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Cotton flowering behavior, fiber traits and gene expression under water-shortage stress

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Abstract: Two accredited cotton (*Gossypium hirsutum* L.) cultivars (Aleppo 118 and Deir Al-Zour 22) have been investigated to assess physiological, morphological, and molecular responses under water shortage conditions. Both cultivars have shown early flowering. However, higher percentage of treated 'Aleppo 118' plants kept flowering towards the end of the growing season compared to treated 'Deir Al-Zour 22' plants. Both cultivars kept consistent micronaire and cohesion under normal and water shortage conditions. The cultivar Aleppo 118 displayed more consistent fiber quality between control and treated plants, while 'Deir Al-Zour 22' showed variation in fiber length and strength between control and treated plants. Results have demonstrated an increase in fold expression of *DREB1A*, *TPS* and *HSPCB* genes in the flowering stage of treated plants compared to the controls. Results also showed a continuous activation of *DREB1A* gene in the two critical growing stages of a cotton life cycle, flowering and boll development in treated plants of 'Deir Al-Zour 22'. This work illustrates the different responses of two cotton cultivars under water shortage stress and its impact on flowering and fiber traits.

1. Introduction

The supply of groundwater in agriculture is reduced due to intersecting environmental, industrial and domestic sectors. Groundwater-dependent agro-economies are mostly affected by drought stress, water deficits and aquifer depletion, specifically in the arid and semi-arid regions. Drought is considered a major constraint to crop productivity and yield stability around the world. It is therefore, a major challenge to farmers in drought subjected regions. Water saving and stable crop production are the main challenges that urge researchers to develop new cultivars and irrigation strategies for a sustainable use of water in agriculture.

Developing new cultivars that tolerate water stress has been assisted and supported by wide range of strategies and procedures that enable the selection of candidate parents and an efficient screening of targeted progenies. Genomics, marker-assisted selection, the manipulation of QTLs using genetic engineering and transcriptomics can contribute in the release of improved, drought-tolerant cultivars (Tuberosa and Salvi, 2006). The metabolomics-assisted breeding is another candidate approach for the development of crops with increased tolerance to abiotic stresses (Fernie and Schauer, 2009). Improving drought tolerance in crops requires understanding the biochemical responses under water deficit (Reddy *et al.*, 2004) and the characterization of drought patterns in the growing regions along with the physio-morphological traits of these crops under such environments (Fukai and Cooper, 1995; Khan *et al.*, 2010). Plants have evolved their drought-adaptive strategies (Meyre *et al.*, 2001), between drought escape (early flowering) and drought tolerance. Comprehending the flowering pattern of each crop under stressed and non-stressed environments is a key to which drought-adaptive strategy the crop plant follows. Early flowering and maturity can help in saving groundwater for irrigation and can help in avoiding early rainfall that usually affects the harvest of certain crops such as cotton (Jawdat *et al.*, 2012). Therefore, a vigilant assessment of crop responses to alteration in irrigation regimes is needed. At any rate, amendments and management of irrigation and water saving methods has a great potential in saving water sources in the arid and semi-arid regions (Oweis *et al.*, 2011; Ünlü *et al.*, 2011).

Cotton is a major cash oilseed and fiber crop with a world production estimated to be 25.01 million tonnes in 2013-2014 as announced at the official website of the International Cotton Advisory Committee (ICAC) (https://www.icac.org). The widely famous, allotetraploid *Gossypium hirsutum* L. has a distinct growth manner and can be useful in monitoring growth and developmental changes under diverse conditions (Jawdat *et al.*, 2012). Cotton is grown in around 70 countries where the irrigated system dominates in arid regions; and is used to supplement rainfall in the humid regions to ensure yield stability. Seed cotton production in Syria (mostly semi-arid where cotton is irrigated) was around one million tonnes in 2000. A decrease in production from around 670 K tonnes in 2009 to around 472 K tonnes in 2010 and then up to about 671 K tonnes in 2011 in the same cultivation area (~200 K hectares), was attributed to the drought wave that hit the country in 2010. A rapid decline in production was recorded between 2011 and 2014, from around 670 to around 162 K tonnes respectively. This was concurrent with the unfortunate events taking place in Syria.

The principal objective of this paper is to assess physiological, morphological, molecular, and biochemical responses of two accredited cotton cultivars in Syria under deficit water regime, in benefits of future breeding programs.

2. Materials and Methods

Plant materials

Plant material consisted of cotton seeds of two *G. hirsutum* L. accredited local cultivars: Aleppo 118 [Aleppo 40 (Aleppo 1 x Acala SJ1) x American cultivar BW 76-31] and Deir Al-Zour 22 (a selected line from Deltapine 41).

Drought stress treatment

Seeds were sown in rows, where the distance between rows and plants were kept at 75 and 25 cm respectively. Experimental plots were designed according to a randomized complete block design (RCBD) with each treatment is replicated once in each block. The experiment site was located in Doubaya in Damascus country side (980 m above sea level, 33° 29´ 36´ N and 36° 04´ 57´ E). The experiment consists of two irrigation treatments (control with full irrigation and treated with 50% of control irrigation), two cultivars (Aleppo 118 and Deir Al Zour 22) and three replicates per treatment. The soil (total N: 0.08%, available P: 12 ppm, K: 1.30 Meq/100 g, and organic matter: 1.19%) was prepared by tractor ploughing deep for 45 cm, followed by two surface ploughings for 15 cm deep. Fertilization was conducted following the Cotton Research Administration (CRA) guidelines and recommendations. Nitrogen fertilizer as urea 46% [$CO(NH_2)_2$] was applied at a rate of 415 Kg/ha in four unequally split applications, 20% before sowing, 40% 30 days after sowing, 20% at floral bud emergence, and 20% at bolls initiation phase. Phosphorus fertilizer [$Ca(H_2PO_4)_2$] was applied at a rate of 130 Kg/ha before sowing. Potassium fertilizer was applied at the rate of 170 Kg/ha in the form of potassium sulphate (K_2SO_4). Drip irrigation was applied at 250 m^3/ha, at first, for both control and treated blocks. Consequent irrigations were applied during the growing season of cultivated plants once a week at 250 m^3/ha (full irrigation) in control blocks, and once in every 10 days at 125 m^3/ha (deficit irrigation; receiving water at 50% of control treatment) in the treated blocks. Except for one week where temperatures reached around 45 degrees in the afternoon, we had to irrigate both control and treatment blocks with emergency full irrigation. The distance between emitters was 25 cm and the emitter discharge was 8 L/h. Thinning of plants was conducted around 4 weeks of germination.

