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PHYSIOLOGICAL, MORPHOLOGICAL, BIOCHEMICAL AND MOLECULAR BASIS OF DROUGHT TOLERANCE IN COTTON

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ABSTRACT

Water is the limiting factor for plant growth, it severely affects the crop productivity. Cotton has been resistant to drought, yet it is one of the biggest factors, behind its low productivity. The current study is a comparative analysis to evaluate the physiological, morphological, biochemical and molecular parameters between two cotton varieties, FDH-786-*Gossypium arboreum* and CIM-496-*Gossypium hirsutum*, under different levels of drought stress. Plant height, root length, fresh and dry biomass and total leaf area were found to be decreased under drought stress in both the varieties, but significant reduction was observed in case of CIM-496-*Gossypium hirsutum*. Other morphological parameters like root, stem and leaf weight ratio and leaf area ratio were also considerably reduced in CIM-496 as compared to FDH-786. Leaf Relative water, proline and chlorophyll content, cell membrane stability, transpiration and photosynthesis rate and *Stomatal* conductance were also sharply decreased in CIM-496 as compared to FDH-786. Relative expression level of drought responsive genes (TPS, PIP, Gh-POD and LHCP-PSII) were observed to be expressed at higher levels in FDH-786 under different drought stress conditions. In epitome, the current study concluded with the remarks that CIM-496-*Gossypium hirsutum*, variety of cotton, is drought prone while FDH-786-*Gossypium arboreum* is drought resistant.

Keywords: *Gossypium hirsutum*,*Gossypium arboreum*, Drought tolerance, Abiotic stress, Cotton

INTRODUCTION

Drought is an abiotic stress, it has drastic affect on plant growth and crop productivity **[1]**.The severity of drought is unpredictable as it depends upon the several factors, among them occurrence and distribution of rain fall, intensity of radiation, moisture storing capacity of soil, low annual rain fall and unavailability of irrigation water are worth mentioning **[2]**. For plants, this scarcity of water is a severe environmental constraint, it affects photosynthesis, major physiological process, by disrupting the functioning of *Stomatal*, which ultimately rule out the bumper production of crop productivity **[3]**. Plants are responsive to drought at physiological, morphological, biochemical as well as at molecular levels **[4,5]**. Plant height, root length, fresh and dry biomass, chlorophyll and proline content, rate of photosynthesis and expression of drought responsive genes are reliable indicators of plant response to drought stress **[6,7]**.

Plants activate diverse set of metabolic activities to defend themselves against drought stress **[8]**. They respond to this extreme environmental condition by increasing chlorophyll production, biosynthesis of osmoprotectants, detoxification of reactive oxygen species, stability of proteins and by increasing water uptake **[9]** and all these processes are triggered by several genes at cellular level. Involvement of multiple genes at genetic level in the initiation of defensive mechanism makes it difficult to fully understand the genetic basis of this system **[10]**. Cotton crop is grown around the world and Pakistan is the 4th largest grower after China, India and USA. Its contribution to our national economy is significant because it provides raw material to textile industry. It share in GDP is 1.6% and its value addition in agriculture is 7.8% **[11]**.

The diploid cotton species are not only the reservoir of improved agronomic and other important traits but also offers better opportunities to analyze gene structure and functions. Drought tolerance mechanism is not completely understood but it can be predicted by observing the performance of crop while studying the various growth factors, physiological, morphological adaptations **[12]** and expression of drought responsive genes **[5, 13]**. Current study was designed to gain a better understanding of the response to drought stress and a comparison was made between CIM-496-*Gossypium hirsutum* and FDH-786-*Gossypium arboreum* to identify the morphological, biochemical,

physiological and molecular parameters, which are playing role under drought stress.

METHODOLOGY

Seed germination, growth conditions and drought stress treatment:

Seeds of CIM-496-*Gossypium hirsutum* and FDH-786-*Gossypium arboreum* were delinted, sterilized and were grown in plastic pots having the soil in green house. Plants were drought stressed after 45 days of germination. First time data and samples were collected after 45 days of germination, before drought stress. Second time samples were obtained after 50 days of germination, after 5 days of stress initiation (5DS). Similarly, another 3rd round of sampling was carried out at 10, 15 and 20 DS respectively. The plants of both varieties kept under controlled conditions, provided regular water, were considered as control plants. Percentage of germination was determined by using the following formula.

Germination percentage = (Number of germinated seeds) / (Total number of seeds) × 100

Morphological Studies

Different growth attributes **[14]** like plant height, root and shoot length, root shoot ratio, total leaf area, plant growth analysis, fresh and dry biomass, root, stem and leaf weight ratio, specific leaf area and its ratio were measured for both the varieties by using the following formula.

$$\text{Percent Increase in Plant Height} = \frac{\text{Final Height} - \text{Initial Height}}{\text{Initial Height}} \times 100$$

$$\text{Percent reduction in biomass} = \frac{\text{Fresh Biomass} - \text{Dry Biomss}}{\text{Fresh Biomass}} \times 100$$

Physiological studies

Relative water content: (RWC)

To determine the relative water content (RWC) leaves from control and stressed plants at each stage of multiple stress condition (5, 10, 15 and 20DS) were taken. Turner method **[15]** was followed and RWC was calculated by using the following formula.

$$\text{Leaf Relative Water Content (RWC)} = \text{FW} - \frac{\text{DW}}{\text{TW}} - \text{DW} \times 100$$

Cell Membrane Stability: (Cms)

Cell membrane stability of control and stressed pants was determined by following the method described by **[16]** and CMS was calculated by using the following formula.

$$\text{CMS\%} = \frac{\text{EC1} - \text{EC0}}{\text{EC2} - \text{EC0}} \times 100$$

Gas exchange parameters

Gas exchange parameters like rate of photosynthesis, (Pn, µmol CO2/m2s) and transpiration, (E, µmol H2O/m2s) and Stomatal conductance (C, µmol/m2s) were measured from the control and stressed leaves by using handheld IRGA (340 Bioscientific Ltd., UK).

