

IDENTIFICATION OF POTENTIAL PLANT MATERIAL AND GENETIC ANALYSIS FOR DROUGHT TOLERANCE IN UPLAND COTTON BASED ON PHYSIOLOGICAL INDICATORS

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Abstract

Climate change has drastically reduced the water availability which negatively impacted the cotton production. Cotton crop is sensitive to drought as it may affect its growth and development. For this purpose, hydroponic culture was used to evaluate 37 cotton genotypes under both control and drought-imposed (PEG-6000) conditions to identify drought tolerant (MNH-886 and MNH-988) and sensitive genotypes (FH-114 and FH-Kehkshan) based on reliable physiological indicators *i.e.*, relative water content, excised leaf water loss and cell membrane stability using k-means cluster analysis and biplot analysis. These genotypes were hybridized to develop F₁ populations (MNH-886 × FH-114 and MNH-988 × FH-Kehkshan) and segregating populations (F₂, BC₁ and BC₂). Both cross combinations along with their generations were planted in field area using Randomized Complete Block Design under normal and drought conditions. Results reflected complex quantitative type of inheritance. Larger influence of non-additive effects along with epistatic interactions of aforementioned traits and seed cotton yield showed significant importance for suggesting delayed selection in segregating populations. Additionally, presence of low to moderate narrow sense heritability and undesirable association of physiological indicators with seed cotton yield suggested the same *i.e.*, selection in later segregating generations to improve drought tolerance in further breeding programs.

Key words: Polyethylene glycol, Genetic effects, Dominance effect, Generation mean analysis, Epistatic effects.

Introduction

Cotton (*Gossypium hirsutum* L.) is the most popular and commercially grown fiber crop worldwide. It is serving as a raw material in textile industry which has about \$600 billion of yearly impact on world's economy. Total production of cotton in the world is about 25 M tons annually (Khan *et al.*, 2020). In Pakistan, it is an important cash crop and a big source of foreign exchange earnings. Though, there was 22.8% decline in cotton production over the last year 2019-20 due to the abiotic stresses (Climate change, heat stress and variable extreme rainfall) and biotic stresses (Anon., 2020-21). Among abiotic stresses, scarcity of water is the severe one. It is a major factor responsible for yield losses in agriculture as compared to other abiotic factors and causes high rate of reduction in productivity and growth (Lambers *et al.*, 2008; Arain *et al.*, 2022). Drought affects the plant life cycle through physiological, morphological and biochemical disturbance of characters (Farooq *et al.*, 2009a). The major effect is the reduction in leaf size, cell expansion, water use efficiency, plant relations with nutrients, water for crop productivity, stomatal size, stomatal frequency, growth of stem and root (Farooq *et al.*, 2009b).

The risk of drought has been increased because of the climatic changes. Moreover, water deficiency making this condition more adverse (Paloti *et al.*, 2017). In Pakistan, climate change has a negative impact on cotton production due to reduced water availability (for irrigation) and high temperatures. Indus River provides irrigation to cultivated area of cotton crop. It would carry less water as the snowfields and glaciers are diminishing in magnitude at Himalayas and Tibet. Though, water from

Indus-river has vital importance to cotton production and agriculture in Pakistan (Batool & Saeed, 2018). The water sector of Pakistan always remained defenseless to the impact of climate change facing challenges to accommodate increasing water requirements. Moreover, water quality degradation and losses are reflecting to the limited water conservation practices. Increasing climatic variability and water scarcity would dry out water resources of Pakistan (Habib, 2021). In year 2015, United States Department of Agriculture reported that there would be an expected decline in cotton production due to drought stress in Pakistan (Rehman, 2015). Consequently, cotton production was declined in 2015-16 by 34% in Pakistan due to drought and heat stress (Rana, 2016). The production remained stagnant afterwards.

