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Physiological and Molecular Responses of Common Cotton Cultivars under Water-Deficient Conditions

Casey R. Meek[1] • Derrick M. Oosterhuis[2*] • James M. Stewart[2]

[1] Oklahoma City Community College, 7777 South May Avenue, Oklahoma City, OK 73159, USA
[2] Department of Crop, Soil, and Environmental Sciences, University of Arkansas, Fayetteville, AR 72701, USA

Corresponding author: * oosterhu@uark.edu

ABSTRACT

Water-deficit stress is a major limiting factor in cotton (*Gossypium hirsutum* L.) production, but understanding of the level of drought tolerance among current cultivars is still lacking. To obtain an estimate of the diversity in drought tolerance in commercial cotton, seven cultivars representative of most of the major cotton areas were chosen for evaluation. These included 'Maxxa' (west), 'Sphinx' (southwest), 'Fibermax' (midsouth), 'Deltapine Nu33B', 'Stoneville 747, 'Sure-Grow 474' (Mississippi Delta), and 'Paymaster 1218' (east). An Australian cultivar, 'Siokra L-23', was included for its known level of drought tolerance. Physiological characterization under water-deficit stressed conditions was performed in the field and growth chamber. Osmotic adjustment was measured 12 hours after re-watering. One week after rehydration, leaf epicuticular wax content and carbon isotope discrimination were measured. Photosynthesis was measured at 16 hours, three and seven days after re-watering. Significant differences in osmotic adjustment and carbon isotope discrimination were encountered among cultivars. Stressed plants discriminated less than control plants. Generally, cultivars with high levels of osmotic adjustment exhibited smaller differences in carbon discrimination between water treatments. Several cultivars showed significantly greater photosynthetic rate at three days after stress cessation compared to control plants, especially 'Siokra L-23' and 'Sphinx'. Leaf epicuticular wax content was significantly higher in stressed plants. 'Siokra L-23' was screened via northern analysis for gene expression related to the compatible solutes, proline and trehalose which are reported to accumulate during drought stress. Slight up-regulation was observed in Δ^1-pyrroline-5-carboxylate reductase (P5CR) and Δ^1-pyrroline-5-carboxylate synthetase (P5CS), while proline dehydrogenase (PDH) was down-regulated. Trehalose-related genes [trehalose-6-phosphate phosphatase (*TPP*), trehalose-6-phosphate synthase (*TPS*), and *trehalase*] were up-regulated in response to water deficit stress. The overall results did not indicate major differences in water-deficit stress tolerance between commercial cultivars, whereas differences in proline and trehalose-related gene expression were observed between water treatments.

Keywords: epicuticular wax, osmotic adjustment, photosynthetic rate, proline, trehalose, water-deficit stress

INTRODUCTION

Cotton (*Gossypium hirsutum* L.) is vulnerable to a variety of adverse environmental conditions, such as drought, chilling or freezing, and salinity. While these factors are diverse, they all result in cellular water-deficit stress. Although water-deficit stress is generally accepted as the most limiting factor to cotton productivity, the literature is lacking regarding the water-deficit stress tolerance of the current commercial cultivars. Rahman *et al*. (2008) screened 32 cotton cultivars in the field in Pakistan, and reported genotypic variation in osmotic adjustment that was positively associated with seedcotton yield. However, an understanding is needed about the physiological explanation of water-deficit tolerance amongst commercial cotton cultivars. If cotton production under adverse conditions is to be improved, all aspects of the water-deficit stress response must be understood. Basic studies in controlled environments and field conditions are necessary to understand the innate physiological tolerance present in current germplasm and provide a foundation upon which to build. Additionally, information on the underlying genetic mechanisms of the stress response will aid in understanding.

Plants have evolved novel strategies for surviving water-deficit stress. However, in cotton and other agronomically important plants, survival alone is inadequate, but yields must be sustained when water deficits are encountered. Traditional selection approaches have generally proved unsuccessful in cotton due to interactions between genotype and environment (Quisenberry *et al.* 1983). If cotton yield capacity is to be maintained or improved, traits associated with maintenance of growth under water deficient conditions must be identified. Although drought tolerance has been shown to exist in the cotton genome (Oosterhuis *et al*. 1987), a comprehensive evaluation of existing drought tolerance capabilities is lacking (Quisenberry *et al*. 1983).

Water-deficit stress triggers a physiological cascade of events and affects a plant at many different levels (Blum 1996). These responses are dictated by the duration and intensity of the stress (Bradford and Hsiao 1982), as well as genotype and other environmental factors. At the cellular level, cell expansion is dependent on turgor pressure, therefore any stress severe enough to significantly decrease turgor can have a negative impact on growth (Hsiao 1973). Carbon fixation is inhibited not only by lack of CO_2 from stomatal closure, but also by the detrimental effects of free radical production (superoxide anion, hydrogen peroxide, and hydroxyl radicals) on photosynthetic apparati (Bohnert and Sheveleva 1998). If the stress is severe enough, plants will be smaller and less vigorous (Kramer and Boyer 1995).