Biochemical studies

Proline content

Proline content was estimated to ascertain the degree of drought in stressed and control plants. To measure the proline content method described by Samaras*et al.*, **[17]** is followed.

Malondialdehyde (MDA) Estimation

Malondialdehyde (MDA) level was measured according to the method described by Quan **[47]** and the final concentration of MDA was calculated with the help of the following formula.

$$C(\mu mol\ L^{-1}) = 6.45(OD_{532} - OD_{600}) - 0.560D_{450}$$

Chlorophyll content

The concentration of a, b and total chlorophyll were determined according to the method described by Arnon and Whatley **[18]** with the help of following formula.

$$\text{Chlorophyll a} = 12.7\,(OD_{663}) - 2.6\,(OD_{645}) \times ml\frac{acetone}{mg}\text{leaf tissue}$$

$$\text{Chlorophyll b} = 22.9\,(OD_{645}) - 4.7(OD_{663}) \times ml\frac{acetone}{mg}\text{leaf tissue}$$

$$\text{Total Chlorophyll} = \text{Chlorophyll a} + \text{Chlorophyll b}$$

Molecular Analysis

Total RNA was extracted **[19]** from leaves of plants stressed at different stages and quantified with Nanodrop (ND-1000) at A260/280 and A260/230. cDNA was synthesized (RevertAidTMH minus first strand cDNA synthesis kit, Fermentas) and the real time quantitative PCR was set up to amplify the TPS, PIP, Gh-POD and LHCP-PSII genes by using the following set of primers.

TPS F' 5'-TGCCTGCCTGAGATTAAGGT-3', R' 5'-CTAGCAAGTGCCAATCACGA-3';

PIP F' 5'-TTCACATGTACCCGTGTTGG-3', R' 5'-GGTCCAACCCAGAATATCCA-3';

Gh- PODF' 5'-GTGGCCGAATAAAATGGTTG-3', R'5'-TACATCCACTACGCCCATGA-3'; **LHCP-PSII** F' 5'GAAAACCACCAAGCCTGTTC-3', R'5'GCTCACGGTTCCTAGCAAAG3'

Reaction mixture contained 0.5 µl cDNA, 1µl each primer (10pm), and 7.5µl 2x SYBER green master mix. Reaction conditions were; Denaturation at 94°C for 3 min, 35 cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec, final extension at 72°C for 10 min and held at 4°C.

RESULTS

Germination Percentage

The germination rate of FDH-786-*Gossypium arboreum* was 70.1%, while CIM-496-*Gossypium hirsutum* showed 67.7% rate of germination.

Morphological parameters

Percentage increase in plant height

The plant height of both the cotton varieties under control and stressed conditions was measured and % increase was calculated. The % increase in plant height of CIM-496 plants under different drought stress levels, 5, 10, 15

and 20DS was 11.9, 11.9, 14.1 and 15.7%, while under control conditions the % increase in plant height was in the range of 10.34 to 86.2%. On the other hand the % increase in plant height of stressed FDH-786 plants in multiple drought stress levels was 6.1, 11.3, 15.1 and 20.5%, respectively, while under control conditions the % increase in plant height of FDH-786 was in the range of 19.2% to 84.7% as shown in **Table 1**. The results indicated that FDH-786 is more drought tolerant as compared to CIM-496 because 4.8% difference in plant height of both the varieties is the strongest evidence in this regard (Table:1). Increase in plant height was rapid in FDH-786 as the stress level increases.

Percent Reduction in Plant Biomass

Reduction in plant biomass was calculated to study the effect of drought stress on both the cotton varieties. Plant biomass was variable at different stress levels in both the varieties. The % reduction in biomass of CIM-496plants was 44.5% and was 73.04 % in FDH-786 before the application of stress treatment. Likewise, the percent reduction in biomass ofCIM-496 plants growing under control conditions was 71, 62.5, 67.7 and 72.3% at 5, 10, 15, 20DS respectively, and for control plants of FDH-786, it was74.7, 77.14, 75.21 and 73.62% respectively. At multiple drought stress levels, 5,10,15 and 20DS the % reduction in biomass of stressed CIM-496 *G. hirsutum* plants was 72.38, 71.94 , 76.01 and 75.25% respectively, while the same in FDH-786 *Gossypium arboreum* plants at 5,10,15 and 20DS was74.52, 74.67, 75.59 and 74.49% respectively. Percent reduction in biomass was increased as the stress level was increased in both the genotypes. However, the results revealed that FDH-786 reduced less biomass as compared to CIM-496 **(Table 2)**.