Cotton crop is sensitive to drought stress as its growth and productivity may be affected at every developmental stage (Loka, 2012). Yield of cotton has showed 50-60% losses due to water scarcity (Mahmood & Hussain, 2020) and its growth is critical under drought stress as it is restricted by affecting the morpho-physiological attributes *viz.*, plant height, node number, leaf area index, root and canopy development, stem dry weight, rate of transpiration, rate of photosynthesis, water potential of leaves and stomatal conductance (Loka *et al.*, 2011; Tang *et al.*, 2022). Agronomic traits such as size of bolls, number of bolls per plant and seed cotton yield are also severely affected resulting in reduction of cotton productivity (Malik *et al.*, 2006). Thus, the increase in water scarcity and its effects on cotton crop impules a need for developing plants which can adapt to survive in water deficit condition at morpho-physiological level (Farooq *et al.*, 2009a).

Tolerance to drought stress is a polygenic and complex trait that involves genotype into environment interactions and also the epistatic effects. It is necessary to gather information about the genetic architecture and gene action of polygenic traits related to drought tolerance and yield for improvement of cotton production under water deficit condition (Madhukar *et al.*, 2018). The physiological traits like cell membrane stability (Azhar *et al.*, 2009), relative water content (Rahman *et al.*, 2000) and excised leaf water loss (Basal & Unay, 2006) are important parameters for screening purpose against drought stress. Regarding germplasm evaluation, it is difficult and complex to analyze polygenic traits and select drought tolerant plants under field experiment because of the interference of environment (Schuster, 2011). The prerequisites for evaluating drought tolerant genotypes are accomplished by giving same amount of stress to all the genotypes which cannot be maintained even in pot experiments (Nepomuceno *et al.*, 1998). Therefore, hydroponic culture is considered to be useful for the purpose of screening under drought stress (Kato *et al.*, 2008). Aforementioned traits were considered as reliable indicators for drought stress tolerance under hydroponic culture (Raziuddin *et al.*, 2010; Iqbal *et al.*, 2020). Thus, cotton germplasm may be evaluated against drought stress under hydroponic condition using PEG induction for differentiating cotton lines into groups of

sensitive and tolerant ones (Echer *et al.*, 2010). From breeding point of view, a breeder must develop a plant with desirable traits and study the gene action and linkage relationship among traits related to quality, yield and adaptation of the plants towards its environment (Malik *et al.*, 2006). In this case, generation mean analysis is considered to be useful method for estimation of genes average effect like epistasis, additive and dominance controlling the expression of quantitative traits (Zdravkovic *et al.*, 2011). Moreover, genetic studies of complex traits aid a breeder to design a proper breeding approach for the development of new varieties (Sharmila *et al.*, 2007; Shakoor *et al.*, 2010).

Keeping in view the above-mentioned facts, our objectives were (i) to evaluate available cotton germplasm on the basis of reliable physiological indicators for drought tolerance, (ii) to investigate genetic architecture and linkage association of these indicators along with seed cotton yield under water deficit condition.

Materials and Methods

Plant material: 37 cotton genotypes were collected from the department of Plant Breeding and Genetics, University of Agriculture Faisalabad (PBG-UAF) as well as from their respective institutes and breeding stations situated in different areas of Pakistan (Table 1).

Table 1. List of cotton genotypes under study and their respective institutes.

Sr. No.	Genotype	Research Institute	Sr. No.	Genotype	Research Institute
1.	FH-114	CRI, Faisalabad	20.	FH-634	CRI, Faisalabad
2.	FH-412	CRI, Faisalabad	21.	FH-Kehkshan	CRI, Faisalabad
3.	IUB-222	IUB, Bahawalpur	22.	FH-901	CRI, Faisalabad
4.	FH-942	CRI, Faisalabad	23.	NIAB-545	NIAB, Faisalabad
5.	IR-3701	NIBGE, Faisalabad	24.	FH-1000	CRI, Faisalabad
6.	MNH-886	CCRI, Multan	25.	FH-Lalazar	CRI, Faisalabad
7.	Sitara-008	Agri Farm Service	26.	FH-312	CRI, Faisalabad
8.	CIM-599	CCRI, Multan	27.	MNS-992	CCRI, Multan
9.	CIM-602	CCRI, Multan	28.	FH-118	CRI, Faisalabad
10.	CIM-598	CCRI, Multan	29.	MNH-786	CCRI, Multan
11.	VH-148	CRI, Vehari	30.	FH-326	CRI, Faisalabad
12.	CEMB-33	CEMB, Lahore	31.	MNH-1016	CCRI, Multan
13.	CIM-612	CCRI, Multan	32.	MNH-992	CCRI, Multan
14.	MNH-988	CCRI, Multan	33.	NIAB-1048	NIAB, Faisalabad
15.	IUB-75	IUB, Bahawalpur	34.	NIA-86	NIA, Tandojam
16.	AA-703	Ali Akbar Seeds	35.	NS-181	Neelum Seeds
17.	CRIS-9	CCRI, Sakrand	36.	CYTO-179	CCRI, Multan
18.	PB-896	UAF, Faisalabad	37.	BS-15	Bandasha Seed
19.	VH-363	CRI, Vehari			