While the role of osmotic adjustment in enhanced growth under water-deficit stress conditions has been debated (Munns 1988), the process does occur in cotton (Oosterhuis and Wullschleger 1987). Nepomoceno *et al*. (1998) found significant differences in osmotic adjustment and relative water content between select cotton genotypes indicating that drought tolerance can be related to measureable

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physiological parameters. Not only does osmotic adjustment aid in turgor maintenance (Kramer and Boyer 1995), certain solutes such as proline, and trehalose are thought to protect against water-deficit stress by buffering redox reactions, scavenging free radicals, preventing protein degradation, and maintaining membrane stability (Bray 1993; Bohnert *et al*. 1995; Hare and Cress 1997). There is a significant link between quantitative trait loci for high levels of osmotic adjustment and increased yield capacity (Saranga *et al*. 2001).

Additionally, the process of osmotic adjustment is highly conserved in most organisms, and transgenic studies with model crops have revealed that this phenomenon improves water-deficit stress tolerance, although it is unclear if the actual accumulation of the solute or the processes involved in synthesis and breakdown afford this protection. Two solutes that have recently received much attention in this area are the disaccharide, trehalose, and the amino acid, proline. While proline is well-established as a compatible solute in higher plants, including cotton, trehalose synthesis has only recently been documented as a normal metabolic pathway in higher plants (Goddjin *et al.* 1997; Müller *et al*. 2001). Nepomuceno *et al*. (1998, 2002) found that the gene for trehalose-6-phosphate synthase (*TPS*) was induced by water-deficit stress in all four of the cultivars used in a study to detect genes differentially expressed among water-deficit tolerant and sensitive cotton cultivars. Based on Nepomuceno's work, Kosmas *et al*. (2006) isolated the *TPS* gene from cotton. The goal of the molecular studies reported here was to determine if all of the genes involved in the synthesis and degradation of trehalose and proline are present in cotton and expressed in response to water deficit.

MATERIALS AND METHODS

Genotypes

In an effort to characterize drought tolerance in commercial US cotton germplasm pools, seven cultivars representative of the major cotton areas were carefully chosen based upon consultations with cotton breeders. The cultivars and region of their main of production are: 'Deltapine NU33B' (Mississippi Delta), 'Fibermax 989' (Midsouth US), 'Acala Maxxa' (Western US), 'Paymaster 1218' (Eastern US), 'Siokra L-23' (Australia), 'Sphinx' (Texas), 'Stoneville 474' (Mississippi Delta), and 'Sure-Grow 747' (Mississippi Delta). 'Siokra L-23' was included because it is known to be drought tolerant (Nepomuceno 1998; G. A. Constable, CSIRO, pers. comm.; Voloudakis *et al*. 2002). Pedigree information is given in **Table 1**.

Table 1 Pedigree information for cultivars in the study.

Cultivar	Area of origin	Parent lines
NuCot 33B	Mississippi River Delta	Bt Coker X DP 5415
Fibermax 989	Midsouth	**Not available*
Acala Maxxa	Western US	T7538 X S4959
Paymaster 1218	Eastern US	MSCot T827 X LA660
Siokra L-23	Australia	DP 90 x Siokra 1-4
Sphinx	Texas	MAR-CDP37HPIH-1-1-86
Stoneville 474	Mississippi River Delta	Stoneville 453 X DES 119
Sure-Grow 747	Mississippi River Delta	Re-selection from Sure-Grow 125

*Company will not make details available until proprietary vendor patent is released.

1. Growth room studies

The physiological responses of the cultivars to water-deficit stress were characterized under controlled conditions. Growth chamber studies were repeated five times between October 2000 and November 2001 at the Altheimer Laboratory, University of Arkansas, Fayetteville, AR.

Plant growth

Plants were grown in 2 L black plastic pots containing Sunshine Mix #1 (Sun Gro Canada Ltd., Seba Beach, Canada), a soilless horticultural blend. Approximately one week after planting (two days after emergence) water and essential minerals were supplied to plants in the form of a balanced nutrient solution at pH 6.6 (Henvitt 1963). Plants were grown in growth chamber (Conviron, Model 35, Pembina, ND) with a 12 h photoperiod at 30°C, 45% relative humidity (RH), 500 μmol m^{-2} s^{-1}; and a night period at 25°C, 65% RH). All eight genotypes received two treatments: water-deficit stress and well-watered, with three replications each.

Water deficit

Initially, pots containing 100% water-saturated Sunshine Mix were weighed and values were recorded. Both water treatments were maintained at a well-watered status (≥80%) until three weeks after emergence. At this time, plants receiving the water-deficit stress treatment were subjected to a gradual dry-down (10 % decrease in soil water content every 2-3 days) for 18-21 days. Pots were weighed every one or two days to maintain desired soil water content. The stress was sufficient to cause moderate wilting and stomatal closure, and resulted in a final leaf water potential (Ψ_w) of -2.5 to -3.0 MPa at the point of maximum stress.

Ψ_W, Ψ_π, and Ψ_P measurements

Leaf water (Ψ_W) and osmotic potential (Ψ_π) were measured using end-window thermocouple psychrometers (J.R.D. Merrill Specialty Equipment Company, Logan, UT, USA; 84 series) as described by Oosterhuis and Wullschleger (1989). Turgor pressure (Ψ_P) was estimated using equation 1:

$$(-\Psi_w) - (-\Psi_\pi) = \Psi_P \quad (1)$$

Osmotic adjustment was expressed as the percent decrease in osmotic potential of rehydrated stressed plants compared to well-watered control plants.