Physiological parameters

Relative water content

Relative water content was estimated to determine the effect of drought stress on cotton plants. The relative water content of control CIM-496 plants was 59, 64, 69, 75 and 81% after 45, 50, 55, 60 and 65 days of germination, respectively. Similarly, for FDH-786 control lines it was 63, 67, 74, 79 and 86% respectively as shown in **Table 3**. At multiple stress levels (5, 10, 15 and 20DS) the Relative water content of stressed CIM-496 plants were 58,54,50, 49% and for FDH-786 stressed plants, at multiple stress levels, were 61,60,57 and 53% respectively as shown in **Table 3**. Drought stress left a pronounced effect on both the cotton varieties, however, results indicated that it has a significant effect on CIM-496 plants as the severity of drought was increased.

Cell Membrane Stability

To determine the cell membrane stability, the ion leakage inCIM-496plants before the application of stress treatment was 91% and of FDH-786 plants was 93%. Similarly, the cell membrane stability of CIM-496 control plants was observed as90, 91, 90and 91% and for FDH-786 it was 92, 91, 93 and 92% respectively. At multiple stress levels, 5,10,15 and 20DS, the cell membrane stability of the plants which were grown under drought stress conditions was 83, 72, 58 and 44% respectively. Similarly, the cell membrane stability in FDH-786 under multiple stress conditions was89, 85, 80 and 76% respectively as shown in the **Table 4**. It was concluded from results that all drought stress levels have a significant impact on both varieties. The plants grown under control conditions have high cell membrane stability as compared to the plants grown under multiple stress conditions. Cell membrane stability was decreased as the stress level increased in both varieties. However, it can be said that FDH-786 was found more drought tolerable variety under drought stress treatment as compared to CIM-496.

Photosynthetic Activity

In this study, the reduction in the photosynthetic activity was observed as the drought stress was imposed progressively. The rate of photosynthesis of CIM-496plants before the application of stress treatment was 11.4 µmol/m^2/s and for FDH-786 plants, the rate was 14.4 µmol/m^2/s. Likewise, the rate of photosynthesis of CIM-496 control plants at 5,10,15 and 20DS, was 13.3,15.4,17.7 and 19.4 µmol/m^2/s and forFDH-786 control plants, that was observed as15.9, 17.2, 19.6, 21.3 µmol/m^2/s respectively as shown in Table: 5. At multiple stress levels (5,10,15,20DS) the rate of photosynthesis ofCIM-496 plants grown under stress conditions was 9.4, 7.3, 5.4 and 4.1 µmol/m^2/s respectively. Likewise, for FDH-786 plants, that was 12.4, 8.3, 7.1 and 5.2 µmol/m^2/s respectively as shown in the **Table 5**.

Stomatal Conductance

Before the application of stress treatment, the *Stomatal* conductance of CIM-496 control plants was 3.6 mmol/m^2/s and was 3.4 mmol/m^2/s for control FDH-786 plants. Similarly, the *Stomatal* conductance of CIM-496 at 5, 10, 15 and 20DS under control conditions was 3.9, 4.4, 5.0 and 5.6 mmol/m^2/s and for FDH-786control plants, it was observed at 4.0, 4.3, 5.1 and 5.9 mmol/m^2/s respectively. At multiple levels of drought stress conditions (5,10,15,20DS) the *Stomatal* conductance of CIM-496 plants growing under drought stress was 3.2, 2.8, 2.5

and 2.1 mmol/m^2/s, respectively. Likewise, that was 3.0, 2.8, 2.7 and 2.7 mmol/m^2/s for FDH-786 stressed plants at multiple drought stress treatments, respectively as shown in the **Table 6**.

Transpiration rate

Transpiration rate was decreased when both the genotypes were subjected to the drought stress, but the significant reduction was observed in CIM-496 plants as compared to the FDH-786 plants. Before the application of drought stress, transpiration rate of control CIM-496 plants was4.6, 5.2, 6.7, 9.3 and 10.1 µmol/m^2/s and that was observed as5.0, 5.4, 6.3, 8.4, 10.3 µmol/m^2/s respectively in FDH-786. While at multiple drought stress, transpiration rate of CIM-496 plants was 4.1, 3.2, 2.8, 2.4 µmol/m^2/s and that was observed as4.6, 4.1, 3.7, 3.2 µmol/m^2/s for stressed plants of FDH-786 as shown in the **Table 7**. Results indicated that at multiple drought stress levels FDH-786 plants showed the greater transpiration rate as compared to the CIM-496plants.

Biochemical Parameters

Proline Content

The concentration of proline was found high in plants subjected to drought stress as compared to the plants under control conditions. Proline content of CIM-496 control plants was 16.2, 24.3, 32.7, 49.9, 67.2 mg/g of fresh leaf tissue and for FDH-786 control plants, that was19.4, 23.1, 36.2, 48.7, 61.9 mg/g under control conditions. At multiple drought stress levels, Proline content inCIM-496plantswas 29.4, 38.6, 54.7, 72.6 mg/g. Similarly, Proline content in stressed plants of FDH was 25.4, 39.4, 56.7 and 78.4 mg/g, respectively at 5,10,15 and 20DS as shown in the **Table 8**. This study revealed that the proline content was more inFDH-786 is more under drought stress conditions as compared to CIM-496.

Lipid Peroxidase

To investigate the effect of drought stress on lipid peroxidation, MDA (a product of Malondialdehyde and index of toxic oxygen species generation) content had been measured. As the plants were subjected to drought stress, MDA was found to be more in both the varieties. When comparing the two varieties, the MDA content was more in FDH-786 plants as compared to CIM-496. The MDA content of CIM-496plants before the application of stress treatment was 2.4, 4.4, 6.7, 8.3, 9.2 nmol/g at 5, 10, 15, 20DS, and for FDH-786 control; plants, that was 3.9, 6.3, 7.4, 9.4 10.1 nmol/g respectively as shown in Table: 9. At multiple drought stress level such as5, 10, 15, 20DS, MDA content in CIM-496 was 6.7, 8.1, 10.2 and 11.3 nmol/g respectively. Similarly, MDA content inFDH-

786 at multiple drought stress was 7.4, 9.3, 11.6 and 13.7 nmol/g respectively as shown in **Table 9**.

