Physiological and molecular responses of common cotton cultivars under water-deficient conditions

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ABSTRACT

Water-deficit stress is a major limiting factor in cotton (Gossypium hirsutum L.) production, but the level of drought tolerance among current cultivars is unknown. 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 NuCOTN 33B, 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. Slight up-regulation was observed in ^{1}-pyrroline-5-carboxylate reductase (P5CR), and ^{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. Overall, physiological results did not indicate major differences in water-deficit stress tolerance between cultivars, and differences in proline and trehalose-related gene expression were observed between water treatments.

List of abbreviations

P5CR = Δ^{1}-pyrroline-5-carboxylate reductase, P5CS = Δ^{1}-pyrroline-5-carboxylate synthetase, PDH = proline dehydrogenase, TPP = trehalose-6-phosphate phosphatase, TPS = trehalose-6-phosphate synthase.

Introduction

Cotton (*Gossypium hirsutum* L.) is vulnerable to a variety of adverse environmental conditions, such as drought, chilling or freezing, and saline soils. 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 for cotton productivity, the literature is lacking regarding the water-deficit stress tolerance of the current commercial cultivars. If cotton production is to be improved under adverse conditions, 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 germplasms and provide a foundation to build upon. Additionally, the underlying mechanisms of the stress response must be understood at the molecular level.

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. The goal of the molecular studies was to elucidate patterns of expression of genes directly involved in the metabolism of these osmotica. While proline has been well-established as a compatible solute in higher plants including cotton, trehalose accumulation has only recently been documented in most higher plants.

Experimental procedure

In an effort to characterize drought tolerance in commercial US cotton germplasm pools, seven cultivars representative of the major cotton areas have been carefully chosen based upon consultations with cotton breeders. The cultivars and region of origin are: Deltapine NuCOTN 33B (Mississippi Delta), Fibermax 989 (Midsouth US), Acala Maxxa (Western US), Paymaster 1218 (Eastern US), Siokra L-23 (Australia), Sphinx (Western US), Stoneville 474 (Mississippi Delta), and Sure-Grow 747 (Mississippi Delta). Siokra L-23 was chosen because studies have revealed the cultivar to be drought tolerant (Nepomuceno, 1998; G. A. Constable, CSIRO, personal communication; Voloudakis *et al.*, 2002).

Growth room studies

The physiological responses to water-deficit stress in the chosen cultivars were characterized under con-

trolled 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 30EC, 45 % relative humidity (RH), 500 μmol m^{-2} s^{-1}; and a night period at 25 EC, 65 % RH). All eight genotypes received two treatments: water-deficit stress and well-watered, with three replications each.

Ψ_w, Ψ_π, and Ψ_p measurements

Leaf water (Ψ_w) and osmotic potential (Ψ_π) were measured after re-watering 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:

$$(-\Psi_w) - (-\Psi_\pi) = \Psi_p \qquad (1)$$

Osmotic adjustment was expressed as the percent decrease in osmotic potential at full turgor of 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 saturating solar radiation.

Leaf epicuticular wax content

Leaf epicuticular wax content was quantified one week after rehydration using one uppermost fully expanded leaf per plant. 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 from plants were collected and dried at harvest. Composite samples of leaf tissue were ground to a fine powder with liquid nitrogen, 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 ($^{13}C_{sample}$) 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:

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

Field studies

Plant growth Seed were cone-planted into a Captina silt loam (fine-loamy, mixed, thermic Rhodic Paleudalfs) 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. Generally, degree of stress in un-irrigated 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:

$$RWC=[(FW\text{-}DW)/(TW\text{-}DW)] \times 100 \qquad (3)$$

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 for the northern analyses due to difficulties in obtaining intact RNA from the other cultivars. Plants were grown in the growth chamber at the Altheimer Laboratory, University of Arkansas, Fayetteville, Arkansas. Plant growth conditions and water-deficit stress induction were performed as previously described. Leaf tissue samples were collected when plants were at the point of maximum stress, approximately five weeks after planting. Approximately 5

g fresh leaf material was collected as whole leaves from both water treatments. Extraction of RNA was immediately performed to prevent degradation. Total RNA was extracted and purified from the leaf tissue 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, and were labeled with 40 μC of ^{32}P. For northern hybridization, 10 μg total RNA was run on a 1% denaturing agarose gel and transferred to a Hybond-N+ hybridization membrane (Amersham, Arlington Heights, IL) using a Turbo-blot transfer system (Ambion, Austin, TX). Hybridization occurred overnight (at least 12 hours) at 42 μC. Membranes were then washed under low-stringency conditions, followed by high stringency conditions. The membrane was then exposed to X-ray film for 24-72 hours prior to manual development in a darkroom.