Photosynthetic rate

Gas exchange parameters were measured within one hour of solar noon with an LI-6200 portable photosynthetic system (LICOR, Lincoln NE). Photosynthetic rate was determined on undamaged, uppermost fully-expanded main-stem leaves (node four or five from the terminal) directly exposed to incoming radiation.

Leaf epicuticular wax content

Leaf epicuticular wax content was quantified one week after rehydration using one uppermost fully expanded leaf per plant as described by Oosterhuis *et al*. (1991). Leaf areas were determined and leaves were washed with reverse osmosis treated water and allowed to dry before wax extraction. For wax extraction, leaves were submerged in chloroform for 30 seconds in pre-weighed glass vials. The extracts were then filtered and evaporated to dryness under a stream of N_2. Wax content was determined by weighing the glass vials.

Carbon isotope discrimination

All leaf tissue was collected and dried at harvest. Composite samples of leaf tissue were ground to a fine powder in liquid nitrogen with a mortar and pestle, and approximately 1 mg of tissue from each sample was submitted to the University of Arkansas Stable Isotope Laboratory for carbon isotope discrimination determination. Carbon isotope composition was measured with an elemental analyzer-isotope ratio mass spectrometer (EA-IRMP). The carbon isotope ratio ($\delta\ ^{13}C_{sample}$) was calculated by comparing the ^{13}C to ^{12}C composition of a sample (R_{sample}) relative to a calcium carbonate (PDB - Pee Dee Belemnite) standard ($R_{standard}$) as shown in equation 2 (Boutton 1991):

$$(\delta\ ^{13}C_{sample} = [(R_{sample}/R_{standard})\text{-}1 \times 1000] \quad (2)$$

Statistical analysis

The experimental design used was a randomized complete block with five replications. A split-plot was utilized with the main factor "water stress" and the sub-factor "cultivars". The statistical analysis was performed using PROC GLM (SAS Institute Inc., Cary, NC). Interactions and main effects were tested with analysis of variance (ANOVA) at α=0.05.

2. Field studies

A field study was incorporated to verify that the growth chamber results were representative of the responses typical for a production system.

Plant growth

Seed were cone-planted into a Captina silt loam (fine-loamy, mixed, thermic Rhodic Paleudalf) on May 25, 2001 at the University of Arkansas Research and Extension Center in Fayetteville, AR. Six replications were arranged in a split plot with water as the main effect and cultivar as the second factor. PVC pipe was used to construct an in-furrow irrigation system. Irrigation, fertilization, and pest control were maintained according to Arkansas Cooperative Extension recommendations (Bonner 1995).

Stress imposition

Well-watered plants were irrigated when daytime leaf water potential approached -1.8 MPa as determined by thermocouple psychrometry. At first-flower (FF) + 1 week and FF + 3 weeks, Ψ_w, Ψ_π and relative water content (RWC) were measured. Sampling was conducted at these stages as they represent important stages in yield development that are particularly sensitive to stress. Generally, degree of stress in unirrigated treatments was low due to significant rainfall throughout most of the growing season.

Relative water content

Determination of RWC was performed using the technique described by Weatherley (1950) and calculated according to equation 3:

$$RWC = [(FW-DW)/(TW-DW)] \times 100 \quad (3)$$

where FW = fresh weight, DW = dry weight, and TW = turgid weight (full saturation) (Weatherley 1950).

Ψ_W, Ψ_π, and Ψ_P measurements

In the field study, Ψ_W and Ψ_π were measured with thermocouple psychrometers at FF + 1 and FF + 3 weeks on leaf disks taken within 1 hour of solar noon in three replications. Details of thermocouple psychrometer techniques were used as previously described. Because full rehydration, necessary for accurate determination of osmotic adjustment, is not feasible under field conditions, osmotic adjustment could not be calculated. However, Ψ_π and Ψ_P were determined. As previously described, Ψ_P was calculated from Ψ_π and Ψ_W values.

Northern analyses

The Australian cultivar, 'Siokra L-23', was used to determine expression of genes related to trehalose and proline catabolism and metabolism in response to water-deficit stress. Plants were grown in a growth chamber at the Altheimer Laboratory, University of Arkansas, Fayetteville, Arkansas under growth and water-deficit stress induction previously described under growth room studies. Leaf tissue samples were collected approximately five weeks after planting when plants were at the point of maximum stress. Approximately 5 g fresh leaf material were collected as whole leaves from both water treatments. To prevent degradation total RNA was extracted and purified immediately according to a revised hot borate method (Wan and Wilkins 1994; Nepumuceno *et al*. 1998). Probes for the northern analyses were obtained from cDNA clones corresponding to the gene of interest (**Table 2**), and were labeled with 40 μC of ^{32}P using a Redi-Prime Random Priming Labeling kit (Amersham, Arlington Heights, IL).

For northern hybridization, 10 μg total RNA was resolved electrophoretically on a 1% denaturing agarose gel and blotted to a Hybond-N+ hybridization membrane (Amersham, Arlington Heights, IL) using a Turbo-blot transfer system (Ambion, Austin, TX). The probes were allowed to hybridize with the RNA overnight (at least 12 hours) at 42°C. Membranes were washed under low-stringency conditions, followed by high stringency conditions. Kodak X-omat X-ray film was then exposed to the membrane for 24-72 hours at -80°C prior to development in a darkroom.