Chlorophyll Content

The chlorophyll content of both the varieties was decreased as the drought stress was imposed progressively. In control plants of CIM-496before the application of stress treatment, the chlorophyll content was 10.1 mg/g and in FDH-786-*Gossypium arboreum* plants before the application of stress treatment was 11.3 mg/g. Similarly, Chlorophyll content of CIM-496 control plants at 5,10,15,20DS, were 12.4, 13.6, 15.3, 17.9 mg/g and in FDH-786control plants, that was observed as14.4, 15.6, 17.3 and 17.2 mg/g, respectively as shown in Table: 10. The chlorophyll content of CIM-496 plants grown under multiple drought stress (5,10,15, 20DS) was 8.4, 6.6, 4.7 and 4.0 mg/g respectively. Likewise, chlorophyll content inFDH-786stressed plants was 9.7, 7.3, 5.4 and 5.1 mg/g respectively as shown in the **Table 10**.

Molecular Analysis

The expression level drought response genes like TPS (Gene Bank Accession # EU750912.1), PIP (Gene Bank Accession # FJ646597.1.1), Gh-POD (Gene Bank Accession # L08199.1) and LHCP-PSII (Gene Bank Accession # L07119.1) genes were determined by using real time quantitative RT-PCR. Expression analysis studies showed that under stress condition, the CIM-496plants were sensitive to drought condition than FDH-786.

Expression of *TPS* (trehalose-6-phosphate-synthase)

Trehalose is a non-reducing disaccharide it serves as stress protectant from denaturation. The relative expression of *TPS*-gene in the plants of CIM-496 was increased as the drought stress level was increased. On the other hand, the expression level of *TPS*-gene was decreased in FDH-786plants as the stress level was increased. Relative high expression of the *TPS* gene in control and stressed plants of FDH-786 showed their sensitivity to water deficiency. However, its relative high expression in CIM-496 plants, under stress conditions showed its tolerance to drought stress as compared to FDH-786 as shown in the **Figure 1**.

Expression of PIP (plasma membrane intrinsic proteins):

Plasma membrane intrinsic proteins (PIPs), a type of aquaporins, mediate water transport in many plant species. In this study, interestingly, the contradictory result has been found as in the case of *TPS*-gene analysis. The CIM-496 plants experienced more drought stress, so, the expression level of the *PIP*s-gene decreased in CIM plants as

compared to the FDH-786 plants. The relative expression level of *PIP*s-gene CIM-496 plants was 1.9 under control conditions while in FDH-786control plants its expression was 1.4 as shown in Table: 12.At multiple stress treatment the relative expression level of the *PIP*s-gene in CIM-496 plants was 1.9, 2, 2.1 and 2.3 respectively. Likewise, the relative expression level of the *PIP*s-gene in FDH-786stressed plants, at multiple stress levels (5, 10, 15 and 20) was 1.8, 2.4, 2.9, and 3.4 respectively as shown in **Figure 2**. The critical analysis showed that the relative expression of *PIP*s-gene increased as the drought stress level increased. Before the application of stress there was no significant difference in both control and stressed plants of both varieties of cotton, but at a higher stress level (15,20DS) relative expression of *PIP*s-gene was increased significantly in stressed plants.

Peroxidase (*GH-POD*) Gene Expression

Drought stress in addition to dehydration also induces the oxidative stress. For example generation of Reactive Oxygen Species (ROS) including super oxide radical, (O^{2-}) nascent oxygen, (O) hydrogen peroxide, (H_2O_2) hydroxyl ion (OH^-). To fight with these reactive oxygen species, plants produce antioxidant enzymes, especially *Gossypium hirsutum* Peroxidase (Gh-POD). It was found that the relative expression level of *Gh-POD*-Gene was remaining nearly constant in the plants of both varieties under control conditions. At multiple stress treatments the expression level of the *Gh-POD*-gene in CIM-496plants was 4.3, 6.7, 9.4 and 9.4 respectively. Similarly, FDH-786 plants the relative expression level was 4.9, 7.8, 10.6, and 12.9 respectively as shown in the **Figure 3**.

Expression Level of LHCP-PSII -Gene

It has been found from this study that *LHCP-PS*II-gene down regulated in stressed plants, but its expression intensity up regulated in control plants of both the varieties. Under control conditions its relative expression in CIM-496plants was almost 5.5 and in FDH-786was almost 5.8. At multiple stress levels the relative expression level of this *g*ene in CIM-496 stressed plants was 5.2, 4.7, 4.0 and 3.3 respectively. However, the relative expression level of the *LHCP-PS*IIin FDH-786 remained nearly 5.9, 5.3, 4.9, and 4.6 respectively (**Figure 4**).