Results and Discussion

Growth chamber

Osmotic adjustment Several significant differences ($P \geq 0.05$) existed in osmotic adjustment between cultivars (Table 1), with Sphinx showing the highest (44%) and Acala Maxxa the lowest (12%) level of osmotic adjustment. Absolute Ψ_π values (Table 1) demonstrate the magnitude of Ψ_π. These values ranged from -1.12 to -1.58 MPa and significant differences among 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 (Figure 1) at 16 hours (day one), three and seven days after rehydration. Means of the well-watered plants for all three measurement timings are displayed as one value (Figure 1) for ease of interpretation. The cultivar x 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 ($P=0.0006$) effects when analyzed without interactions. The only significant differences as determined by LSD ($P \leq 0.05$) 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 *et al*. (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 re-watering 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 (Figure 2) showed large significant differences (cultivar x water: $P \leq 0.001$) between well-watered and water-deficit stressed plants of all cultivars. Significant differences were also observed in cultivar ($P=0.0016$) and water treatment effects ($P \leq 0.0001$) 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.

Carbon isotope discrimination Water-deficit stressed plants in cultivars had significantly (cultivar x water: $P=0.0013$) lower carbon isotope composition values than well-watered control plants (Table 2). Significant differences were also observed in cultivar ($P=0.0166$) and water treatment effects ($P \leq 0.0001$) 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 highest osmotic adjusting cultivar, exhibited the smallest change in carbon discrimination between water treatments within a cultivar.

Field study

At FF + 1 week, the cultivar x water interaction was significant for Ψ_w (<0.0001), Ψ_π ($P \leq 0.0001$), and RWC ($P=0.0001$) (Table 3). Significant differences were encountered in cultivar alone for Ψ_w ($P=0.007$), and water alone for Ψ_w (<0.0001), Ψ_π ($P \leq 0.0001$), and RWC ($P \leq 0.0001$). No significant differences were observed in turgor pressure. Similar results were obtained at FF + 3 weeks, with significant cultivar*water interactions observed in Ψ_w ($P \leq 0.0001$), Ψ_π ($P \leq 0.0001$), and RWC ($P=0.005$) (Table 4). Also at FF + 3, significant differences were encountered in cultivar alone for Ψ_π ($P=0.003$), and water alone for Ψ_w (<0.0001), Ψ_π ($P \leq 0.0001$), and RWC ($P \leq 0.0001$). In agreement with the first measurements, no significant differences were observed in turgor pressure at FF + 3 weeks.

As expected, Ψ_w, Ψ_π values turgor pressure and RWC values were greater in irrigated plants at both measurement times, demonstrating a greater degree of hydration in these plants. However no plants experienced a Ψ_w value less than 1.85, or a RWC value of less than 74%, indicative of 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, in the un-irrigated plants the opposite trend was observed, with plants in all cultivars exhibiting an increase in RWC between the two timings. 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.

The lack of significant differences ($P \leq 0.05$) 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; Morgan 1984). The significant decreases in Ψ_π and not in turgor pressure among the water-deficit stressed plants further supports this idea.