Table 2 Origin and identification gene clones used in probe synthesis for northern blot analyses.

Gene	Source	GenBank accession number	Probe size (bp)
P5CR	UC Davis	BG443368	745
P5CS	CUGI	BQ406862	743
PDH	CUGI	BQ404249	418
TPP	CUGI	AI055662	674
TPS	UA	AF056946	309
Trehalase	TAIR	AL109619	436

Table 3 Mean values of osmotic potential (Ψ_π) and percent osmotic adjustment of cultivars with and without water-deficit stress from five experiments in the growth chamber.

Cultivar	Water status	Ψ_π (MPa)	Adjustment[1] (%)
DeltaPine Nu33B	WW	-1.24	19 c
	WS	-1.48	
Fibermax 989	WW	-1.24	18 c
	WS	-1.44	
Acala Maxxa	WW	-1.23	12 d
	WS	-1.43	
Paymaster 1218	WW	-1.22	21 c
	WS	-1.48	
Siokra L-23	WW	-1.12	24 bc
	WS	-1.36	
Sphinx	WW	-1.12	44 a
	WS	-1.51	
Stoneville 474	WW	-1.21	23 bc
	WS	-1.47	
Sure-Grow 747	WW	-1.21	31 b
	WS	-1.58	
L.S.D. (0.05)	Water	0.07	
	Cultivar	0.14	
Cultivar within Water		0.17	

[1] percent decrease in osmotic potential of stressed plants compared to well-watered control plants.

RESULTS AND DISCUSSION

1. Growth chamber

Osmotic adjustment

Several significant differences ($P \geq 0.05$) existed in osmotic adjustment between cultivars (**Table 3**), with 'Sphinx' showing the highest (44%) and 'Acala Maxxa' the lowest (12%) level of osmotic adjustment. Absolute Ψ_π values (**Table 3**) demonstrate the magnitude of Ψ_π. These values ranged from -1.12 to -1.58 MPa and significant differences among cultivars were again evident. Well-watered 'Sphinx' plants exhibited the highest (least negative) Ψ_π values, hence lowest solute concentrations, compared to the other cultivars. However, under water-deficit stress conditions, 'Sphinx' accumulated a greater percentage of solutes via osmotic adjustment. It is possible that these two components influenced each other, and the increased accumulation observed in 'Sphinx' was necessary to bring the solute concentration to the appropriate level of drought tolerance.

Photosynthetic rate after recovery

Gas exchange was measured for one of the repetitions (**Fig. 1**) at 16 hours (day one), 3 and 7 days after rehydration. Means of the well-watered plants for all three measurement timings are displayed as one value (**Fig. 1**) for ease of interpretation. The cultivar × water interaction was significant at day three (P = 0.0089) and day seven (P = 0.0335). At day three, significant differences were observed in cultivar (P = 0.0082) and water (P = 0.0006) when analyzed without interactions. The only significant differences as determined by LSD (P < 0.05) between cultivars of a given water treatment existed at day three. Among well-watered plants, 'Sphinx' had the lowest and 'Sure-Grow 474' had the highest photosynthetic rates. At day one, all cultivars except for 'Paymaster 1218', showed a numerical reduction in photosynthetic rate in the water-stressed plants compared to the well-watered control plants, although this difference was not significant. At day three, all cultivars except 'Siokra L-23' showed an increase in photosynthetic rate above that of the well-watered plants. At day seven, wide variability in the water-stressed treatment was encountered, with most cultivars showing a stabilization of photosynthetic rate. Photosynthetic rate of 'Siokra L-23' remained stable in both treatments at each measurement, thus providing further evidence to support the observation of Nepomuceno (1998) of its ability to maintain high photosynthetic rates during periods of water-deficit stress. Water-deficit stressed 'Paymaster 1218' showed steady increases with time after rewatering above the well-watered control plants in photosynthetic rate at all three measurement intervals.

Leaf epicuticular wax content

Leaf epicuticular wax content measured one week after rehydration (**Fig. 2**) showed large significant differences (cultivar × water: P < 0.001) between well-watered and water-deficit stressed plants of all cultivars. Significant differences were also observed in cultivar (P=0.0016) and water treatment (P < 0.0001) when analyzed separately. 'Paymaster 1218' contained the most wax among well-watered plants. 'Siokra L-23' and 'Paymaster 1218' had the most wax among stressed plants. The significant (P ≤ 0.05) increase in wax content in stressed plants of all cultivars compared to control plants was not surprising, as water-deficit stressed cotton leaves commonly exhibit an increase in the waxy cuticular layer of the leaf (Weete *et al*. 1978; Oosterhuis *et al*. 1991).

Carbon isotope discrimination

Carbon isotope (^{13}C) discrimination provides a measure of water use efficiency in crops (Farquhar and Richards 1984) and was used in this study to measure cultivar response to water deficit. Across cultivars water-deficit stressed plants had significantly (cultivar × water: P = 0.0013) less ^{13}C discrimination values than well-watered control plants (**Table 4**). Significant differences were also observed among cultivars (P = 0.0166) and between water treatments (P < 0.0001) when analyzed separately. Generally, cultivars with higher levels of osmotic adjustment exhibited less difference in discrimination between the two water treatments, suggesting that stomates remained open longer in the water-stressed plants with more osmotic adjustment. 'Sphinx', the cultivar with highest osmotic adjustment, exhibited the smallest change in carbon discrimination between water treatments within a cultivar.