Table 1: Percentage Increase in Plant Height of CIM-496-*G.hirsutum* andFDH-786-*G.arboreum* under Control and Multiple Drought Stress

Multiple drought stress condition	% Increase in Plant Height of CIM-496-*G. hirsutum*		% Increase in Plant Height of FDH-786-*G.arboreum*	
	Control	Stress	control	Stress
Before stress	-	-	-	-
5DS	10.34	11.9	19.2	6.1
10DS	31	11.9	30.8	11.3
15DS	58.62	14.1	48.5	15.1
20DS	86.2	15.7	84.7	20.5

Table2: Percent Reduction in Biomass of CIM-496-*G.hirsutum* and FDH-786-*G.arboreum* at the Control and Multiple Drought Stress

Multiple drought stress condition	Reduction in Biomass of CIM-496-*G.hirsutum* (%)		Reduction in Biomass of FDH-786-*G.arboreum* (%)	
	Control	Stress	Control	Stress
Before stress	44.5	-	73.04	-
5DS	71	72.38	74.7	74.52
10DS	62.5	71.94	77.14	74.67
15DS	67.7	76.01	75.21	75.59
20DS	72.3	75.25	73.62	74.49

Table 3: Relative water content of CIM- 496 *G.hirsutum* and FDH-786 *G.arboreum* under control and Multiple Drought Stress

Multiple drought stress condition	Relative Water Content of CIM-496-*G.hirsutum* (%)		Relative Water Content of FDH-786-*G. arboreum* (%)	
	Control	Stress	Control	Stress
Before stress	59	-	63	-
5DS	64	58	67	61
10DS	69	54	74	60
15DS	75	50	79	57
20DS	81	49	86	53

Table 4: Cell Membrane Stability of CIM- 496- *G.hirsutum* FDH-786-*G.arboreum* under Control and Multiple Drought Stress

Multiple drought stress condition	Cell Membrane Stability of CIM-496-*G.hirsutum* (CMS %)		Cell Membrane Stability of FDH-786-*G.arboreum* (CMS %)	
	Control	Stress	Control	Stress
Before stress	91	-	93	-
5DS	90	83	92	89
10DS	91	72	91	85
15DS	90	58	93	80
20DS	91	44	92	76

Table 5: Comparison of Rate of Photosynthesis in CIM- 496 *G.hirsutum* and FDH-786 *G.arboreum* under Control and Multiple Drought Stress

Multiple drought stress condition	Rate of Photosynthesis in CIM-496-*G.hirsutum* (µmol/m^2/s)		Rate of Photosynthesis of FDH-786-*G.arboreum* (µmol/m^2/s)	
	Control	Stress	Control	Stress
Before stress	11.4	-	14.4	-
5DS	13.3	9.4	15.9	12.4
10DS	15.4	7.3	17.2	8.3
15DS	17.7	5.4	19.6	7.1
20DS	19.4	4.1	21.3	5.2

Table 6: Comparison of Stomatal Conductance in CIM- 496- *G.hirsutum* and FDH-*786-G.arboreum* under Control and Multiple Drought Stress

Multiple drought stress condition	*Stomatal* Conductance in CIM-496-*G.hirsutum* (mmol/m^2/s)		*Stomatal* Conductance in FDH-786-*G.arboreum* (mmol/m^2/s)	
	Control	Stress	Control	Stress
Before stress	3.6	-	3.4	-
5DS	3.9	3.2	4.0	3.0
10DS	4.4	2.8	4.3	2.8
15DS	5.0	2.5	5.1	2.7
20DS	5.6	2.1	5.9	2.7

Table 7: Comparison of Transpiration Rate in CIM- 496 *G.hirsutum* and FDH-786 *G.arboreum* under Control and Multiple Drought Stress Levels

Multiple drought stress condition	Transpiration rate in CIM-496-*G.hirsutum* (µmol/m^2/s)		Transpiration rate in FDH-786-*G.arboreum* (µmol/m^2/s)	
	Control	Stress	Control	Stress
Before stress	4.6	-	5.0	-
5DS	5.2	4.1	5.4	4.6
10DS	6.7	3.2	6.3	4.1
15DS	9.3	2.8	8.4	3.7
20DS	10.1	2.4	10.3	3.2

Table 8: Comparison of Proline Content of CIM- 496- *G.hirsutum* and FDH-786-*G.arboreum* under Control and Multiple Drought Stress

Multiple drought stress condition	Proline Content of CIM-496-*G.hirsutum* (mg/g)		Proline Content of FDH-786-*G.arboreum* (mg/g)	
	Control	Stress	Control	Stress
Before stress	16.2	-	19.4	-
5DS	24.3	29.4	23.1	25.4
10DS	32.7	38.6	36.2	39.4
15DS	49.9	54.7	48.7	56.7
20DS	67.2	72.6	61.9	78.4

Table 9: Comparison of MDA Content of CIM- 496- *G.hirsutum* and FDH-786-*G.arboreum* under Control and Multiple Drought Stress

Multiple drought stress condition	MDA contents of CIM-496-*G.hirsutum* (nmol/g FW)		MDA contents of FDH-786-*G.arboreum* (nmol/g FW)	
	Control	Stress	Control	Stress
Before stress	2.4	-	3.9	-
5DS	4.4	6.7	6.3	7.4
10DS	6.7	8.1	7.4	9.3
15DS	8.3	10.2	9.4	11.6
20DS	9.2	11.3	10.1	13.7

Table 10: Comparison of Chlorophyll Content of CIM- 496- *G.hirsutum*and FDH-786-*G.arboreum* under Control and Multiple drought Stress