Physiological data

Chlorophyll a and b content. Leaves samples (100 mg/sample) were each immersed in 4 ml of 95% acetone and incubated at 6-8°C for 24 hrs. Chlorophyll a and b concentration was determined spectrophotometrically by measuring the absorbance (optical density-OD) at 662 and 644 nm respectively. The concentrations of Chlorophyll a and Chlorophyll b (µg. g^{-1} FW) in leaf tissues were calculated using the following equations (Cha-Um *et al.*, 2006):

$$\text{Chlorophyll a} = 9.784 * D_{662} - 0.99 * D_{644}$$

$$\text{Chlorophyll b} = 21.426 * D_{644} - 4.65 * D_{662}$$

Where Di is an optical density at the wavelength i.

Osmotic potential OP) and osmotic adjustment (OA). Osmometer readings in mOsm/kg, were taken using the freezing point osmometer, the Micro-Osmoette™ (Precision systems Inc., USA). Frozen 100 mg of leaf samples in liquid nitrogen were ground (a pool of three leaves per treatment). Two milliliters of autoclaved d.d. water were added to each sample and samples were vortexed briefly and centrifuged for one minute at 13000 rpm. Fifty microliters of the supernatant were used for each reading. The osmotic potential Ψs was calculated using the following formula:

$$\text{OP (MPa)} = - \{R * T * \text{osmometer reading}\}/1000$$

where R is the gas constant (0.008314), and T is the laboratory temperature in Kelvin (298). The osmotic adjustment (OA) was calculated as the difference between the osmotic potential of non-stressed plants and the water stressed plants.

Morphological data

Twenty plants of each replicate were randomly tagged and flowering behavior data for days after planting (DAP) were recorded such as: first floral bud emergence, plant height at first flower, and nodes above white flower (NAWF). The percentage of plants bearing flowers and plants bearing floral buds were recorded towards end of season. End of season data also included: plant height, root depth (randomly selected plants from each replicate were carefully pulled out of ground), and number of secondary roots.

Physical and chemical fiber properties

Fiber quality testing. Thirty bolls of each replicate were picked and sent to the fiber quality lab at the Cotton Research Administration (CRA) for testing: micronaire, fiber length, fiber strength, fiber cohesion, and ginning percentage. CRA fiber quality lab conducts its testing according to the international testing standards with a temperature of 22±2°C and relative humidity of 64%±4%. The lab uses the following instruments: Micronaire 775, Digital Fibrograph, Pressley tester and Stelometer for fiber testing.

Fourier Transform Infrared (FTIR) acquisition spectra of cotton fiber

FTIR was conducted to study the chemical variance, if any, between fibers from control and treated plants of the two studied cultivars. The infrared spectra were recorded on Nicolet 6700 in the range 4000 to 400 cm^{-1} with a resolution of 4 cm^{-1} at 32 scans as direct measurements of the cotton fibers (three replicates per treatment) samples using universal attenuated total reflectance technique FTIR-ATR with KRS-5 crystals. The instrument is equipped with DTGS detector and KBr beam splitter. The operating system used was the OMNIC software (Version 7.3, Thermo Nicolet, USA). The infrared spectra of fiber samples for control and treated plants of Aleppo 118 and Deir Al Zour 22 cultivars were recorded. The recorded spectra were repeated three times for each investigated sample.

Measurement of crystallinity by conventional X-Ray powder diffractometry (XRD)

XRD was applied to determine the crystallinity of fiber samples from control and treated plants of 'Aleppo 118' and 'Deir Al-Zour 22' samples. X-ray powder diffraction patterns were obtained on a STOE transmission Stadi-P diffractometer with monochromatic Cu $K_{\alpha 1}$ radiation (λ = 1.5406 Å) selected using an incident-beam curved-crystal germanium Ge (111) monochromator, with the STOE transmission geometry (horizontal set-up) and a linear position-sensitive detector (PSD).

The determination of crystallinity of the samples was performed with the program *WinXPOW* (Stoe and Cie, 1999) using single-sample method. This method requires defining the portions of the diagram due to air scatter and inelastic scattering, amorphous scattering and Bragg or crystal scattering. If $I_c(2q)$ and $I_a(2q)$ are known for the whole diagram, the crystallinity index is calculated from the relation:

$$x_c = (\sum I_c(2\theta) / (LP \cdot F \cdot T) / (\sum [(I_c(2\theta) / T + I_a(2\theta)] / (LP \cdot F) \qquad \textit{Eq. 1}$$

The summing is over Bragg angle (*2q*). The Lorentz-Polarization factor *LP*, the average form factor *F* and the temperature factor *T* are all 2q-dependent functions:

$$LP(2\theta)= 1 + \cos^2 (2\theta) / (\sin^2 (2\theta) \cos\theta \qquad \textit{Eq. 2}$$

$$F(2\theta)= \sum f \ (n, 2\theta) \qquad \textit{Eq. 3}$$

$$T(2\theta)= \exp(-2B \sin^2 2\theta) / \lambda^2) \qquad \textit{Eq. 4}$$

The summing in Eq. 3 is over all atoms in the formula unit. The sample's overall formula and average temperature factor (*B*) have to be known in order to be able to apply this method. It's noteworthy to mention that sample absorption is always neglected; therefore, this method works well for transmission data of organic samples within ± 3% error of the true crystallinity. The formula of cellulose ($C_6H_{12}O_5$) and *B* = 4.0 Å^2 was used for our cotton fiber samples.

Thermogravimetry analysis (TGA) and differential scanning calorimetry (DSC)

The dynamic weight loss of fiber samples as a function of increasing temperature tests were conducted using a Mettler instrument (TG50). The tests were carried out in a nitrogen atmosphere, purged (30 ml/min) using sample weights of 10-15 mg at a heating rate of 10^{o}C/min. The resolution of the balance is given, as 1 microgram for weights less than 100 milligrams, and the temperature precision of the instrument is ± 2^{o}C.

DSC was used to determine the glass transition temperatures of the prepared samples. A Mettler instrument (DSC20) was utilized to record the DSC spectra. All samples were tested in aluminum pans at a heating rate of 10^{o}C/min over a temperature range from room temperature to 400^{o}C. The precision of the used instrument is ± 0.2^{o}C.

Scanning electron microscopy (SEM)

Scanning electron microscopy (VEGAII XMU, TESCAN, Czech Republic) with an accelerating voltage of 30 Kv, was operated to capture SEM images of cotton fibers coming from control and treated plants of both cultivars.