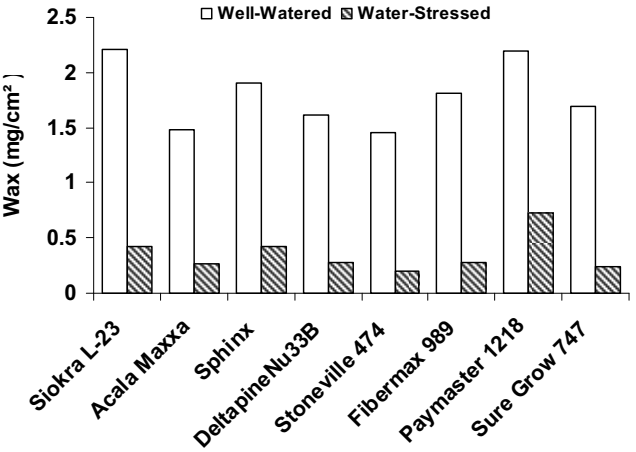

Fig. 2 Leaf epicuticular wax content of cultivars with and without water-deficit stress one week following rehydration.

Table 4 Carbon isotope discrimination expressed as composition of cultivars with and without water-deficit stress measured in the growth chamber studies.

Cultivar	Well-watered (‰)	Water-stressed (‰)	Difference within cultivars
DeltaPine Nu33B	-31.1	-30.1	1.0
Fibermax 989	-31.8	-31.0	0.8
Acala Maxxa	-32.0	-31.2	0.8
Paymaster 1218	-31.2	-30.8	0.6
Siokra L-23	-31.8	-31.3	0.5
Sphinx	-31.3	-31.1	0.2
Stoneville 474	-31.7	-30.6	1.1
Sure-Grow 747	-31.6	-31.3	0.3
L.S.D. Cultivar	0.4		
(0.05) Water	0.2		
Cultivar within Water	0.7		

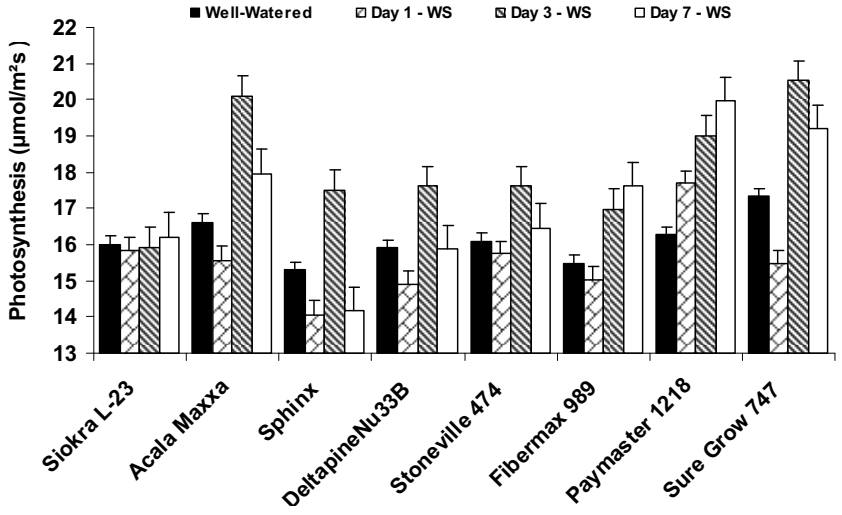

Fig. 1 Photosynthesis of cultivars with and without water-deficit stress after 1, 3 and 7 days following rehydration in the growth chamber. The mean values for the three measurements are displayed for well-watered plants. Error bars represent ± standard error of the mean.

2. Field study

At FF + 1 week, the cultivar × water interaction was significant for Ψ_W (<0.0001), Ψ_π (P<0.0001), and RWC (P = 0.0001) (**Table 5**). Significant differences were encountered among cultivars alone for Ψ_W (P = 0.007), and between water treatments for Ψ_W (P < 0.0001), Ψ_π (P < 0.0001), and RWC (P < 0.0001). No significant differences were observed in turgor pressure. Similar results were obtained at FF + 3 weeks with significant cultivar*water interactions in Ψ_W (P < 0.0001), Ψ_π (P < 0.0001), and RWC (P = 0.005) (**Table 6**). Also at FF + 3, significant differences occurred among cultivars alone for Ψ_π (P = 0.003), and between water treatments for Ψ_W (P < 0.0001), $\Psi\pi$ (P < 0.0001), and RWC (P < 0.0001). No significant differences were observed in turgor pressure at either date.