CRI; Cotton Research Institute, IUB; Islamia University of Bahawalpur, NIBGE; National Institute of Biotechnology and Genetic Engineering, CCRI; Central Cotton Research Institute, CEMB; Center for Excellence in Molecular Biology, UAF; University of Agriculture Faisalabad, NIAB; Nuclear Institute for Agriculture and Biology and NIA; Nuclear Institute of Agriculture

Evaluation of cotton germplasm: Collected genotypes were evaluated under hydroponic culture in May 2018 at wire-house (PBG-UAF). For this purpose, plant material was sown in Styrofoam cups (10×6.5 cm) filled with mixture of soil and sand as a culture medium. Seedlings were grown to first true leaf stage and transferred to hydroponic medium in split plot arrangement under Completely Randomized Design with two replications in each treatment. Two treatments *i.e.*, control and drought-

impose (PEG-6000) were organized in main plot and genotypes were placed in subplots. Four plastic tubs (30 liters capacity) were arranged for control condition while two plastic tubs (100 liters capacity) were used for drought-impose condition. These tubs were filled with Hoagland solution (Hoagland & Arnon, 1950) as nutrient medium. Seedlings shifted to plastic tubs were suspended in nutrient medium using Styrofoam sheet. Air-pipes connected to air-pump were set for maintaining constant

aeration in medium at root zone. Nutrient medium was replaced with fresh one on weekly basis. Seedlings were allowed to grow for two weeks for adaptation to the medium. Afterwards, PEG-6000 of 17% concentration (Zhang *et al.*, 2007; Carlos *et al.*, 2011) was dissolved in 100 liters tubs to impose drought stress. Experiment was conducted for 45 days from date of emergence to data recording. Data were recorded for Relative water content (RWC), Excised leaf water loss (ELWL) and Cell membrane stability (CMS).

Selection of parents, development of segregating populations and field trial: From screening experiment, two highly drought tolerant genotypes (MNH-886 and MNH-988) and two highly sensitive ones (FH-114 and FH-Kehkshan) were selected as parents. These parents were crossed to produce F₁ populations (MNH-886 × FH-114 and MNH-988 × FH-Kehkshan). For this purpose, selected parents were sown in loamy soil filled earthen pots in green house facility to produce F₁ hybrids. The temperature of green house at day time was maintained as 28-30°C and 20-25°C at night time. Relative humidity was 50-60% with 16hrs of day length approximately. Subsequently, F₁ populations along with their parents were grown in the field area to develop backcrosses and F₂ population. Pictorial representation of crossing scheme is given in (Fig. 1).

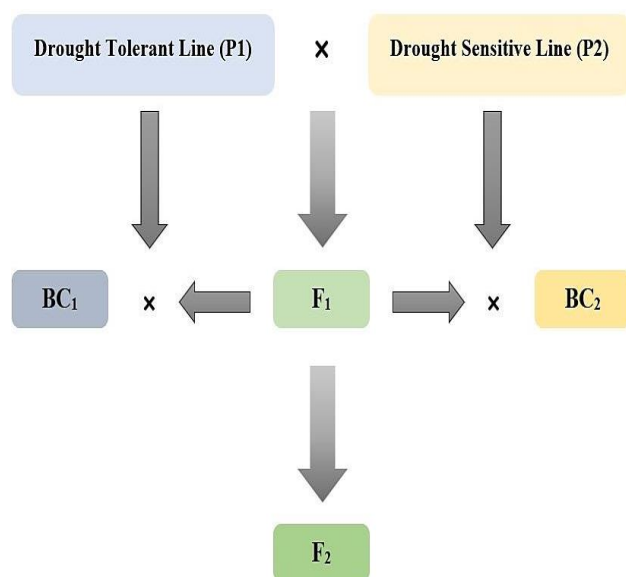


Fig. 1. Pictorial representation of crossing scheme of selected upland cotton genotypes.

