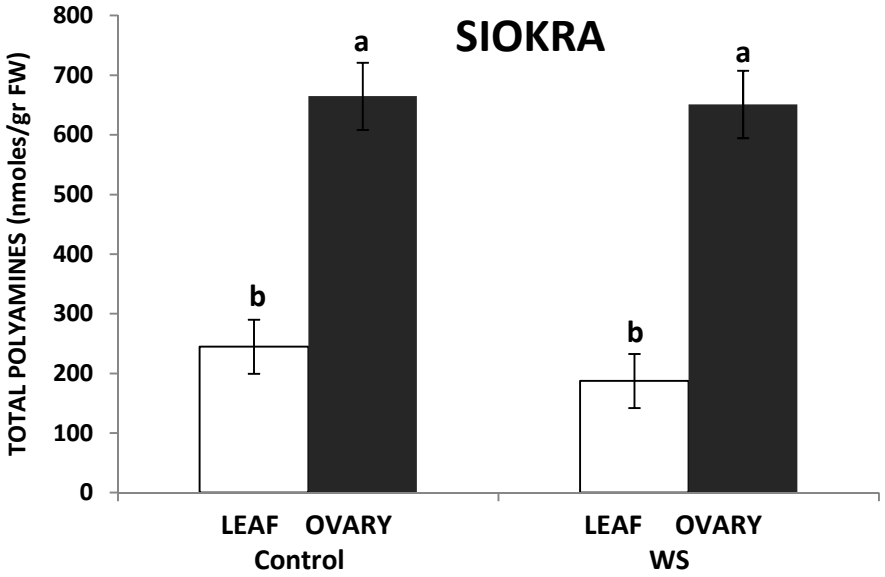

Figure 5: Effect of water-deficit stress on total polyamine content in leaf and ovary of Siokra L23. Columns connected with the same letter are not significantly different (P=0.05). Error bars represent ±1 standard error.

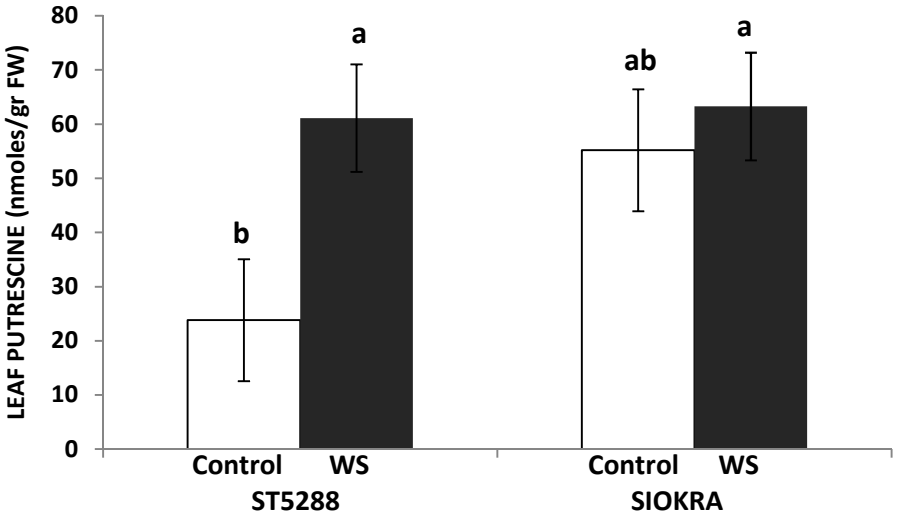

Figure 6: Effect of water-deficit stress on leaf putrescine concentrations of ST 5288 and Siokra L23. Columns connected with the same letter are not significantly different (P=0.05). Error bars represent ±1 standard error.