Gene expression analysis

RNA isolation and cDNA synthesis. Total RNA was isolated from leaves of cotton plants using the modified hot borate method (Jawdat and Karajoli, 2012). Quality of total RNA was tested by agarose-formaldehyde gel electrophoresis using standard protocols. The iScript^{TM} Select cDNA Synthesis kit (BioRad, USA) was used to obtain first strand cDNA starting from 1 µg total RNA following the manufacturer's protocol. The cDNA was diluted 4X and stored at -20°C.

Relative quantification of gene expression

The cDNA samples (control and treated) were used as a template to quantify target genes (*DREB1A*, *TPS* and *HSPCB*) expression level. Real-Time PCR was performed in two replicates in a 25 µl of a reaction mixture composed of 12.5 µl IQ SYBR super mix (BioRad, USA), 1 µl of cDNA, 1 µl of each of the forward and reverse primer (10 µM), and 9.5 µl of d.d. water.

The quantification of mRNA levels was normalized with the level of mRNA for *GhEF1α5* (Artico *et al.,* 2010). The relative expression (fold expression) was calculated using the -ΔΔC$_t$ method as follows:

$$2^{-\Delta\Delta Ct} = 2^{-[(Ct\ treated_{target\ gene} - Ct\ treated_{reference\ gene}) - (Ct\ untreated_{target\ gene} - Ct\ untreated_{reference\ gene})]}$$

Specific target and reference gene primers are listed in Table 1.

3. Results

Physiological, morphological, molecular and chemical data were recorded to identify main changes in two cash cultivars of cotton. The physiological and molecular analysis were carried out on leaf material from control and treated plants of 'Aleppo 118' and 'Deir Al-Zour 22', harvested 45 DAP

Table 1 - Target and reference genes accession numbers and primers sequences

Gene	GenBank accession No.	Forward and reverse primers (5'-3')
Dehydration responsive element binding gene (DREB1A)	AY321150.3	F-AGCTATAGCACTGAGAGGGAAG
		R-GCTTCTTCGTCCAAGTAAAACC
Trehalose-6-phosphate-synthase gene (TPS)	AY628139.1	F-TTCACTACATGCTGCCCATGTG
		R-GGCTGTGGAGGAAAAAACCAAG
Heat-shock protein calmodulin binding gene (HSPCB)	AY819767.1	F-CTCCTTGAATGTATTTACTGCC
		R-GTGCGTCCTCTAGTGTCTTT
Elongation Factor 1-alphagene (Ef1α5)	DQ174254	F-TCCCCATCTCTGGTTTTGAG
		R-CTTGGGCTCATTGATCTGGGT

in the course of floral bud initiation stage.

Chlorophyll a and b content and osmotic potential

The chlorophyll a content showed a non-significant decreasing trend in treated plants compared to control plants in both cultivars. However, the chlorophyll b content showed a small increment in the treated plants. The chlorophyll a/b ratio showed a reduction in treated plants of both cultivars. A larger reduction (18%) was observed in treated plants of ‘Deir Al-Zour 22’ compared to the small reduction (4%) in treated plants of ‘Aleppo 118’ (Fig. 1 inset graph). A non-significant drop in the osmotic potential of leaves in treated plants was observed in both cultivars and the osmotic adjustment was higher (0.016 MPa) in ‘Aleppo 118’ compared to ‘Deir Al-Zour 22’ (0.006 MPa) (Fig. 1).

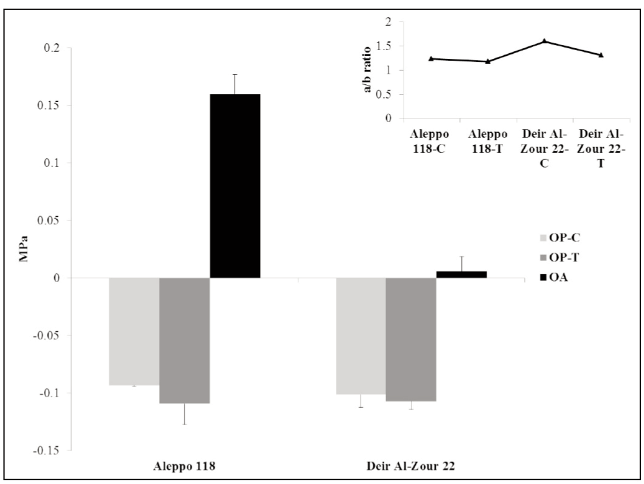

Fig. 1 - The inset graph is chlorophyll a/b ratio in leaves of control (C) and treated (T) ‘Aleppo 118’ and ‘Deir-Al Zour 22’ plants. Main graph represents osmotic potential and osmotic adjustment in leaves of control and treated ‘Aleppo 118’ and ‘Deir Al-Zour 22’ plants. Plants were under drip irrigation system, where the control plants were given full irrigation each week, and treated plants have been given deficit irrigation each 10 days. Data represent means and standard error of three replications.

Plant morphology

Early floral bud emergence was observed in both treated cultivars compared to control plants (Fig. 2 inset graph I). The control plants of Aleppo 118 cultivars flowered earlier than control plants of ‘Deir Al-Zour 22’. NAWF counts, another indicator of flowering earliness, was also recorded in the third week of first flower emergence (Fig. 2 inset graph II). Recorded NAWF counts showed a significant decrease in treated plants of both cultivars. A final record of plants with flowers, and plants with floral bud percentages were also taken five months after planting towards the end of season (Fig. 2). End of season flowering counts have shown ‘Aleppo 118’ tendency to keep initiating floral buds (~ 21%) in treated plants compared to control plants (5.6%), and produce fully opened flowers (~ 31%) in treated compared to control plants (~ 2%). However, a similar but less potent flowering counts pattern was observed in Deir Al-Zour 22 cultivar.

End of season measurements have also included plant stem height, taproot depth and number of secondary roots (Table 2). Treated plants in both cultivars showed a slight increase in plant stem height and minor decrease in root depth. However, non-significant increase in number of secondary roots was observed in treated plants of both cultivars.