As expected, average values for Ψ_W, Ψ_π, turgor pressure, and RWC values were greater in irrigated plants at both measurement times (**Tables 5, 6**), demonstrating a greater degree of hydration in these plants. However no plants experienced a Ψ_W value less than -1.88 MPa, for a RWC value of less than 73%, indicating that they experienced only a mild stress. The RWC values showed an interesting trend for the two sampling periods. Well-watered plants in all cultivars exhibited a numerical decrease in RWC values from FF + 1 week to FF + 3 weeks, even though irrigation had been administered four days prior to both sampling periods. However, the unirrigated plants showed the opposite trend, with all cultivars exhibiting an increase in RWC between the two periods. This phenomenon narrowed the difference in RWC values between the two water treatments, suggesting that cotton has an acclimation mechanism when exposed to a mild water-deficit stress. 'Sphinx', the cultivar showing the greatest degree of osmotic adjustment under controlled conditions, also exhibited the largest difference in Ψ_π between the two water treatments at FF + 1 week. The cultivar showing the least degree of osmotic adjustment under controlled conditions, 'Acala Maxxa', showed differences in Ψ_π between the two water treatments similar to other cultivars at both sampling periods. Degree of change of Ψ_π among the two water treatments under field conditions showed no remarkable trends. It is possible that the field conditions influence osmotic accumulation in ways which cannot be predicted or accounted for in the growth chamber.

Table 5 Leaf water (Ψ_w) and osmotic potential (Ψ_π), turgor (Ψ_p), and RWC of cultivars with and without water-deficit stress at FF + 1 week in the field.

Cultivar	Water Status	Ψ_w (MPa)	Ψ_π (MPa)	Ψ_p (MPa)	RWC (%)
DeltaPine	WW	-0.97	-1.38	0.41	91.1
Nu33B	WS	-1.51	-1.96	0.45	74.5
Fibermax 989	WW	-0.82	-1.34	0.52	92.6
	WS	-1.67	-2.00	0.34	73.4
Acala Maxxa	WW	-0.81	-1.46	0.56	92.7
	WS	-1.15	-1.96	0.45	78.2
Paymaster 1218	WW	-1.17	-1.56	0.39	91.2
	WS	-1.83	-2.11	0.29	77.5
Siokra L-23	WW	-0.81	-1.37	0.56	92.6
	WS	-1.15	-1.61	0.46	76.8
Sphinx	WW	-1.00	-1.35	0.35	91.0
	WS	-1.88	-2.30	0.42	75.7
Stoneville 474	WW	-0.94	-1.46	0.52	91.4
	WS	-1.26	-1.81	0.55	76.7
Sure-Grow 747	WW	-0.84	-1.29	0.44	90.8
	WS	-1.34	-1.34	0.64	77.7
L.S.D. (0.05)	Water	0.14	0.12	0.12	1.6
	Cultivar	0.29	0.25	0.24	3.2
Cultivar within Water		0.40	0.33	0.36	4.7

Table 6 Leaf water (Ψ_w) and osmotic potential (Ψ_π), turgor (Ψ_p), and RWC of cultivars with and without water-deficit stress at FF + 3 in the field.

Cultivar	Water Status	Ψ_w (MPa)	Ψ_π (MPa)	Ψ_p (MPa)	RWC (%)
DeltaPine	WW	-1.01	-1.76	0.75	86.3
Nu33B	WS	-1.22	-1.67	0.45	80.3
Fibermax 989	WW	-0.89	-1.46	0.57	80.6
	WS	-1.85	-2.01	0.16	78.8
Acala Maxxa	WW	-0.58	-1.31	0.72	84.5
	WS	-1.47	-1.91	0.44	83.7
Paymaster 1218	WW	-0.84	-1.61	0.78	81.5
	WS	-1.84	-2.22	0.38	81.1
Siokra L-23	WW	-0.72	-1.36	0.64	85.0
	WS	-1.11	-1.42	0.30	78.3
Sphinx	WW	-0.97	-1.48	0.51	85.5
	WS	-1.36	-1.92	0.56	78.3
Stoneville 474	WW	-0.70	-1.53	0.83	84.6
	WS	-1.33	-1.99	0.65	80.3
Sure-Grow 747	WW	-0.99	-1.56	0.57	84.0
	WS	-1.47	-2.17	0.70	79.7
L.S.D. (0.05)	Water	0.14	0.12	0.14	1.6
	Cultivar	0.28	0.24	0.29	3.2
Cultivar within Water		0.12	0.52	0.14	3.2

The lack of significant differences (P ≤ 0.05) in turgor pressure when Ψ_W and RWC was significantly lower indicates that cotton has the ability to overcome water deficient periods. The importance of maintaining adequate turgor pressure during periods of water-deficit stress has long been recognized (Kramer and Boyer 1995). When Ψ_W decreases, it is necessary for a cell to also decrease Ψ_π (increase in solute accumulation) to maintain adequate turgor (Bohnert 1995). The significant decreases in Ψ_π and not in turgor pressure among the water-deficit stressed plants further supports this idea.

Role of neutral compounds

Two neutral compounds in particular have been examined in cotton, namely trehalose (Nepomuceno 1998) and proline (Hare and Cress, 1997). Nepomuceno (1998) observed that thalose-6-phosphste synthase (*TPS*) was induced in all cotton genotypes he examined (two drought-resistant and two drought-susceptible genotypes). Since trehalose does not accumulate in cotton, we assumed that the other enzymes involved in trehalose metabolism and catabolism [trehalose-6 phoshate phosphatase (TPP), and trehalase] should also be present (up-regulated) in drought-stressed plants so that a metabolic cycle results. Likewise, proline does not accumulate to the extent that the need for osmotic adjustment is satisfied (Hare *et al*. 1999). These authors suggested that the cycling of proline was more important than accumulation. For the cycle to be complete, the enzymes Δ^1-pyrroline-5-carboxylate synthetase (P5CS), and Δ^1-pyrroline-5-carboxylate reductase (P5CR) and proline dehydrogenase (PDH) must be present. In neither case have the transcripts for the complete cycles been shown to be present in cotton.