Multiple drought stress condition	Chlorophyll Content of CIM-496-*G.hirsutum* (mg/g)		Chlorophyll Content of FDH-786-*G.arboreum* (mg/g)	
	Control	Stress	Control	Stress
Before	10.1	-	11.3	-
5DS	12.4	8.4	14.4	9.7
10DS	13.6	6.6	15.6	7.3
15DS	15.3	4.7	17.3	5.4
20DS	17.9	4.0	17.2	5.1

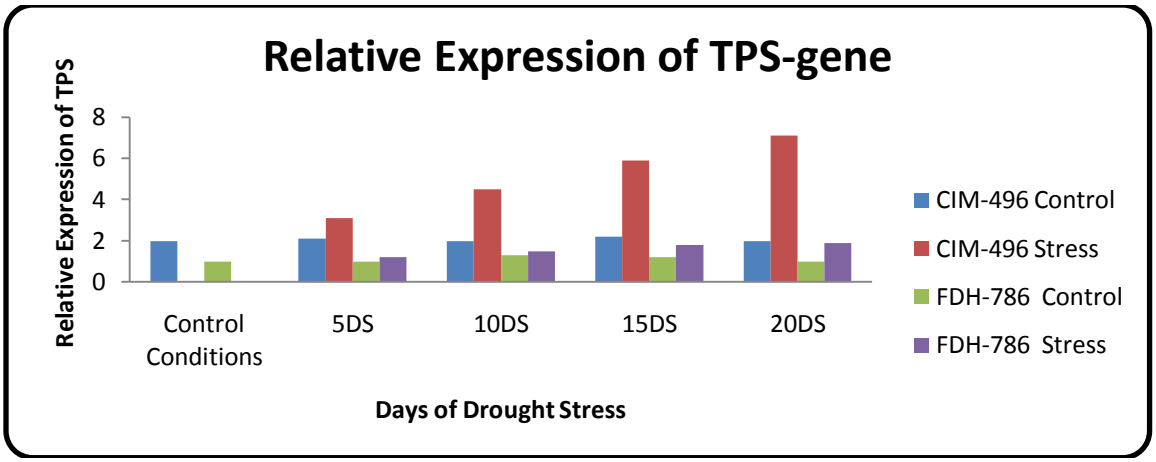

Figure 1: Relative Expression Level of *TPS*-Gene in CIM-496-*G hirsutum*and in FDH-786-*G arboretum* at Multiple Drought Stress

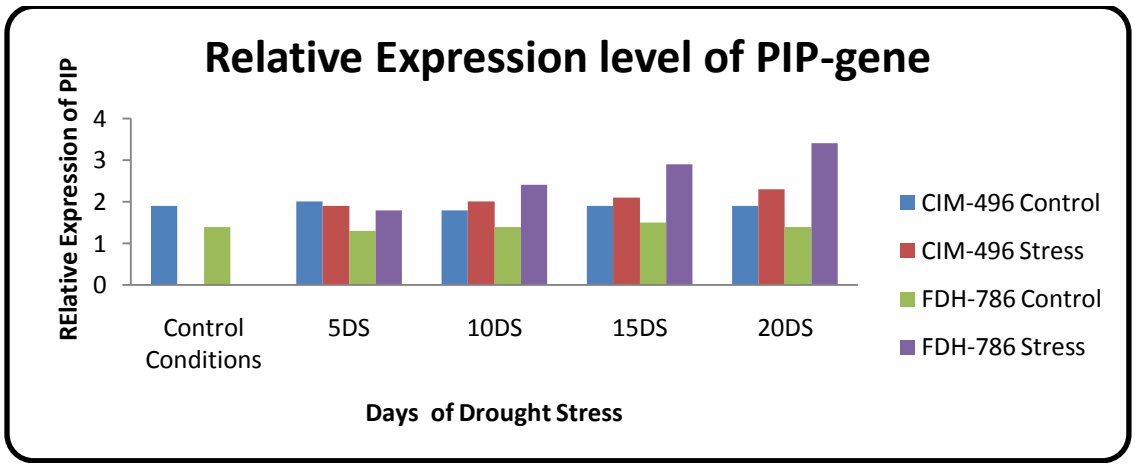

Figure 2: Relative Expression Level of *PIP*-Gene in CIM-496-*Gossypium hirsutum* and FDH-786-*Gossypium arboreum*

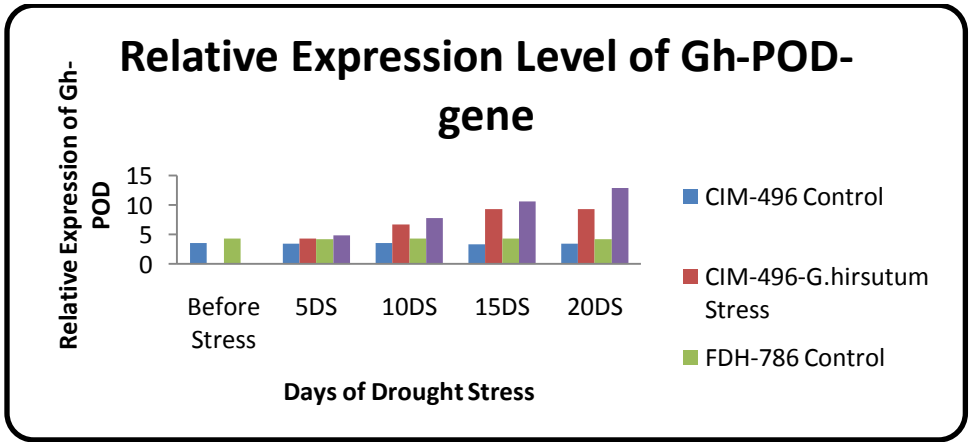

Figure 3: Relative Expression Level of *GH-POD*-Gene in CIM-496-*Gossypium hirsutum* and FDH-786-*Gossypium arboreum*