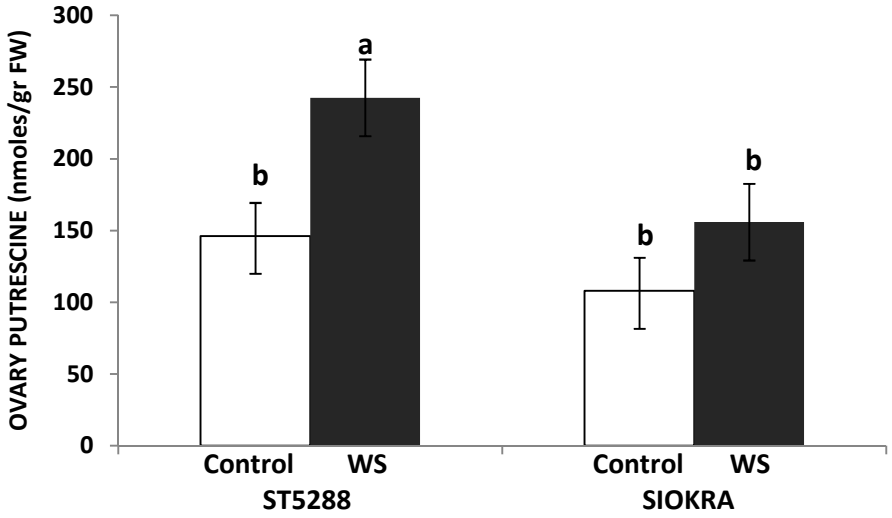

Figure 7: Effect of water-deficit stress on ovary putrescine concentrations of ST 5288 and Siokra L23. Columns connected with the same letter are not significantly different (P=0.05). Error bars represent ±1 standard error.

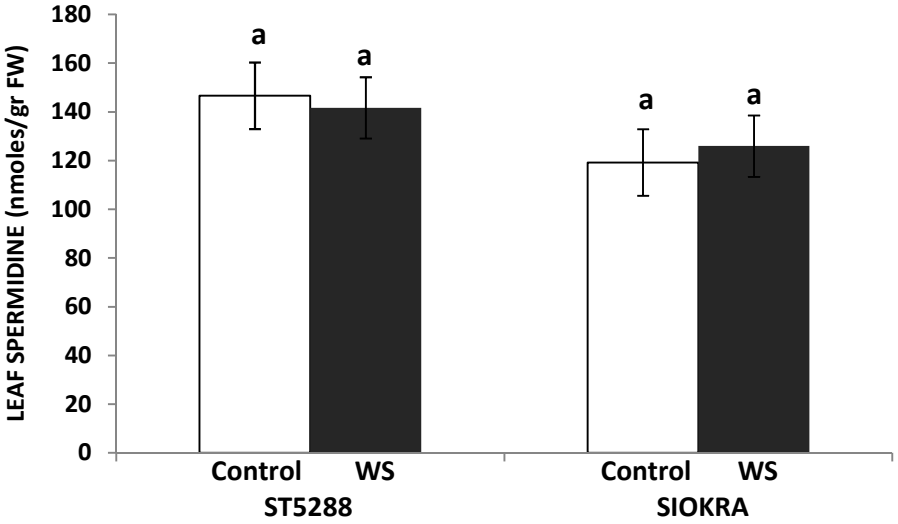

Figure 8: Effect of water-deficit stress on leaf spermidine concentrations of ST 5288 and Siokra L23. Columns connected with the same letter are not significantly different (P=0.05). Error bars represent ±1 standard error.