Trehalose-related genes

One gene transcript of approximately 1.5 kb was obtained for *TPS* and this gene was up-regulated under water-deficit stress conditions (**Fig. 3**). Two transcripts of *TPP* (~ 2 and 3 kb) were obtained (**Fig. 3**). Expression of the larger transcript (<3 kb) was similar between water treatments, but the lower molecular weight transcript (<2 kb) was induced by water deficit. Three gene transcripts were detected for trehalase (~1, 2, and 4 kb). No changes in expression of the largest (4 kb) transcript occurred in response to water treatment, but the 2 kb band was slightly up-regulated under water-deficit stressed conditions, while the smallest fragment was strongly up-regulated.

The *TPS* probe (**Table 2**) was an EST fragment identified and cloned by Nepomuceno *et al*. (2002). In their study, water-deficit stress was applied to four cotton cultivars, including 'Siokra L-23', with contrasting responses to water-deficit. They did not detect expression of the *TPS* fragment

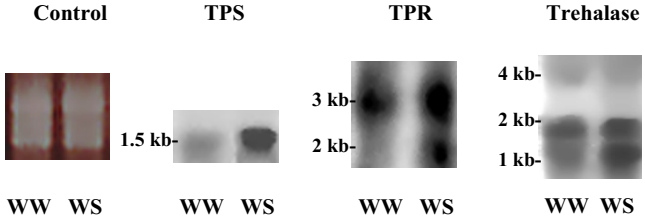

Fig. 3 Northern analysis of expression in Siokra L-23 of genes involved in trehalose metabolism under well-watered (WW) and water-deficit stressed (WS) conditions. Left to Right: 1) EtBr stained membrane prior to hybridization; hybridization with cDNA probes for 2) *TPS*, 3) *TPP*, 4) *trehalase*.

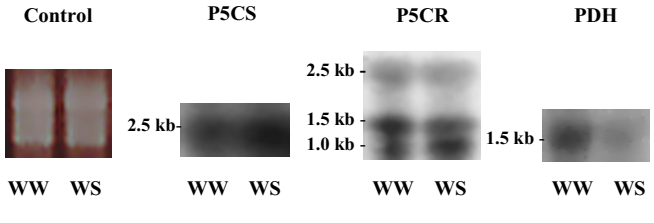

Fig. 4 Northern analysis of expression in Siokra L-23 of genes involved in proline metabolism under well-watered (WW) and water-deficit stressed (WS) conditions. Left to right: 1) EtBr stained membrane prior to hybridization; hybridization with cDNA probes for 2) *P5CR*, 3) *P5CS*, 4) *PDH*.

in well-watered control plants, but the gene was induced in all four of the cultivars by water-deficit stress. Since this transcript was induced in both drought tolerant and drought sensitive genotypes the assumption was made that cotton, in general, would respond to drought- stress this way. Voloudakis *et al*. (2002) also examined gene expression of 'Siokra L-23' under PEG-induced water-deficit conditions based on PCR oligonucleotide primers designed from the sequences reported by Nepomuceno *et al*. (2002). They failed to detect expression of *TPS* in 'Siokra L-23', or in any of the other cultivars in that study regardless of the water status, however, a more recent paper by this group (Kosmas *et al*. 2006) reported the isolation of the *TPS* gene from 'Siokra L-23'. They reported that expression of *TPS* was constitutive but that it also was strongly up-regulated by water deficit. Our results from northern analysis agree with the pattern of expression in this latter report. The differences in gene expression patterns detected among these studies could be related to differences in plant growth environments, plant ages and/or methods of stress imposition.

Kosmas *et al*. (2006) found a conserved region of *TPP* on the cotton *TPS* gene, so it is possible that the lower molecular weight transcript induced by water-deficit in this study was the TPS gene. Whether or not the smaller transcript codes for a protein with TPP activity, TSP activity or both, remains to be determined. Nevertheless, presence of the heavier *TPP* transcript in 'Siokra L23' (**Fig. 3**) indicates that both of the enzymes for synthesis of trehalose are present and expressed in the cotton cultivar, especially during water-deficit stress. Up-regulation of a unique isozyme of *TPP* in response to water-deficit stress in cotton is particularly noteworthy in that this is the first report of this enzyme in cotton. The clone used as a probe for these gene transcripts (**Table 2**) was an expressed sequence tag (EST) from *G. arboreum* and was also up-regulated in response to water-deficit stress (R. Wing, pers. comm., 1998).