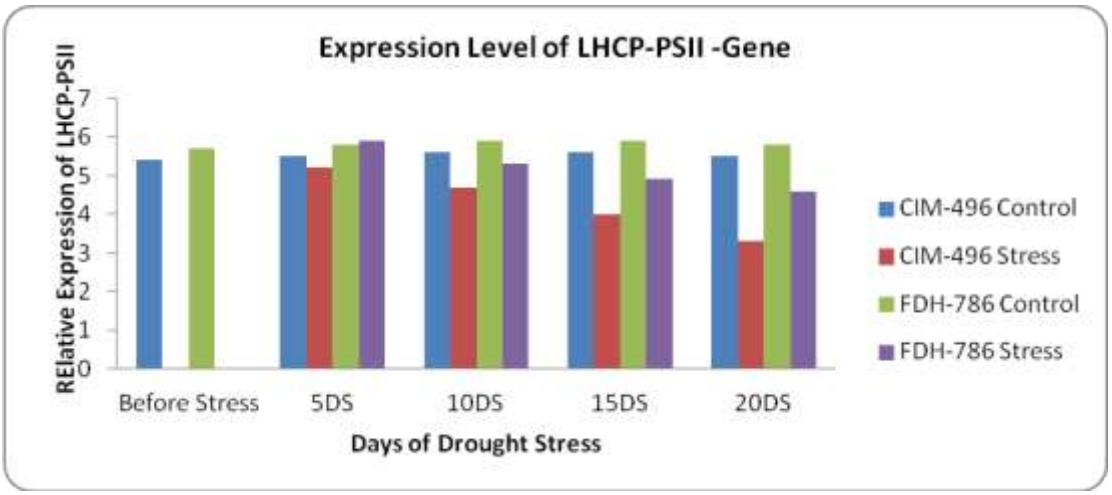

Figure 4: Relative Expression Level of *LHCP-PS*II in CIM-496-*Gossypium hirsutum* and FDH-786-*Gossypium arboreum*

DISCUSSION

Drought stress is adverse environmental condition that seriously affects crop productivity around the world. Drought tolerance mechanisms has remained a major challenge to plant biologists because several factors are involved in **[20]**. Economic survey of Pakistan (2012-13) also revealed that low yield of certain crops is because of one of an important factors such as scarcity of water, it demands new varieties with more tolerance to drought stress. Recently, research into the molecular mechanisms of stresses responses has started to bear fruits and genetic modification of stress tolerance has also shown promising results that may ultimately apply to ecologically important plants **[21]**. The current study has been conducted to evaluate and compare the drought tolerance between two varieties of cotton, CIM-496and FDH-786, under control and multiple stress conditions (5,10,15 and 20DS).

Drought stress affects the germination of seeds. Although most plants are tolerant during germination, however, drought stress delayed the process of germination **[22]**. The germination rate of FDH-786was 70.1%, while the germination rate of CIM-496 was 67.7%. The results indicated, that drought stress had a significant impact on the germination rate of both the varieties. It is supposed that seeds could not germinate at drought stress because embryo needs enough water to initiate germination. Other variables such as seed viability, dormancy, seed coat pretreatment and permeability to water may complicate data interpretations and comparisons with other crops or to other developmental stages. Percent increase in plant height could be considered an easy parameter to evaluate and compare different

crop varieties for drought tolerance **[23,24]**. We found a significant reduction in plant height of both the varieties under drought stress. However, when we compared the percent increase in plant height then it was revealed that plant height of FDH-786 lines is least affected by drought stress as compared to the CIM-496.In this study, continuous % reduction plant in plant biomass was observed as the stress level was increased in both the varieties but it was also obvious that drought has multiplied effect on stressed CIM-496 plants where the % reduction in biomass was rapid as compared to the stressed FDH-786 plants. It is supposed that reduction in dry and fresh biomass is because of decline in photosynthetic activity and other metabolic reactions because of drought stress. Under drought stress,reduction in biomass was observed in sugar beet **[25]**. Reduction in dry weight of sugar cane **[26]** was also found. Similarly, reduction in biomass was observed by Wu and Xia **[27]** and in *Petroselinum crispum* by Petropoulos *et al.,* **[9]**

A positive co-relation exist between leaf water content and relative water content (RWC), however, RWC progressively recovered within 48 hours as long as stress is disappeared **[28]**. Reductions in RWC result in loss of turgidity, which leads to Stomatal closure and reduced photosynthetic rate **[29]**. We also found the same behavior in both control and stressed lines of both the varieties but the stressed plants of CIM-496 showed more reduction in the Leaf water content as compared to the stressed plants of FDH-786. Several biochemical reactions are taking place on the interior surface of the cell membrane, so, its stability is imperative for all metabolic reactions. Both biotic and abiotic stresses affects the stability of cell membrane **[30].** Drought stress alters the structures of the membrane proteins **[31]** and in turn leakage of ions increases, which would be indirect measure of drought stress in diverse plant species **[32]**. A similar trend was found in both the varieties under drought stress, which caused severe ion leakage in stressed plants. However, the ion leakage was observed maximum in CIM-496 lines where the FDH-786 lines were much less affected.