Northern analyses

Trehalose-related genes One band (~1.5 kb) was obtained for TPS and was up-regulated under water-deficit stress conditions (Figure 3). For TPP, two bands (~2 and 3 kb) were present in the water-deficit stressed plants, but the lower molecular weight band was absent in the well-watered plants. Expression of the heavier fragment was similar between both water treatments. Three bands were present for trehalase (~1, 2, and 4 kb). The ~3 kb band was slightly up-regulated under water-deficit stressed conditions, with a greater degree of up-regulation occurring in the lighter fragment. The cDNA fragment used to design the TPS probe was identified through differential display (Nepomuceno *et al.*, 2002). In their study, water-deficit stress was applied to four cotton cultivars with contrasting responses to water-deficit, including Siokra L-23. They did not detect expression of the putative TPS fragment in control plants, but only in those subjected to water-deficit stress. In Nepomuceno's study, a gradual stress was applied by reducing water over a six-day period when plants were approximately four-weeks old. The stress induction in the current study was similar, except it was applied over the course of 18-21 days, terminating when the plants were six-weeks old. In addition to the plants being younger in the previous study, they were also grown in the greenhouse, as opposed to the growth chamber in the present study. Often, plants in the growth chamber develop faster when compared to those grown under greenhouse conditions, resulting in a more pronounced difference in size. In both studies, plants were grown in 2 l pots, therefore it is possible that a stressful environment was encountered even though the control plants in the present study were maintained in a well-watered status throughout the study (pots were never allowed to dry, and Ψ_w was near -0.8 MPa as determined by thermocouple psychrometry). Also, it is possible that the larger plants in the current study experienced a mild stress imposed by growth in the 2 l pot even though no there was no indication of this. Voloudakis *et al*. (2002) also examined gene expression of Siokra L-23 under PEG-induced water-deficit conditions. Oligonucleotide primers were designed based on the sequence of the original putative TPS fragment from Nepomuceno *et al*., (2002), and used in RT-PCR. They failed to detect expression of TPS in Siokra L-23, or any of the other cultivars in this study regardless of the water status. Again, plant age could possibly account for the lack of detection of expression of TPS, as they used young seedlings.

Expression of an 824 bp fragment with high homology [P(N)1e-44, 104 amino acid identities (69%)] to the *Arabidopsis thaliana* TPP gene has also been reported as an expressed sequence tag (EST) in *Gossypium arboreum* and was up-regulated in response to water-deficit stress (R. Wing personal communication, 1998). The cDNA probe for the current analysis was created from a clone of Wing's putative TPP fragment. The expression of TPS, TPP, and trehalase provide the possibility that trehalose cycling may occur in cotton. It is possible that trehalose is degraded too rapidly for detection. Recently, low levels of trehalose have been detected in tobacco (Goddijn *et al.*, 1997) and *Arabidopsis* (Muller *et al.*, 2001) when trehalase was inhibited by validimycin A. More recently, regulated over expression in *Escherichia coli* of trehalose biosynthesis genes (otsA and otsB) as a fusion gene in rice, has resulted in 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. 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). It is hypothesized that the cycling of trehalose metabolism in cotton is an important protective process during cellular dehydration. It is, however, conceivable that trehalose, or its precursor, trehalose-6-phosphate, 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 alone of the genes involved in trehalose metabolism may not necessarily constitute activity of the product, as post-transcription processing may occur. Future biochemical studies are needed using a trehalase inhibitor, such as validimycin A, to determine if the protein is actually being accumulated.

Proline-related genes Results of northern analyses for genes involved with proline metabolism are shown for both water treatments of Siokra L-23 in Figure 4. Only one band was detected for P5CR (~2.5 kb), and expression was similar in both water treatments, with the stressed plants showing a very slight degree of up-regulation. Three bands were obtained for P5CS (~1, 1.5, and 2.5 kb), which is responsible for the rate-limiting step in proline formation. While no dramatic differences between band intensity were observed between water regimes, the two larger fragments (~1.5 and 2.5 kb) appeared to be slightly down-regulated in the water-deficit stressed plants, while the smallest band (~1 kb) was slightly up-regulated in this treatment. Water-deficit stress resulted in a drastic down-regulation of PDH, with only one band being present for this gene (1.5 kb). Most expression studies have shown increased expression of P5CS (Hu *et al.*, 1992; Delauney and Verma, 1993; Kishor *et al*., 1995; Zhang *et al*., 1995; Peng *et al.*, 1996) and P5CR (Verbruggen *et al.*, 1992; Hua *et al*., 2001) in response to water-deficit stress in plants. Although differences in expression of these two genes were marginal between water treatments, the trend was in the direction of earlier reports. As mentioned previously, it is possible that a mild stress was imposed by growth in the 2 l pot even though there were no visible indications of this. The down-regulation of PDH under water-deficit stressed conditions was not surprising. 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.*, 1996). This also indicates that the decline in proline breakdown is under genetic control (Verbruggen *et al*., 1996), and does not result from stress-induced inactivation of PDH (Hare *et al*., 1999). It was beyond the scope of this project to determine 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