Since trehalose is not known to accumulate in cotton, expression of the genes for the enzymes involved in synthesis (*TPS*, *TPP*) and degradation (trehalase) of trehalose suggest the possibility that trehalose cycling is important. Low levels of trehalose were detected in tobacco (Goddijn *et al*. 1997) and *Arabidopsis* (Müller *et al*. 2001) when trehalase was inhibited by validamycin A. Also, regulated over-expression of *E. coli* trehalose biosynthesis genes (*ots*A and *ots*B) as fusion genes in rice, resulted in recovery of several lines with increased tolerance to water-deficit stress (Garg *et al*. 2002). A positive correlation between trehalose accumulation and increased soluble carbohydrates and photosynthetic capacity was observed under both stressed and unstressed condition, but trehalose levels remained less than 1 mg/g fresh weight. Also, Avonce *et al*. (2004) observed an increase in dehydration tolerance related to over-expression of TPS in *A. thaliana* transformed with *TPS* but only small increases in trehalose and T-6-P. Because high concentrations of trehalose were not observed in any of these examples, it is unlikely that it is important as an energy reserve or acts as a compatible osmolyte (Garg *et al*. 2002). We propose that the cycling of trehalose or trehalose-6-phosphate in cotton is an important protective process during water-deficit stress. Conceivably, trehalose or its precursor plays a role in signal transduction in plant metabolism and development (Vogel *et al*. 2001), and aids in the regulation of carbohydrate metabolism and sugar sensing (Goddijn and Smeekens 1998; Muller *et al*. 2000; Garg *et al*. 2002). This is supported by the finding that exogenously-supplied trehalose can replace sucrose as a regulatory compound in barley (*Hordeum* sp.) (Muller *et al*. 2000).

Alternatively, expression of the genes involved in trehalose metabolism alone may not necessarily constitute activity of the product, as post-transcriptional processing may occur. Future biochemical studies are needed using a trehalase inhibitor, such as validamycin A, to determine if the protein is actually being synthesized.

Proline-related genes

Results of northern analyses for 'Siokra L-23' genes involved in synthesis and catabolism of proline are shown for both water treatments in **Fig. 4**. Only one transcript of ~2.5 kb was detected for *P5CS*, the gene coding for the rate-limiting enzyme in proline formation. Gene expression was present in the well-watered control but appeared to increase in response to water-deficit stress. Three transcripts were obtained for *P5CR*, with molecular sizes slightly less than 1.0, 1.5, and 2.5 kb. While no dramatic differences between transcript levels (band intensity) were observed between water regimes, the two heavier transcripts (<1.5 and 2.5 kb) appeared to be slightly down-regulated in the water-deficit stressed plants, while the lightest band (< 1 kb) was slightly up-regulated in this treatment. Only one size transcript was present for PDH (~1.5 kb), and this gene was down-regulated in response to water-deficit stress. Most expression studies have shown increased expression of *P5CS* (Hu *et al*. 1992; Delauney and Verma 1993; Kishor *et al*. 1995; Peng *et al*. 1996; Zhang *et al*. 1996) and *P5CR* (Verbruggen *et al*. 1993; Hua *et al*. 2001) in response to water-deficit stress in plants. Although we observed only marginal differences in expression of these two genes between water treatments, the trend was in agreement with previous reports. Perhaps the confined roots of the control plants (2 L pots) resulted in stress that triggered physiological responses that were not morphologically apparent. Such a response would reduce the apparent degree of induction of the enzymes by water-deficit stress.

The down-regulation of PDH under water-deficit stressed conditions was not surprising in as much as a decrease in this enzyme activity could aid in proline accumulation. Transcript levels of *PDH* have repeatedly been shown to decrease during long periods of water-deficit stress (Kiyosue *et al*. 1996; Peng *et al*. 1996). Verbruggen *et al*. (1993) indicated that the decline in proline breakdown has a strong genetic component rather than stress induced inactivation of pre-existing PDH (Hare *et al*. 1999). This research does not address the relative importance of proline cycling versus proline accumulation. These findings, however, contribute to the basic understanding of proline metabolism outside the realm of model crops.

CONCLUSION

Variability of several physiological responses associated with water-deficit stress tolerance were detected, although it remains unclear if these differences impart yield maintenance under water-deficient conditions. Several cultivars showed evidence of enhanced recovery of photosynthetic rate following water-deficit stress. The physiological studies also revealed a trend for osmotic adjustment to be negatively correlated with the change in carbon isotope discrimination between water treatments of a given cultivar, suggesting that stomates remained open longer in the water-stressed plants with more osmotic adjustment, therefore enabling entrance of CO_2 for continued photosynthesis.

All three of the genes for enzymes (TPP, TPS, and trehalase) directly involved in trehalose metabolism were shown to be up-regulated in response to water-deficit stress. Enhanced expression of the *TPP* and *trehalase* genes by water-deficit stress in cotton is reported for the first time. Recent studies in tobacco and *Arabidopsis* have revealed low levels of trehalose accumulation when trehalase was inhibited. Prior to these investigations, trehalose was thought not to be synthesized in most higher plants. Since the trehalase gene is present, inhibition of this enzyme might result in trehalose accumulation in cotton. Interestingly, the genes for both synthesis and breakdown of trehalose are up-regulated by water-deficit stress. This suggests that the cycling of trehalose or trehalose-6-phosphate may be important in stress adaptation.

Although the proline cycle has been well-characterized in model systems and other higher plants, research at the level of gene expression is lacking for cotton. Only a slight up-regulation was shown for *P5CR*, and the results were ambiguous with regard to expression of *P5CS*, whose product is responsible for the rate-limiting step in proline production. However, notable down-regulation of *PDH*, which catabolizes proline, was observed.

As more traits are revealed that impart tolerance to water-deficit stress, both wild and cultivated varieties can be screened for these features and used in breeding efforts. In order to meet the increasing demands for cotton products, and as water resources are depleted, it is crucial that commercial cultivars have the capability to maintain yields during times of water-deficit stress.

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