Physical and chemical fiber properties

Fiber quality testing. The two tested cultivars have presented slightly different behavior in terms of fiber properties. Seed cotton yield showed no significant difference between treated and control plants in each cultivar. The cultivar Deir Al-Zour 22 showed a significant increase in both fiber length and strength in the treated plants compared to control ones. Fiber

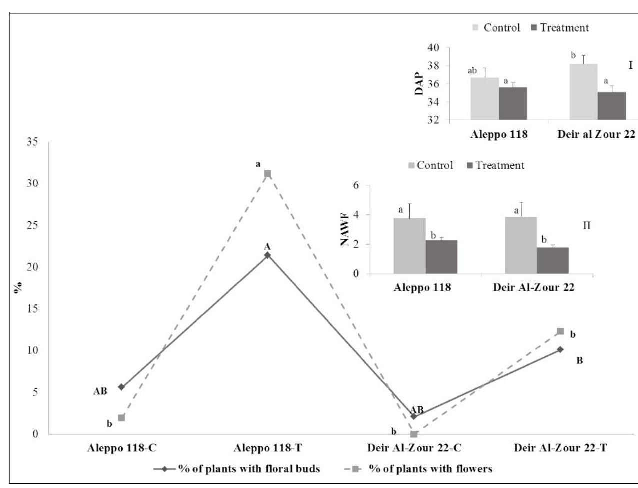

Fig. 2 - The inset graph-I shows floral buds emergence in days after planting (DAP) in control and treated ‘Aleppo 118’ and ‘Deir Al-Zour 22’ plants. The inset graph-II represents NAWF counts in control and treated plants of both cultivars. Data were subjected to Duncan’s test with a confidence level of 95% using STATISTICA program. Columns sharing a letter are not significantly different. Data represent means and standard error of three replications. The Original graph is the percentages of plants with flowers and plants with floral buds of both control and treated Aleppo 118 and Deir Al-Zour 22 cultivars, five months after planting. Data were subjected to Duncan’s test with a confidence level of 95% using STATISTICA program. Line markers sharing a letter are not significantly different (capital letters for plants with floral buds and small letters for plants with flowers.

Table 2 - End of season measurements of plant stem height, taproot depth and number of secondary roots in control and treated plants of Aleppo 118 and Deir Al-Zour 22 cultivars

Cultivar	Plant height (cm)		Root depth (cm)		No. secondary roots	
	Control	Treatment	Control	Treatment	Control	Treatment
Aleppo 118	99.3±3.5 ab	105.9±5.5 a	19.8±2.2	17.7±2.1	10.4±0.8 AB	13.1±0.8 A
Deir Al-Zour 22	80.3±3.8 c	87.6±5.1 bc	18.3±2.8	17.0±0.7	9.9±0.9 B	11.4±1.1 AB

Data were subjected to Duncan's test with a confidence level of 95% using STATISTICA program. Numbers in rows and columns sharing a letter in each block (Plant height and No. secondary roots) are not significantly different. Data represent means and standard error of three replications.

elongation showed no significant difference between control and treated plants in each cultivar. Furthermore, no significant difference was recorded in the cultivar Aleppo 118. Two fiber properties, fiber cohesion and micronaire, exhibited no significant difference between cultivars and between treatments. A significant increase in fiber maturity was observed in the treated plants of Aleppo 118 cultivar, whereas, a non-significant increase was recorded in treated plants of 'Deir Al-Zour 22' (Fig. 3).

FTIR-ATR spectra acquisition of the cotton fiber. The FTIR-ATR spectra of fiber samples from control and treated plants of Aleppo 118 and Deir Al-Zour 22 cultivars were recorded. The spectra showed a typical characteristic reflectance bands for common cotton fiber substances. The spectra of fiber samples from control and treated 'Aleppo 118' plants were aligned for comparison and showed minimum variation (Fig. 4 A). The spectra of fiber from 'Aleppo 118' control plants showed 15 distinctive bands centered at 3310, 2890, 1740, 1605, 1429, 1372, 1315, 1203, 1160, 1103, 1055, 1020, 675, 554 and 431 cm^{-1}. The vibration at 1429 cm^{-1} is designated and assigned as a "crystalline" absorption band of the fiber (Abidi *et al.*, 2014). The band at 1372 cm^{-1} vibration is assigned to C-H bending, and it may be most suitable for indicating cellulose crystallinity associated with the band at 2890 cm^{-1}. The spectra of control and treated samples were highly similar except for the band at 1740 cm^{-1} which was absent in the treated sample.

Similar spectra range was observed in Deir Al-Zour 22 cultivar. However, few noticeable new bands with distinctive structures were observed in the treated sample at 2890, 1740, and 1605 cm^{-1}. The band centered at 2890 cm^{-1} contains two prominent peaks vibrations at 2910 and 2847 cm^{-1}, which are assigned to CH_2 asymmetrical and symmetrical stretching,

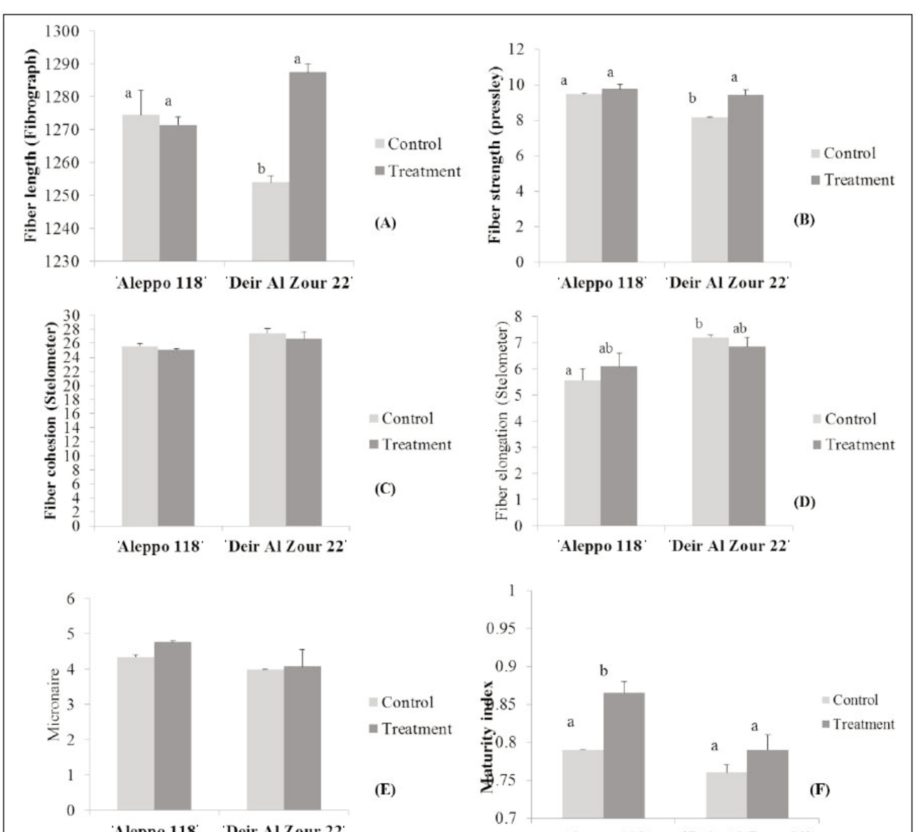

Fig. 3 - Fiber properties of control and treated Aleppo 118 and Deir Al-Zour 22 cultivars. Fiber length (A), fiber strength (B), fiber cohesion (C), fiber elongation (D), micronaire (E), and maturity index (F). Data were subjected to Duncan's test with a confidence level of 95% using STATISTICA program. Columns sharing a letter are not significantly different. Data represent means and standard error of three replications.

respectively. As for the second band at 1740 cm^{-1}, which is assigned to a carboxylic ester group (C=O stretching), a clearer structure is noticed in the treated sample in comparison with the control. Another distinctive banding pattern is observed at 1605 cm^{-1} (adsorbed water), which is neither seen in the treated Aleppo 118 cultivar and nor observed in Deir Al-Zour 22 cultivar control sample. This band contains two prominent peaks vibrations at 1577 and 1540 cm^{-1} (Fig. 4 B).