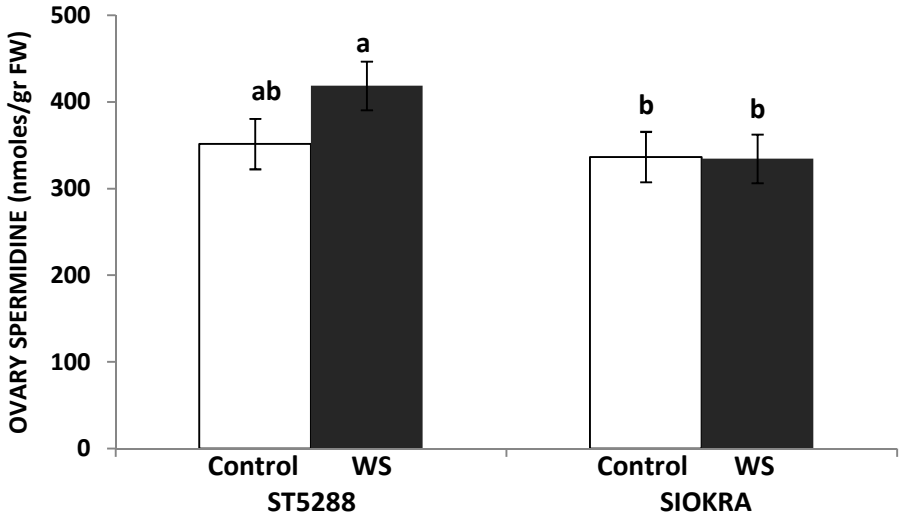

Figure 9: Effect of water-deficit stress on ovary spermidine concentrations of ST 5288 and Siokra L23. Columns connected with the same letter are not significantly different (P=0.05). Error bars represent ±1 standard error.

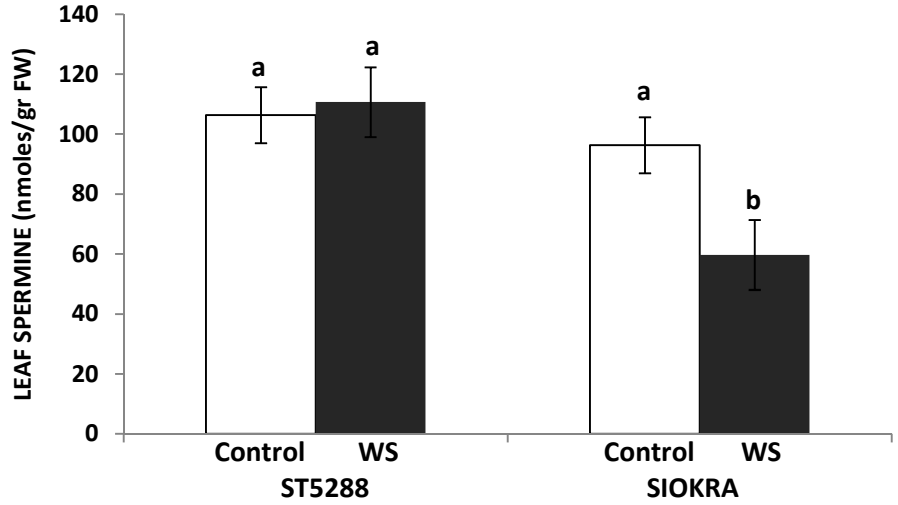

Figure 10: Effect of water-deficit stress on leaf spermine concentrations of ST 5288 and Siokra L23. Columns connected with the same letter are not significantly different (P=0.05). Error bars represent ±1 standard error.

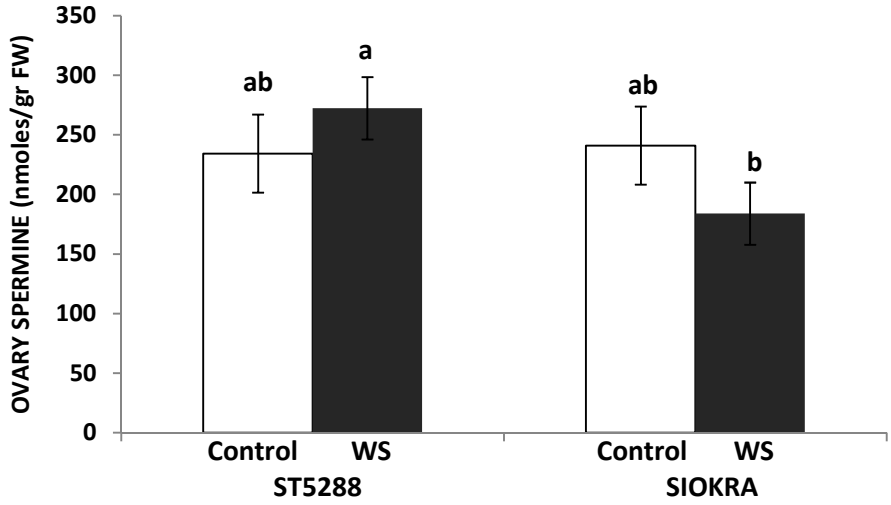

Figure 11: Effect of water-deficit stress on ovary spermine concentrations of ST 5288 and Siokra L23. Columns connected with the same letter are not significantly different (P=0.05). Error bars represent ±1 standard error.