Overall, results indicated variability of several physiological responses associated with water-deficit stress tolerance, 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 that osmotic adjustment was 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 directly involved in trehalose metabolism (TPP, TPS, and trehalase) were shown to be up-regulated in response to water-deficit stress. Although expression of TPP and TPS in water-deficit stressed cotton has been shown, no previous reports of trehalase expression in cotton have been found in the literature. Recent studies in tobacco and *Arabidopsis* have revealed low levels of trehalose accumulation when trehalase was inhibited. Prior to these investigations, trehalose was not thought to accumulate in most higher plants. Possibly, inhibition of trehalase might result in trehalose accumulation in cotton. However, expression alone does not guarantee that the product will be formed, as post-transcriptional processing often prevents formation of the protein. Although the proline cycle has been well-characterized in model systems and other higher plants, research at the expression level is lacking for cotton. Only a slight up-regulation was shown in P5CR. Results were ambiguous in regards to expression of P5CS, whose product is responsible for the rate-limiting step in proline production. However, drastic 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 current commercial cultivars have the capabilities to maintain yields during times of water-deficit stress.

References

- Blum, A. (1996). Crop responses to drought and the interpretation of adaptation. *Plant Growth Regulation,* **20**: 135-148.
- Bohnert, H.J., Nelson, D.E. and Jensen, R.G. (1995). Adaptations to environmental stresses. *Plant Cell*, **7**: 1099-1111.
- Bonner, C.M. (1995). Cotton production recommendations. AG 422-4-95. Univ. of Ark. Coop. Ext. Ser., Little Rock, AR.
- Delauney, A.J. and Verma, D.P.S. (1993). Proline biosynthesis and osmoregulation in plants. *Plant J.*, **4**: 215-223.
- Garg, A.K., Kim, J.K., Owens, T.G., Ranwala, A.P., Choi, Y.D., Kochian, L.V. and Wu, R.J. (2002). Tre-

halose accumulation in rice plants confers high tolerance levels to different abiotic stresses. *P.N.A.S. (Online Journal)*, **99**: 15898-15903.