During cotton growing season in May 2020, developed populations (F₁, F₂ and backcrosses) along with parents were raised in the field area (PBG-UAF) in Randomized Complete Block Design using three replications under drought and normal conditions. Cross 1 (MNH-886 × FH-114) and Cross 2 (MNH-988 × FH-Kehkshan) along with populations were planted and analyzed separately. Plant to plant and row to row distance was maintained at 30 cm and 75 cm, respectively. Normal cultural and agronomic practices were also

conducted for both experiments. Trial maintaining normal condition was irrigated with 22 acre-inches whereas drought condition trial received 12 acre-inches of water. 13.59 inches precipitation was received during field trial. Recorded data for average rainfall during experiment was collected on daily basis from meteorological unit, department of Crop physiology, UAF, Pakistan.

Data recording of traits

Cell membrane stability (CMS): Collected leaf samples were used to take leaf discs having diameter of 10mm. Six leaf discs were taken from each genotype in each replication and placed it into a falcon tube. Deionized water was used in falcon tubes. Each tube was filled with 20ml of deionized water and kept in room temperature for two hours. Afterwards, initial conductance (C₁) reading was noted using EC (Electrical conductivity) meter after shaking each tube. Falcon tubes containing leaf discs were autoclaved for 15mins at 121°C. and kept overnight at room temperature to obtain the reading of second conductance (C₂) (Petcu and Terbea, 1995).

$$\text{CMS} = 1 - \% \text{ Injury} = 1 - (C_1/C_2) \times 100$$

% Injury = $(C_1/C_2) \times 100$ is the formula of membrane injury index

Relative water content (RWC): Fully expanded leaf samples were put into polythene bags right after excised from plants and stored in ice filled container. Samples were taken to the laboratory and fresh weight was recorded using electronic balance. Afterwards, turgid weight was recorded by dipping leaf samples in water for overnight. Leaf samples were then put in oven to measure dry weight. Samples were dried for 72hrs at 70°C and dry weight was recorded (Barrs & Weatherly, 1962).

$$\text{RWC} = \frac{(\text{Fresh weight} - \text{Dry weight})}{(\text{Turgid weight} - \text{Dry weight})} \times 100$$

Excised leaf water loss (ELWL): Fully expanded leaf samples were put into polythene bags right after excised from plants and stored in ice filled container. Samples were taken to the laboratory and fresh weight was recorded as early as possible using electronic balance. Afterwards, wilted weight was recorded by keeping samples at room temperature for six hours. Leaf samples were then placed in oven for 72hrs at 70°C and dry weight was measured after oven dried (Clarke & McCaig, 1982).

$$\text{ELWL} = (\text{Fresh weight} - \text{Wilted weight}) / \text{Dry weight}$$

Seed cotton yield (SCY): Total bolls picked up in two pickings (1st picking after 120 days and 2nd picking after 160 days) from a plant constitutes to seed cotton yield of a plant. It was measured by using electronic balance in grams. Weighed seed cotton yield of selected plants of a population per replication was averaged to obtain yield of a population in a replication.

Statistical Analysis

In hydroponic experiment, analysis of variance was conducted to check variability among collected genotypes, treatments (Control and drought) and genotype \times treatment in experiment (Steel *et al.*, 1997). The response of cotton genotypes under control and drought stress was identified by k-means cluster analysis (Forgy, 1965) and principal component biplot analysis (Jolliffe, 2002) performed using computer-based statistical software named *XLSTAT 2021*.