Measurement of crystallinity by conventional x-ray powder diffractometry. Fiber analysis using the XRD procedure showed a typical cellulose pattern in both control and treated plants for each cultivar (Fig. 5). The amount of crystalline cellulose in studied samples was between 66-75% (Table 3). As seen in Table 3, there was a minor difference between the CI values of the treated and control 'Deir Al-Zour 22' samples. However, there is no variation between control and treated samples of Aleppo 118 cultivar.

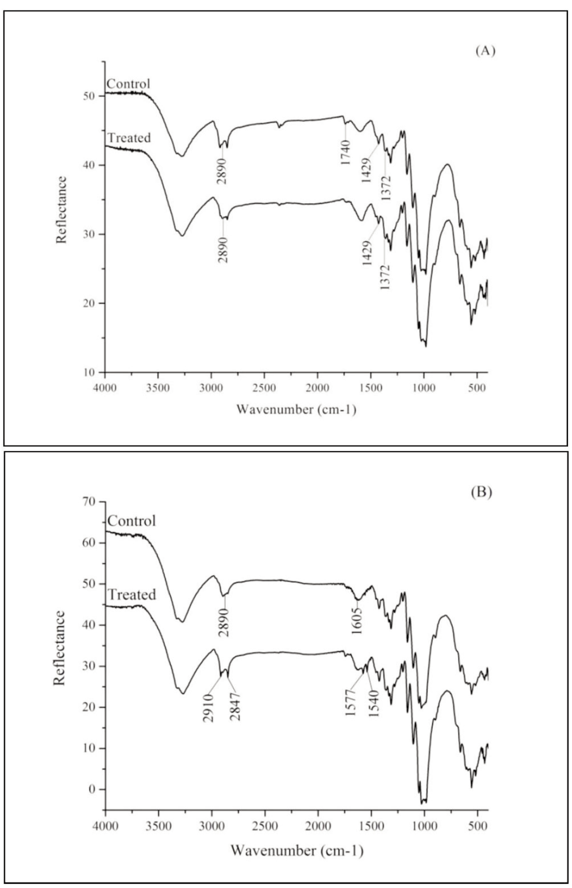

Fig. 4 - FTIR-ATR spectra of the cotton fibers obtained from (A) 'Aleppo 118' control and treated plants and (B) 'Deir Al-Zour 22' control and treated at room temperature in the range 400-4000 cm^{-1}. The recorded spectra were repeated three times and showed a typical and characteristic absorption bands for common cotton fibers substances.

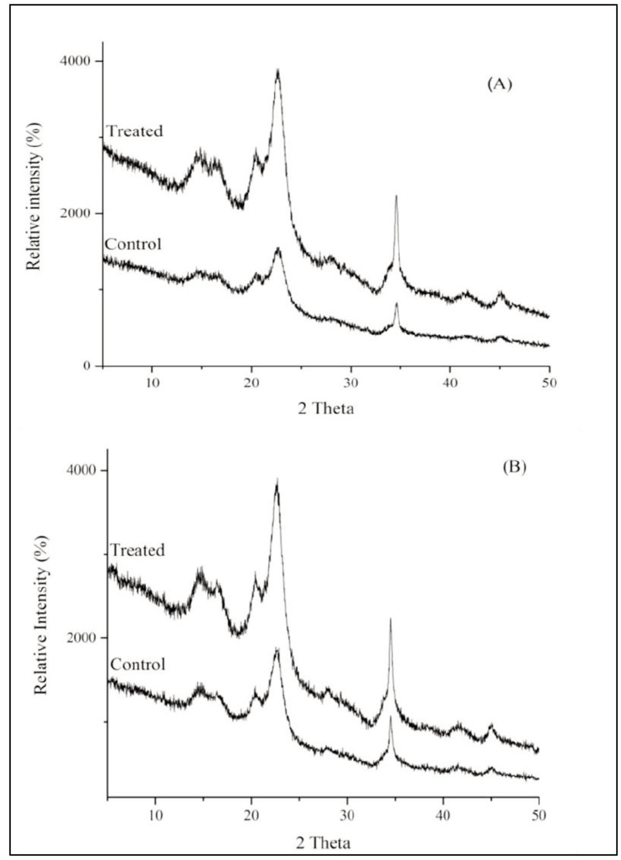

Fig. 5 - X-ray powder diffraction patterns of fibers in control and treated Aleppo 118 and Deir Al-Zour 22 cultivars.

Table 3 - Fiber crystalline index in control and treated Aleppo 118 and Deir Al-Zour 22 cultivars

Cultivar	Crystallinity Index (%)	
	Control	Treatment
Aleppo 118	66.5±3.0	66.8±3.0
Deir Al-Zour 22	73.7±3.0	68.5±3.0

Thermogravimetry analysis (TGA) and differential scanning calorimetry (DSC). Typical dynamic TGA thermograms of the studied samples have been recorded and are shown in figure 6 A. The TGA thermograms show a slight decrease in the weight and one significant step at high temperature.

Differential scanning calorimetry (DSC) was used to locate a possible glass transition temperature (T_g) of the samples. The glass transition temperature could not be observed in all DSC thermograms. The endothermic peak at around 100°C could be due to evaporation of adsorbed water (Fig. 6 B).