Variability in net photosynthesis and Stomatal conductance have been suggested as tools for the comparison and evaluation of different plant varieties for drought tolerance **[33]**. Drought stress leads to a significant reduction in net photosynthesis because of Stomatal closure, which restricts the diffusion of CO_2 into the leaf **[34]**.Similarly, in our study, drought stress reduced the photosynthetic rate in both the genotype. The comparative study indicated that CIM-496 is drought prone.

Transpiration rate decreases with decline in moisture stress. The reason behind decline in moisture stress might be because of lowered water potentials in the root zone, it triggers a signal from root to shoot **[35]**. This study showed the similar result in correlation with the previous reports that the drought stress induces the reduction in photosynthesis associated with the other physiological markers i.e. Stomatal conductance and transpiration rate **[36]**. Therefore, it could be hypothesized that the maintenance of Stomatal conductance, relative water content, and high photosynthetic rate in FDH-786 is only because this variety of cotton is resistant to wilting

Proline is a major osmoregulant, it is produced in larger amount under stress as compared to the normal conditions **[37]**. Proline protects the plant tissues by producing stress responsive proteins **[38]**. The results of our study are in accordance with the findings of Krieg, Mohammadian and Moghaddam, **[26, 33]** that sugar and proline content increased under drought stress conditions in drought tolerant plants. The proline was found higher in FDH-786 plants as compared toCIM-496 plants. Super oxide dismutase, catalases, glutathione peroxidase and methadone reductase are antioxidant enzymes in plant tissues, inhibit the oxidation of other molecules by removing free radicals **[39]**. So, antioxidants are reducing agents. Dehydration stress which may result from drought lead to the disruption of cellular membranes, making them more permeable to ions by increased solubilization and peroxidation of membrane lipids under stress conditions **[22, 40]**. Drought stress caused severe ion leakage in CIM-496 plants, whereas the FDH lines were least affected by drought stress. To further understand this result, we estimated the lipid peroxidation by determining MDA levels in leaf tissues. The MDA levels in FDH-786plants were significantly higher than that of CIM-496 plants under drought stress. Thus, the minimum ion leakage took place in FDH-786plants may be due to more lipid peroxidation under drought stress. Simultaneously, the more lipid peroxidation under drought stress in the FDH-786plants reflects a maximum level of reactive oxygen species (ROS) as compare to CIM-496, where low level of MDA threats its susceptibility to ROS. By measuring the chlorophyll content of a plant tissue, a reliable estimate of photosynthetic rate in green tissues of a plant can be gagged **[41]**. Drought stress imposed the significant decline in chlorophyll a, b and total chlorophyll content. The results of this study are according to the findings of Ackerson *et al*, **[41]** as they described a

significant reduction in chlorophyll a and b content caused by drought stress in *Triticum aestivum* cultivars. A decrease in total chlorophyll under drought stress implies a lowered capacity for light harvesting. Since, the production of reactive oxygen species is mainly driven by excess energy absorption in the photosynthetic apparatus, this might be avoided by degrading the absorbing pigments.

Molecular analysis study was conducted to evaluate relative expression level of different drought responsive genes in both the varieties of cotton. Literature is available to correlate the changes in gene expression with physiological process. Trehalose has a protective role during abiotic stresses **[42]**. Expression level of *TPS* is increased which is one of the indicators of drought stress condition and its accumulation was high in drought sensitive plants as compared to the drought tolerate plants **[43-45]**. Our finding about the expression of *TPS* gene showed significant similarities with the above mention reports. *TPS*-Gene was increased in CIM-496-*Gossypium hirsutum* plants as the stress level increases while it was decreased in FDH-786-*Gossypium arboreum* variety with the increase in stress level. It is believed that the Plasma intrinsic protein (*PIP*) family plays an important role in the transportation of water. Several *PIP*s have been known to act as aquaporins, facilitating the permeation of water across membranes driven by differences in water potential **[46-48]**. We found high relative fold expression of the *PIP* gene in stressed plants of FDH-786 and CIM-496as compared to control plants. However, the expression level was found relatively higher in stressed plants of FDH-786as compared to CIM-496.

The effect of drought stress was found on the activities of antioxidant enzymes especially in *Gossypium hirsutum* Peroxidase (*Gh-POD*) where its expression participates in the scavenging of ROS **[49-54]**. This study revealed a gradual increase in *POD* activities in stressed plants of both the varieties and remained nearly constant in control plants of both the varieties. The comparison analysis indicates, FDH-786 is drought tolerant variety of cotton. *LHCP*s plays role in collecting and transferring light energy to photosynthetic reaction centers. Several studies have postulated that the *LHCP* genes were down-regulated in stress conditions. The results of this study show the significant similarity with pervious finding. *LHCP-PS*II was down regulated in stressed plants as compared to the control plants of both the varieties. However, comparative analysis shows that the expression of *LHCP-PS*II was decreased in CIM-496 plants as compared to the FDH-786.

It indicates that FDH-786-*Gossypium arboreum* is drought resistant variety.

CONCLUSION

Drought tolerance is a complex trait, selecting, evaluating and comparing a drought tolerant variety via selection process, conventional breeding or via transgenic technique, it becomes a challenging prospect. The results of this study support the higher responses of tolerance in FDH-786, under drought stress condition. The plants of FDH-786 showed improved physiological, morphological, biochemical and high expression of drought responsive genes as compared to CIM-496. This study has agricultural importance, since the incidence of stress is unpredictable and plants may be exposed to drought stress at any time during their life under field conditions.

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