In field trial, all six generations of both cross combinations in field trial under normal and drought conditions were analyzed for genetic variability following similar method mentioned above (Steel *et al.*, 1997). Generation mean analysis was used to determine genetic effects of traits under study using weighted least square analysis (Mather & Jinks, 1982) (Supplementary Table 2). Components of variance from weighted least squares analysis (best fit model) were utilized for estimation of narrow sense heritability (h_{ns}^2). Correlation coefficients were calculated from individual plants data of F_2 populations (Dewey & Lu, 1959).

Results

Identification of drought tolerant and sensitive genotypes

Genetic variability among available cotton germplasm: Analysis of variance revealed that there was significant genetic variability among thirty-seven genotypes, treatments and genotype \times treatment interaction indicating possible potential for drought tolerance among genotypes for reliable indicators viz., relative water content (RWC), excised leaf water loss (ELWL) and cell membrane stability (CMS) (Table 2).

This would help to initiate further biometrical analysis for screening of germplasm *i.e.*, k-means cluster analysis and principal component biplot analysis.

K-means cluster analysis: K-means cluster analysis classified 37 cotton genotypes into six clusters on the basis of physiological indicators under both treatments *i.e.*, control and drought-imposed (Table 3). In control experiment, the six cotton genotypes viz., IUB-222, MNH-886, MNH-988, IUB-75, VH-363 and MNH-786 in cluster 2 performed well and achieving highest mean values for RWC (74.91%) and CMS (77.12%) but lowest mean values for ELWL (0.956g/g). On the contrary, genotypes in cluster 1 (FH-114, FH-412, IR-3701, CIM-598, VH-148, FH-901 and NIA-86) and cluster 6 (FH-634, FH-Kehkshan, NIAB-545 and CYTO-179) performed poorly with lowest mean values for RWC (66.36% in cluster 1 and 60% in cluster 6) and CMS (59.56% in cluster 1 and 64.94% in cluster 6) while higher values for ELWL (1.291g/g in cluster 1 and 1.497g/g in cluster 6). Under drought-imposed condition, Cluster 4 showed highest mean values for RWC (75.60%) and CMS (75.13%) while lowest mean value for ELWL (1.001g/g). The four cotton genotypes namely MNH-886, MNH-988, IUB-75 and FH-118 falling in cluster 4 were directed towards drought tolerance. On the other hand, genotypes in cluster 1 (FH-114, CIM-599, CIM-598, FH-634, FH-Kehkshan, FH-901 and NS-181) with lowest mean values for RWC (52.58%) and CMS (54.42) but highest mean value for ELWL (1.845g/g) performed poorly and identified as drought sensitive. Genotypes like MNH-886, MNH-988 and IUB-75 performed well under both conditions would be identified as drought tolerant ones. Five genotypes viz., FH-114, CIM-598, FH-901, FH-634 and FH-Kehkshan were considered as drought sensitive ones as these genotypes were identified as least performer under both conditions.

Supplementary Table 1. Meteorological data during the cotton growing season 2020.

Parameters	Months, 2020							
	April	May	June	July	August	September	October	November
Rainfall (mm)	28.30	29.80	49.20	87.80	183.30	9.00	0.00	1.40
Mean Max. Temp. (°C)	34.50	40.00	40.30	39.64	38.00	37.38	36.32	27.85
Mean Min. Temp. (°C)	18.20	23.30	26.50	28.79	28.61	26.31	17.27	10.78
Mean Relative Humidity (%)	43.00	50.80	58.50	64.50	73.03	69.16	59.09	63.26

Source: Meteorological unit, department of Crop physiology, University of Agriculture Faisalabad, Pakistan.

Supplementary Table 2. Parameters for the weighted least squares analysis of generation means (Mather and Jinks, 1982).