Scanning electron microscopy (SEM). SEM images reveal the longitudinal morphology of fibers coming from control and treated plants of Aleppo 118 and Deir Al-Zour 22 cultivars under three magnifications

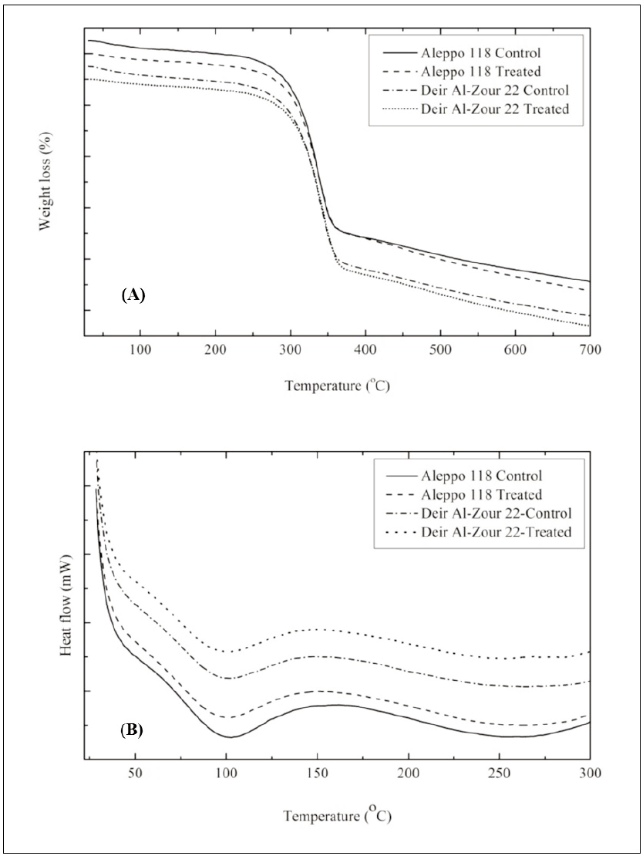

Fig. 6 - (A) TGA thermograms of cotton fibers in presence of nitrogen. (B) DSC thermograms of cotton fibers in presence of nitrogen.

(1.00 kx, 3.00 kx and 20.00 kx) (Fig. 7). Images of the two cultivars show the ribbon fiber shape rolled in a helicoid manner, a typical cotton morphological feature. The spiral and twisting fiber nature is present in both cultivars under both conditions.

Expression analysis of DREB1A, TPS and HSPCB drought-related genes

Fold expression of three drought-related genes in cotton (*DREB1A*, *TPS* and *HSPCB*) was analyzed, assisted by *GhEF1α5* gene expression for normalization (Fig. 8). Leaf samples of control and treated plants of both cultivars were harvested at two points (stages), S1 and S2. Where S1 represents leaf material harvested at 40 DAP (during floral bud to flower transition stage) and S2 represents leaf material harvested at 60 DAP (bolls opening and maturation). Gene expression analysis showed an activation of the studied genes in treated plants compared to the controls of both cultivars during S1. The S2 stage, experienced a drop in genes activity in both treated cultivars. Analysis showed a major significant drop in gene activity (*HSPCB* and *TPS*) in treated 'Deir Al-Zour 22' plants (S2) compared to samples of the earlier stage (S1). Exceptionally, *DREB 1A* gene showed a slight decrease in gene expression keeping its high activity in treated plants ~4 fold higher than the con-

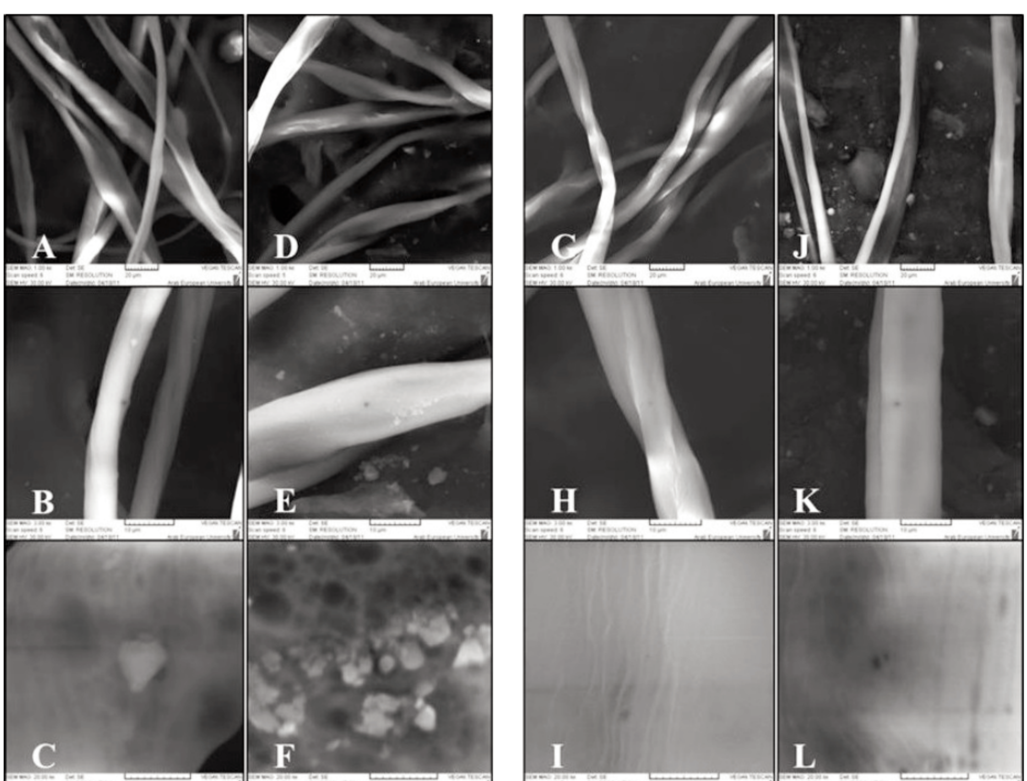

Fig. 7 - SEM images of cotton fibers. A, B and C. fibers of control 'Aleppo 118' under 1.00, 3.00 and 20.00 kx magnifications. D, E and F. fibers of treated 'Aleppo 118' under 1.00, 3.00 and 20.00 kx magnifications. G, H and I. fibers of control 'Deir Al Zour 22' under 1.00, 3.00 and 20.00 kx magnifications. J, K and L. fibers of treated 'Deir Al Zour 22' under 1.00, 3.00 and 20.00 kx magnifications.

trols. On the other hand, Aleppo 118 cultivar showed an almost steady expression pattern of the three genes in the two stages (high expression in S1 and low in S2). This continuous high expression activity of *DREB 1A* needs to be further investigated in 'Deir Al-Zour 22' to study its relation to water deficit tolerance.