INTERPRETIVE SUMMARY

Water-deficit stress is a major abiotic factor compromising plant growth and productivity in more than 30% of cultivated areas around the world (Boyer, 1985; Masacci et al., 2008). Cotton (*Gossypium hirsutum* L.) is a perennial with indeterminate and complex growth pattern that has been reported to be relatively drought tolerant due to its origin from hot and arid areas (Lee, 1984). As a result cotton plants are able to mitigate the negative effects of water-stress through a variety of defense mechanisms such as leaf and root osmotic adjustment (Oosterhuis and Wullschleger, 1987; Nepomuceno et al., 1998), accumulation of compatible osmolytes (Kuznetsov et al., 1999), heat shock protein production (Burke et al., 1985a) or high water use efficiency (Ackerson et al., 1977b). Nevertheless, due to its domestication and cultivation as an annual crop, the effectiveness of those mechanisms has been compromised (Quisenberry and Roark, 1976), ultimately resulting in significant yield losses (Basal et al., 2005) since cotton's physiological and metabolic functions are rendered vulnerable against water stress. Even though considerable debate still exists on the developmental stage that cotton is most susceptible to drought (de Cock et al., 1993) the high sensitivity of cotton to water stress during flowering and boll development is well established (Cull et al., 1981a,b; Turner et al., 1986). However, little attention has been given to the effects of water-deficit stress on the physiology and metabolism of cotton's reproductive units, especially flowers, with the only information existing being on petal water potential (Trolinder et al., 1993) and their hormonal balance (Guinn et al., 1990).

In order to provide more insight into cotton's flower metabolism under conditions of water stress a series of studies was conducted. The objective of the first study was to monitor

gas exchange responses of cotton plants under conditions of limited water supply and evaluate the effect of those conditions on the carbohydrate and antioxidant metabolism of the cotton flower. Growth chamber experiments were conducted in 2008 and 2009 and a widely used cultivar, ST5288B2F, was used to test the hypothesis that water-deficit stress would impair gas exchange functions which would result in a perturbation of carbohydrate and antioxidant metabolism in cotton reproductive units. The data indicated that water-deficit stress resulted in a significant decrease in leaf stomatal conductance. Leaf photosynthetic and respiration rates were similarly decreased compared to the control. Ovary and style water potential of water-stressed plants were significantly higher compared to the water potential of water stressed leaves, indicating that cotton flowers are fairly resistant to changes in the water status of the plant. However, carbohydrate concentrations of water-stressed pistils (ovary and style) were significantly increased compared to the control and a similar pattern was observed in the levels of glutathione reductase of water-stressed pistils. In conclusion, water-deficit stress during flowering resulted in significant decreases in leaf gas exchange functions as well as leaf water potential. Cotton pistils appeared to be less sensitive since they were able to maintain water potential similar to the control under limited water supply and increase glutathione reductase levels. However, pistil carbohydrate metabolism was significantly affected resulting in accumulation of both hexose and sucrose indicating a perturbation in sucrose cleaving and hexose utilizing enzymes that could potentially have as a consequence a compromise in fertilization and seed set efficiency.

In order to determine whether our results were consistent under both controlled and filed conditions, a field study was conducted in 2011 in two locations (Fayetteville, AR, and

Lubbock, TX) to investigate the effect of water-deficit stress during flowering on carbohydrate, glutathione reductase and polyamine metabolism of the cotton flower and its subtending leaf. Treatments consisted of control (well watered) and water-stress (irrigation was withheld for two weeks during flowering) in a split plot design. First position white flowers and their subtending leaves were collected one and two weeks after stress imposition and used to determine carbohydrate levels, glutathione reductase activity and polyamine concentrations. Water-deficit stress resulted in significant increases in pistil and leaf sucrose concentrations, and a similar pattern was observed in leaf glucose and fructose levels, while pistil glucose and fructose concentrations remained similar to the control. Glutathione reductase activity of both pistils and leaves remained unaffected by the limited water supply. Conversely, putrescine and spermidine levels of water-stressed pistils and leaves were significantly higher compared to the control. Pistil and leaf spermine content significantly increased under drought conditions in one location, remaining unaltered in the other one. Leaf and pistil polyamine metabolism appeared to be more responsive under conditions of water-deficit stress compared to glutathione reductase. Nevertheless, the significant increases in polyamine levels, the observed increases in water-stressed pistil and leaf carbohydrate concentrations indicated an inhibition of carbohydrate utilization that could potentially result in yield decreases.