- Goddijn, O.J.M, Verwoerd, T.C., Voogd, E., Krutwagen, R.W., De Graaf, P.T., Poels, J.K., can Dun, Ponstein, A.S., Damm, B. and Pen, J. (1997). Inhibition of trehalase activity enhances trehalose accumulation in transgenic plants. *Plant Physiol.*, **113**: 181-190.
- Goddijn, O. and Smeekens, S. (1998). Sensing trehalose biosynthesis in plants. *Plant J.*, **14**: 143-146.
- Henvitt, E.J. (1963). Mineral nutrition of plants in culture media. p. 97-134. *In* F. C. Stewart (ed.) Plant Physiology. Academic Press, New York, NY.
- Hu, C.A.A., Delauney, A.J. and Verma, D.P.S. (1992). A bifunctional enzyme (^{1}-pyrroline-5-carboxylate synthetase) catalyzes the first two steps in proline biosynthesis in plants. *Proc. Natl. Acad. Sci.*, **89**: 9345-9358.
- Hua, X.J., Van de Cotte, V., Van Montegu, M. and Verbruggen, N. (2001). The 5' untranslated end of At-P5R gene is involved in both transcriptional and post-transcriptional control. *Plant J.*, **26**: 157-169.
- Kavi Kishor, P.B.K., Hong, Z., Miao, G.H., Hu, C.A. and Verma, D.P.S. (1995). Overexpression of ^{1}-pyrroline-5-carboxylate synthetase increases proline production and confers osmotolerance in transgenic plants. *Plant Physiol.*, **108**: 1387-1394.
- Kiyosue, T., Yoshibe, Y., Yamaguchi-Shinozaki, K. and Shinozaki, K. (1996). A nuclear gene encoding mitochondrial proline dehydrogenase, an enzyme involved in proline metabolism, is upregulated by proline but downregulated by dehydration in *Arabidopsis*. *Plant Cell*, **8**: 1323-1335.
- Kramer, P.J. and Boyer, J.S. (1995). Water Relations of Plants and Soils. Academic Press. Orlando, FL. Pp. 495.
- Muller, J., Aeschbacher, R.A., Wingler, A., Boller, T. and Wiemken, A. (2001). Trehalose and trehalase in *Arabidopsis*. *Plant Physiol.*, **125**:1086-1093.
- Muller, J., Aeschbacher, R.A., Sprenger, N., Boller, T. and Wiemken, A. (2000). Disaccharide-mediated regulation of sucrose: fructan-6-fructosyltransferase, a key enzyme of fructan synthesis in barley leaves. *Plant Physiol.*, **123**: 265-273.
- Nepomuceno, A.L. (1998). Physiological and molecular responses of plants to water deficit. 1998. Ph. D. dissertation. Univ. of Arkansas. 269 p.
- Nepomuceno, A.L., Stewart, J.M. and Oosterhuis, D.M. (2000). Isolation of a cotton NADP(H) oxidase homologue induced by drought stress. *Pesq. Agropec. Bras.*, **35**: 1407-1416.
- Peng, Z., Lu, Q. and Verma, D.P.S. (1996). Reciprocal regulation of ^{1}-pyrroline-5-carboxylate synthetase and proline dehydrogenase controls proline levels during and after osmotic stress in plants. *Mol. Gen. Genet.*, **253**: 334-341.
- Verbruggen, N., Villarroel, R. and Van Montagu, M. (1993). Osmoregulation of a pyrroline-5-carboxylate reductase gene in *Arabidopsis thaliana*. *Plant Physiol.*, **103**: 771-781.
- Verbruggen, N., Villarroel, R. and Van Montagu, M. (1993). Osmoregulation of a pyrroline-5-carboxylate reductase gene in *Arabidopsis thaliana*. *Plant Physiol.*, **103**: 771-781.
- Vogel, G., Fiehn, O., Jean-Richard-dit-Bressel, L., Boller, T., Wiemken, A., Aeschbacher, R.A. and Wingler, A. (2001). Trehalose metabolism in *Arabidopsis*: occurrence of trehalose and molecular cloning and characterization of trehalose-6-phosphate synthase homologues. *J. Exp. Bot.*, **52**: 1817-1826.
- Voloudakis, A.E., Kosmas, S.S., Tsakas, S., Eliopoulos, E., Loukas, M. and Kosmidou, K. (2002). Expression of selected drought-related genes and physiological response of Greek cotton varieties. *Funct. Plant Biol.*, **29**: 1237-1245.
- Wan, C.Y. and Wilkins, T.A. (1994). A modified hot borate method significantly enhances the yield of high-quality RNA from cotton (*Gossypium hirsutum*). *Anal. Bioch.*, **223**: 7-12.
- Zhang, C.S., Lu, Q. and Verma, D.P.S. (1995). Removal of feedback inhibition of 1-pyrroline-5-carboxylate synthetase, a bifunctional enzyme catalyzing the first two steps of proline biosynthesis in plants. *J. Biol. Chem.*, **270**: 20491-20496.

Table 1. *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 NuCOTN 33B	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	
	Water	0.07	
L.S.D. (0.05)	Cultivar	0.14	
	Cultivar within water	0.17	

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

Table 2. *Carbon isotope discrimination expressed as composition of cultivars with and without water-deficit stress measured in the growth chamber studies.*

Cultivar	Well-watered ($^0/_{00}$)	Water-stressed ($^0/_{00}$)	Difference within cultivars
Deltapine NuCOTN 33B	-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
	Cultivar	0.4	
L.S.D. (0.05)	Water	0.2	
	Cultivar within water	0.7	

Table 3. *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 NuCOTN 33B	WW	-0.97	-1.38	0.41	91.1
	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
	Water	0.14	0.12	0.12	1.6
L.S.D. (0.05)	Cultivar	0.29	0.25	0.24	3.2
	Cultivar within water	0.40	0.33	0.36	4.7

Table 4. *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 NuCOTN 33B	WW	-1.01	-1.76	0.75	86.3
	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 474	WW	-0.99	-1.56	0.57	84.0
	WS	-1.47	-2.17	0.70	79.7
	Water	0.14	0.12	0.14	1.6
L.S.D. (0.05)	Cultivar	0.28	0.24	0.29	3.2
	Cultivar within water	0.12	0.52	0.14	3.2