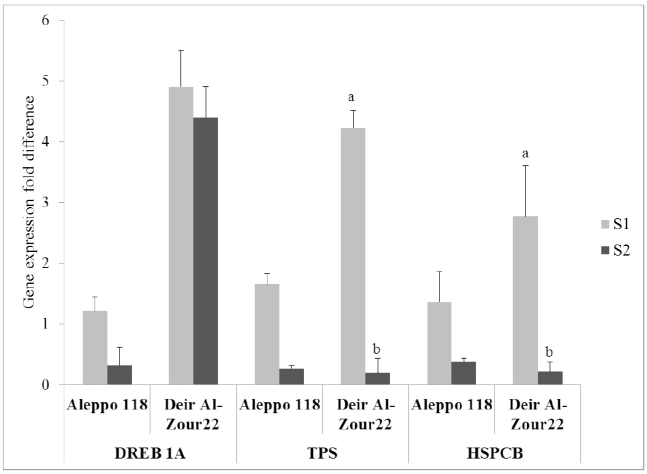

Fig. 8 - Fold expression of DREB1A, TPS and HSPCB genes in leaves of treated 'Aleppo 118' and 'Deir Al-Zour 22' plants. Leaf samples were collected in two stages, S1 (during floral bud to flower transition stage) and S2 (bolls opening and maturation). Data were subjected to Duncan's test with a confidence level of 95% using STATISTICA program. Significant fold expression change of TPS and HSPCB genes was observed between S1 and S2 treated 'Deir Al-Zour 22'. Data represent means and standard deviation of three replications.

4. Discussion and Conclusions

This study overlooks the behavior of two accredited cotton cultivars subjected to two irrigation regimes along their life cycle. It is anticipated that water scarcity in the Mediterranean region will increase under climatic change and this can be addressed by evaluating the impacts of climate change on water resources and their management, the adaptive capacity and the policy responses (Iglesias *et al.*, 2011).

Improving water resources management is challenged by the physiology and biology of the crop, irrigation practices, environment, farmers' perspectives, and government funding. Increasing crop water productivity, i.e. producing more food, income, better livelihoods and ecosystem services with less water (Molden *et al.*, 2010), has been a major interest to agronomists, farmers, environmentalists and economists.

Our research is mainly interested in understanding the responses of two credited Syrian cotton cultivars grown under water-shortage stress conditions, motivated by the concept of reaching a better quality and quantity of cotton fiber coupled with saving ground water resources in a cotton growing country.

Recently, drip irrigation systems have been widely employed, due to their improvement of water use efficiency (Patanè *et al.*, 2011). Hence, our field experiments were irrigated using the drip irrigation system to reduce water runoff losses.

Two credited cotton local cultivars, Aleppo 118 and Deir Al-Zour 22, have been investigated in our study. The two cultivars are assigned by the Ministry of Agriculture to be grown in their pertinent environments in the Syrian land. 'Deir Al-Zour 22' is the featured cultivar for cultivation in Deir Al-Zour Province, along the Euphrates, where high temperature is the dominant feature of the area especially during the growing season. 'Aleppo 118', an early maturity cultivar, is cultivated in the arid and semi-arid Northern-West regions of Syria, mostly in Aleppo province.

Control (full irrigation) and treated (deficit irrigation) plants of both cultivars were tested using some physiological, morphological, molecular, and biochemical parameters. The restricted water regime applied on our treated plants showed mild, non-significant effects on chlorophyll a and b content, which indicates a weak impact of the water-shortage stress regime on the photosynthesis process. However, the cultivar Aleppo 118 showed a slight reduction in chlorophyll a/b ratio in treated plants compared to a larger reduction in treated plants of 'Deir Al-Zour 22', which indicate the presence of higher chlorophyll b content in treated 'Deir Al-Zour 22' plants. This in turn may suggest a slower degradation process of chl b coming from a decrease in the enzymatic activities that are known to render the conversion of chl b to chl a (Folly and Engel, 1999). It is suggested that drought stress has to be prolonged and severe before the chloroplast start to break down (Jiang *et al.*, 2010). Another non-significant drop was also observed in the osmotic potential values of treated plants in both cultivars. However, the cultivar Aleppo 118 showed a greater osmotic adjustment value which may indicate its stronger turgor maintenance mechanism. Osmotic adjustment has been also associated with maintenance of membranes and protein structure, protection against oxidative damage (Dacosta and Huang, 2006). It has been reported that the greater the stress duration (increasing the number of stress cycles), the larger the osmotic adjustment in cotton leaves (Oosterhuis and Wullschleger, 1987).

The morphology and phenology of plants can be significant indicators of drought tolerance. Changes in plant growth rate and/or in flowering time are two common strategies that plants use to cope with drought (Schmalenbach *et al.*, 2014).

The allotetraploid *G. hirsutum* L. species has a distinct growth manner in which the plant keeps a systematic morphological architecture (Khan, 2003) that can be useful in monitoring growth and developmental changes under diverse environmental conditions (Jawdat *et al.*, 2012). In general, physiological processes are induced in plants under stress conditions to reduce the cellular damage and to alter developmental timing to complete their life cycle fittingly (Yaish *et al.*, 2011). Stress-mediated flowering has been reported in several plant species; stresses like ultraviolet C, poor nutrition, low temperature and drought (Wada and Takeno, 2010). Our results noted earliness in treated plants of both varieties and a tendency of 'Aleppo 118' to bear higher percentage of flowers and floral buds toward the end of season. 'Aleppo 118' has been reported to show excessive vegetative growth under excess water and nitrogen compared to other local cultivars (CRA, personal communication). This may suggest that water status (excess and shortage) may hold back or trigger floral cues, and this needs to be investigated fully taking into consideration our result of the higher osmotic adjustment in 'Aleppo 118' compared to 'Deir Al-Zour 22'.

The small increase in stem height in treated plants of both cultivars can be explained that plants accumulate reserves in shoots and stems to cope with water stress (Chavez *et al.*, 2002). The water deficit regime, applied in our study through drip irrigation, encouraged the increase in number of secondary roots in treated plants of both cultivars on the expenses of a decrease in tap root length.