Generations	Components of genetic effects					
	[m]	[d]	[h]	[i]	[j]	[l]
P ₁	1	1.0	0.0	1.00	0.00	0.00
P ₂	1	-1.0	0.0	1.00	0.00	0.00
F ₁	1	0.0	1.0	0.00	0.00	1.00
F ₂	1	0.0	0.5	0.00	0.00	0.25
BC ₁	1	0.5	0.5	0.25	0.25	0.25
BC ₂	1	-0.5	0.5	0.25	-0.25	0.25

[m], coefficients of the mean; [d], additive; [h], dominance; [i], additive \times additive; [j], additive \times dominance and [l], dominance \times dominance

Table 2. Mean squares for relative water content (RWC), excised leaf water loss (ELWL) and cell membrane stability (CMS) of 37 genotypes studied under control and drought conditions.

Source	Df	RWC	ELWL	CMS
Treatment	1	1555.04*	5.4475*	1808.17*
Error 1	1	1.34	0.0132	0.57
Genotypes	36	207.52**	0.5038**	162.09**
Treatment × Genotypes	36	24.04**	0.0492**	17.88**
Error 2	72	0.09	0.0008	0.06
Total	147			

*, $p < (0.05)$; **, $p < (0.01)$

Table 3. K-means cluster analysis of 37 cotton genotypes under control and drought conditions on the base of physiological indicators.

Traits	Control					
	Cluster 1	Cluster 2	Cluster 3	Cluster 4	Cluster 5	Cluster 6
RWC	66.36	74.91	75.61	72.92	64.07	60.00
ELWL	1.291	0.956	1.055	1.127	1.330	1.497
CMS	59.56	77.12	61.82	69.14	75.01	64.94
Genotypes	FH-114	IUB-222	FH-942	Sitara-008	PB-896	FH-634
	FH-412	MNH-886	CIM-599	CIM-602	FH-312	FH-Kehkshan
	IR-3701	MNH-988	CRIS-9	CEMB-33	MNH-1016	NIAB-545
	CIM-598	IUB-75	NIAB-1048	CIM-612	MNH-992	CYTO-179
	VH-148	VH-363		AA-703	NS-181	
	FH-901	MNH-786		FH-1000	BS-15	
	NIA-86			FH-LALAZAR		
				MNS-992		
				FH-118		
				FH326		
Traits	Drought					
	Cluster 1	Cluster 2	Cluster 3	Cluster 4	Cluster 5	Cluster 6
RWC	52.58	60.35	71.17	75.60	54.99	63.66
ELWL	1.845	1.650	1.326	1.001	1.948	1.487
CMS	54.42	54.47	63.04	75.13	62.98	66.47
Genotypes	FH-114	FH-412	IUB-222	MNH-886	CIM-602	MNS-992
	CIM-599	IR-3701	FH-942	MNH-988	PB-896	MNH-1016
	CIM-598	VH-148	SITARA-008	IUB-75	NIAB-545	MNH-992
	FH-634	FH-1000	CEMB-33	FH-118	FH-312	
	FH-Kehkshan	NIA-86	CIM-612		FH326	
	FH-901		AA-703		CYTO-179	
	NS-181		CRIS-9		BS-15	
			VH-363			
			FH-LALAZAR			
			MNH-786			
		NIAB-1048				

RWC; Relative water content, ELWL; Excised leaf water loss and CMS; Cell membrane stability

Principal component biplot analysis: Biplot has displayed the association among physiological indicators with performance of genotypes under control and drought-imposed conditions (Figs. 2 and 3). In control condition, first and second component of graph explained 85.44% variability of the data. Negative association between RWC and ELWL was observed. CMS showed poor association with RWC and ELWL. Interaction of genotypes with traits under study was quite diversified. Cotton genotypes viz., MNH-988, MNH-886, IUB-222, IUB-75 and FH-118 has strong positive interaction with RWC. CMS was interacted positively by MNH-786, VH-363 and PB-896. Cotton

genotypes falling near to ELWL were NIAB-545, FH-Kehkshan, Cyto-179, CIM-602, NS-181, BS-15, FH-634 and NIA-86. Under drought condition, biplot analysis exhibited 92.49% variability of data considering first and second components. There was considerable positive association between CMS and RWC. ELWL exhibited negative association with RWC and CMS. Genotypes like MNH-988, MNH-886, IUB-75, FH-118 and IUB-222 showed strong positive association with RWC and CMS. In ELWL, cotton genotypes viz., FH-Kehkshan, FH-114, FH-901, NS-181, NIAB-545, BS-15 and CIM-602 showed strong positive interaction while these genotypes were negatively associated to RWC and