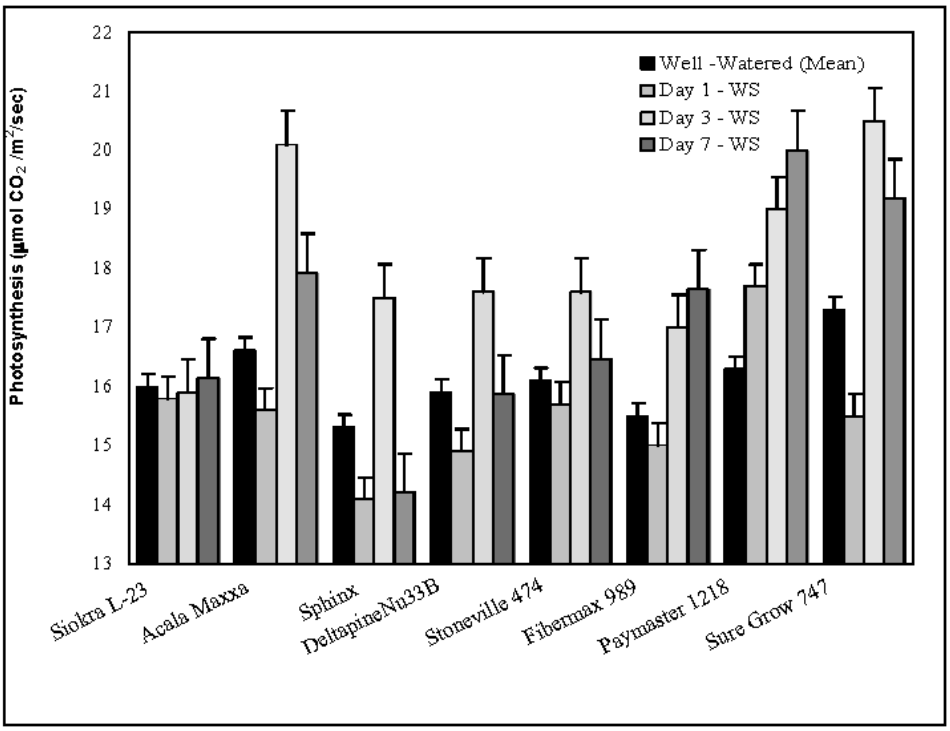

Figure 1. *Photosynthesis of cultivars with and without water-deficit stress after 1, 3 and 7 days after rehydration in the growth chamber. The mean values for the three measurements are displayed for well-watered plants. Error bars represent " standard error of the mean.*