Fiber quality, which is a result of interactions between genetics and environment, controls fiber prices of cotton textile products (Wang *et al.*, 2014). The quest for cotton growers is the balance between fiber quality and quantity. Achieving such a balance in cotton cultivation regions with growing water shortages is a main concern to the agricultural and industrial sectors. An escalating number of publications aim at understanding the mechanism of cotton fiber development and adaptation to abiotic stresses, such as drought, for the improvement of cotton fiber yields and quality (Zhu *et al.*, 2011; Deeba *et al.*, 2012; Padmalatha *et al.*, 2012; Bowman *et al.*, 2013; Riaz *et al.*, 2013; Wang *et al.*, 2013; Zhang *et al.*, 2013; Sekmen *et al.*, 2014; Xie *et al.*, 2015). Cotton growth and yield are severely affected by excessive water-shortage stress, especially at critical growth stages such as the flowering phase (Dağdelen *et al.*, 2009). Soil water was found to be correlated with fiber strength, elongation (Johnson *et al.*, 2002) and fiber maturity (Davidonis *et al.*, 2004). Our results showed that two fiber properties, cohesion and micronaire, had no significant differences between control and treated plants of the two cultivars. It is with fiber cohesion, a property that causes materials to cling together, yarn spinning from staple fiber is possible (Wakelyn, 2007). A previous study showed fiber cohesion stability of 'Deir Al-Zour 22' between seasons and locations; whereas, 'Aleppo 118' showed cohesion stability between seasons in one location (Jawdat *et al.*, 2012). As for fiber micronaire, it reflects fiber fineness and is a measure of internal fiber thickness and deposits of cellulose. It has a desired value range in the international market between 3.5 and 4.9 (Jawdat *et al.*, 2012). Fiber micronaire, showed stability in both 'Aleppo 118' and 'Deir Al-Zour 22' between seasons and locations (Jawdat *et al.*, 2012). This indicates that environmental factors including water deficit have weak impact on the two fiber properties, cohesion and micronaire. Deficit irrigation showed no significant effect on fiber length and strength in Aleppo 118 cultivar. However, it had significant impact on the two fiber properties, causing increment in both strength and length of fiber in treated plants of Deir Al-Zour 22 cultivar. Fiber strength is an indicator of fiber resistance to stretching (Wang *et al.*, 2014) and is determined by environmental conditions and cultivar traits (Zhou *et al.*, 2011). It has been found that fiber strength is correlated with daily mean temperature during fiber development (Hanson and Ewing, 1956; Ma *et al.*, 2006; Wang *et al.*, 2009). This has been also observed on a previous study where a difference of 4-5°C showed a significant effect on fiber strength in both 'Aleppo 118' and 'Deir Al-Zour 22' (Jawdat *et al.*, 2012). However, this temperature difference did not affect fiber length in both cultivars (Jawdat *et al.*, 2012). This shows the impact of each of water deficit and temperature on fiber strength of 'Deir Al-Zour 22' compared to the impact of only temperature on fiber strength of 'Aleppo 118'. Fiber maturity can be defined as the relative wall thickness and wall development (Wakelyn, 2007). In our work, we observed an increase in fiber maturity factor values in treated plants compared to the controls, which suggests that water deficit did not affect the maturity of cotton fiber in a negative way and hence their quality as related to dye-ability and ease of processing.

The FTIR-ATR spectra of the cotton fibers obtained from control and treated Aleppo 118 and Deir Al-Zour 22 cultivars showed similar spectra range except for few noticeable bands. This can be due to a reduction in surface water adsorption which leads to stronger fibers. The minimized accessibility of water molecules to the internal hydroxyl groups can be mostly due to the formation of cellulose macromolecules that induce a re-organization of cellulose and also increase the ordered cellulose (or crystalline) portion to produce a stronger fiber (Liu, 2013) in Deir Al-Zour 22 treated cultivar. Results of XRD showed minor CI variation between fibers from control and treated Deir Al-Zour 22. Both TGA and DSC showed no variation between fibers of treated and control plants in both cultivars.

Our work has also investigated gene fold change in expression of the transcription factor gene (*DREB1A*), the molecular chaperone gene (*HSPCB*) (Sotirios *et al.*, 2006), and the trehalose biosynthesis gene (*TPS*) (Kosmas *et al.*, 2006). The two genes *HSPCB* and *TPS* have been found in few studies to be expressed differently between drought-tolerant and drought-sensitive cotton genotypes. While the *HSPCB* showed differential expression during the drought period in leaves of drought tolerant genotypes and not in the sensitive ones (Nepomuceno *et al.*, 2002; Voloudakis *et al.*, 2002), the *TPS* gene was induced during the water stress period in both tolerant and sensitive cultivars (Nepomuceno *et al.*, 2002). Interestingly, our study showed a significant reduction in *HSPCB* and *TPS* genes activity in leaves during bolls opening and maturation in both cultivars. Whereas, *DREB 1A* gene kept a high active mode in leaves of treated 'Deir Al-Zour 22' plants compared to 'Aleppo 118' treated plants during that stage. Dehydration-responsive element binding proteins (DREBs) are transcription factors known to activate the expression of abiotic stress-responsive genes in divergent species via specific binding to the dehydration-responsive element/C-repeat (DRE/CRT) *cis*-acting element in promoters of target genes (Mizoi *et al.*, 2012). In *Arabidopsis*, the overexpression of *DREB1A* revealed both freezing and dehydration tolerance in transgenic plants (Liu *et al.*, 1998). A better drought and salt tolerance was observed in transgenic wheat plants with *DREB1* from *Glycine max* (Shiqing *et al.*, 2005). The overexpression of *Oryza sativa DREB1* in rice transgenic plants has also showed improved tolerance to drought, salt and low temperature stresses (Dubouzet *et al.*, 2003; Ito *et al.*, 2006). A group of researchers found that *DREB 1A* was induced at a high level in pistils, three days after drought treatment which suggests its possible role as an early drought response regulator in the *Arabidopsis* flower (Su *et al.*, 2013). Our study adds to such findings and points to *DREB 1A* constant up-regulation in leaves of 'Deir Al-Zour 22' under drought stress, during flowering and boll maturation.

Our study has presented the responses of two accredited cotton cultivars, Aleppo 118 and Deir Al-Zour 22 which have shown different behavior under water shortage. Both cultivars have shown early flowering. However, a larger percentage of treated 'Aleppo 118' plants kept flowering towards end of season compared to treated 'Deir Al-Zour 22' plants. This may indicate a potential survival mechanism and fruiting cycle extension in Aleppo 118 cultivar under water deficit regime, compared to the cultivar Deir Al-Zour 22.

Aleppo 118 cultivar displayed more consistent fiber quality between control and treated plants, while 'Deir Al-Zour 22' showed variation in fiber length and strength between control and treated plants. Both cultivars kept consistent micronaire and cohesion under normal and water deficit conditions. Two critical stages in cotton life cycle, flowering and boll development, were screened for *HSPCB*, *TPS* and *DREB 1A* gene activity using leaf material. Results have demonstrated an increase in fold expression of these genes in the flowering stage of treated plants compared to the controls. A large regression in genes activity was noticed during boll development except for *DREB1A* gene in treated 'Deir Al-Zour 22' plants. *DREB1A* gene showed continuous activation in the two stages and can be considered a candidate gene to support water deficit stress tolerance mechanism in this cultivar.

The question of which cultivar is more drought-tolerant than the other is to be deeply investigated in both cultivars, since each cultivar has shown different approach towards water-shortage stress tolerance.

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