Ethylene, an endogenous plant hormone, has often been observed to increase under environmental stress conditions, resulting in abscission of leaves and fruiting forms and ultimately in yield reduction. In cotton, however, the effects of water-deficit stress on ethylene production have been uncertain. In this study it was hypothesized that application of an ethylene inhibitor 1-Methylcyclopropene (1-MCP) would prevent ethylene production and

result in alleviation of water-deficit stress consequences on the physiology and metabolism of cotton flower and subtending leaf. To test this, growth chamber experiments were conducted in 2009-2010 with treatments consisting of (C) untreated, well-watered control, (C +1MCP) well-watered plus 1-MCP, (WS) untreated, water-stressed control, and (WS+1MCP) water-stressed plus 1-MCP. The plants were subjected to two consecutive drying cycles during flowering, approximately 8 weeks after planting, and 1-MCP was foliar applied at a rate of 10 g. ai/ha at the beginning of each drying cycle. The results showed that 1-MCP application had no significant effect on gas exchange functions and did not prevent reductions in leaf photosynthesis, respiration and stomatal conductance. However, application of 1-MCP resulted in a decrease in sucrose of the pistil. These results lead us to speculate that 1-MCP has the potential to interfere in carbohydrate metabolism resulting in a more efficient utilization of carbohydrates.

Polyamines, putrescine (PUT), spermidine (SPD) and spermine (SPM) are ubiquitous components of all living cells. Apart from their participation in numerous physiological and metabolical functions of the plant, they are also implicated in plants' responses under conditions of abiotic stress. Previous research in other crops has indicated that polyamines and changes in their concentrations are associated with drought tolerance under conditions of water-deficit stress, however no information exist for cotton. Growth chamber studies were conducted in 2011-2012, with two cotton cultivars differing in drought tolerance. ST5288B2F (average drought-tolerant) and Siokra L23 (drought-tolerant) were planted in order to investigate the distribution of polyamines, the effect of water-deficit stress on the polyamine metabolism of cotton reproductive units and their subtending leaves, as well as the possible

relationship between polyamines and drought tolerance in cotton. According to our results cotton ovaries contained significantly higher levels of total polyamines compared to their subtending leaves under both control and water stress conditions. Water-deficit stress significantly increased PUT concentrations in ST5288, while SPM levels significantly decreased in Siokra L23. The results indicated that water-deficit stress significantly affected cotton polyamine metabolism in reproductive structures and their subtending leaves, however no clear relationship between drought-tolerance and changes in polyamine accumulation was established. Further research is needed in order to elucidate the mechanism according to which water-deficit stress affects polyamine metabolism.

Overall, our results indicated that water-deficit stress during flowering significantly compromised leaf gas exchange functions resulting in decreased stomatal conductance, photosynthesis, respiration and water potential. However, cotton reproductive units appeared to be less drought-sensitive compared to the leaves possibly due to higher water potential and glutathione reductase activity. Additionally, limited supply of water significantly affected carbohydrate metabolism of both leaf and pistil resulting in carbohydrate accumulation in both tissues. Contrary to our expectations, application of the ethylene inhibitor 1-MCP had no effect on leaf gas exchange function however, it reversed the effect of water stress on pistil sucrose concentrations. Finally, water-deficit stress during flowering had a significant effect on polyamine metabolism of both leaf and pistil, consequently resulting in increases in putrescine, spermidine and spermine in drought-sensitive cultivars. The differential response of polyamine metabolism between drought-sensitive and tolerant cultivars suggests that polyamines could be effective tools not only in selection of drought-tolerant cultivars but also in drought

tolerance engineering, however further research is needed in order to elucidate the exact pathways of their action.