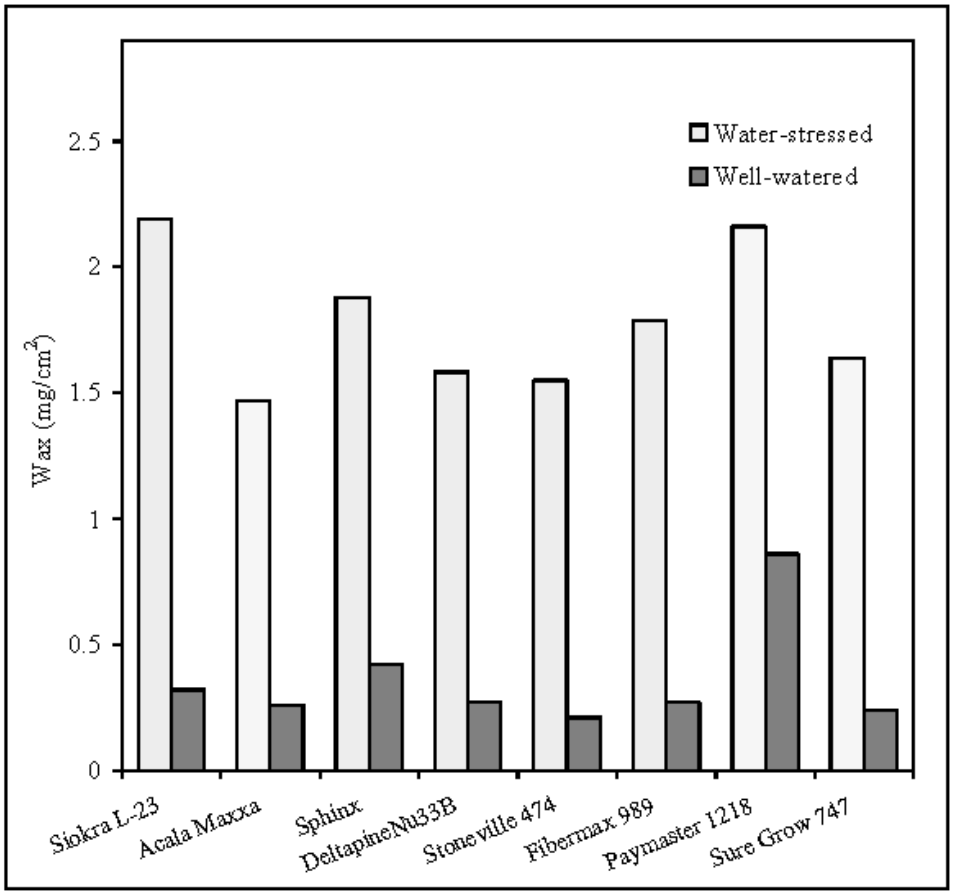

Figure 2. *Leaf epicuticular wax content of cultivars with and without water-deficit stress one week after rehydration.*

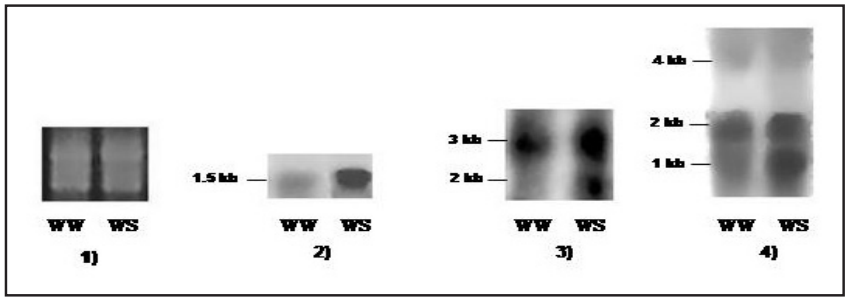

Figure 3.
Results of northern analysis in Siokra L-23 under well-watered (WW) and water-deficit stressed (WS) conditions for genes involved in trehalose metabolism. Left to Right: 1) EtBr stained membrane prior to hybridization; hybridization with cDNA probe corresponding to 2) TPS, 3) TPP 4) Trehalase. (Legend: WW = well-watered, WS=water-deficit stressed, TPS=trehalose-6-phosphate synthetase, TPP=trehalose-6-phosphate phosphatase, P5CR=1-pyrroline-5-carboxylate reductase, P5CS=1-pyrroline-5-carboxylate synthetase PDH=proline dehydrogenase).

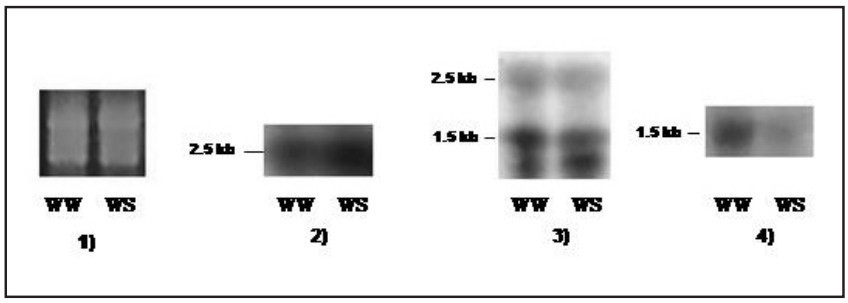

Figure 4.
Results of northern analysis in Siokra L-23 under well-watered (WW) and water-deficit stressed (WS) conditions for genes involved in proline metabolism. Left to right: 1) EtBr stained membrane prior to hybridization; hybridization with cDNA probe corresponding to 2) P5CS, 3) P5CR 4) PDH. (Legend: WW=well-watered, WS=water-deficit stressed, TPS=trehalose-6-phosphate synthetase, TPP=trehalose-6-phosphate phosphatase, P5CR=1-pyrroline-5-carboxylate reductase, P5CS=1-pyrroline-5-carboxylate synthetase PDH=proline dehydrogenase).