CMS. Cotton genotypes existed between vectors of RWC and CMS with greater distance from central point would be selected as drought tolerant (MNH-886 and MNH-988). However, genotypes fell opposite to the vectors of RWC and CMS and, near to vector of ELWL with greater distance would be identified as drought sensitive (FH-114 and FH-Kehkshan). Principal component biplot analysis has validated and confirmed the results observed in k-means cluster analysis and identified drought tolerant and sensitive genotypes. In conclusion to the results, two highly drought tolerant genotypes (MNH-886 and MNH-988) and two highly drought sensitive ones (FH-114 and FH-Kehkshan) were selected and hybridized to develop segregating populations for field trial and genetic analysis.

Field trial and genetic analysis

Genetic variability among generations of cross combinations: Variability analysis showed significant differences among generations in both cross combinations (MNH-886 × FH-114 and MNH-988 × FH-Kehkshan) under both water treatments *i.e.*, normal and drought. However, ELWL showed no considerable differences among generations in Cross II under normal condition (Table 4). The variability among generations of both cross combinations would help to initiate genetic analysis of cross combinations using generation mean analysis and correlation coefficient analysis on the base of physiological indicators and seed cotton yield under normal and drought conditions.

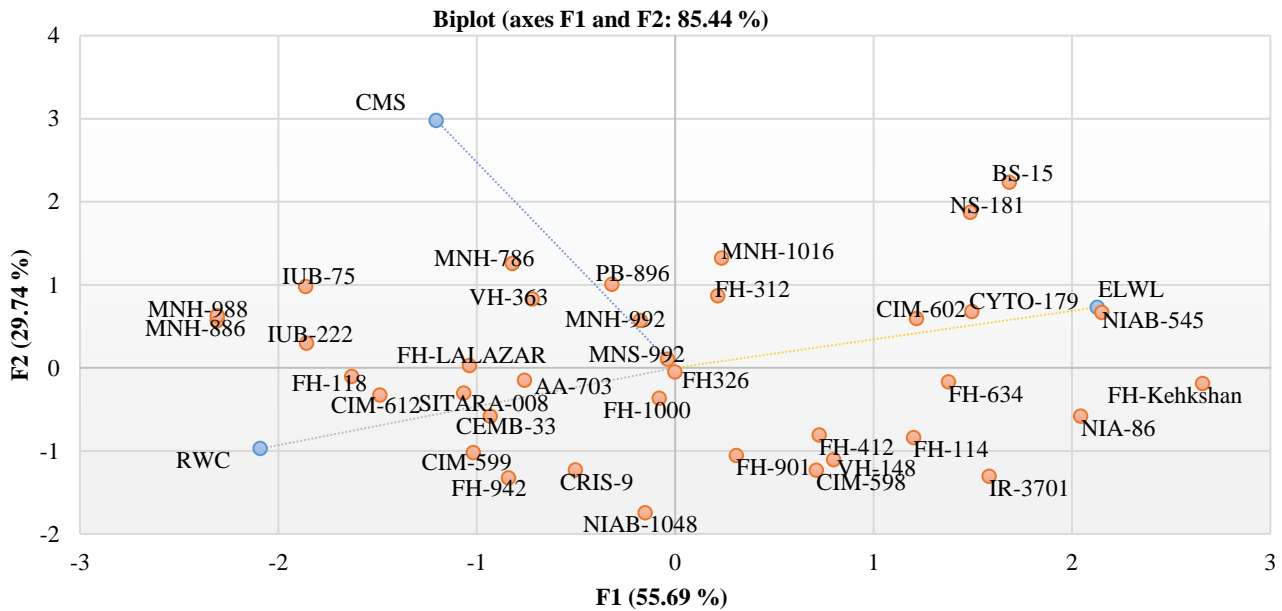


Fig. 2. Biplot analysis of 37 cotton genotypes on the base of relative water content (RWC), excised leaf water loss (ELWL) and cell membrane stability (CMS) under control condition.

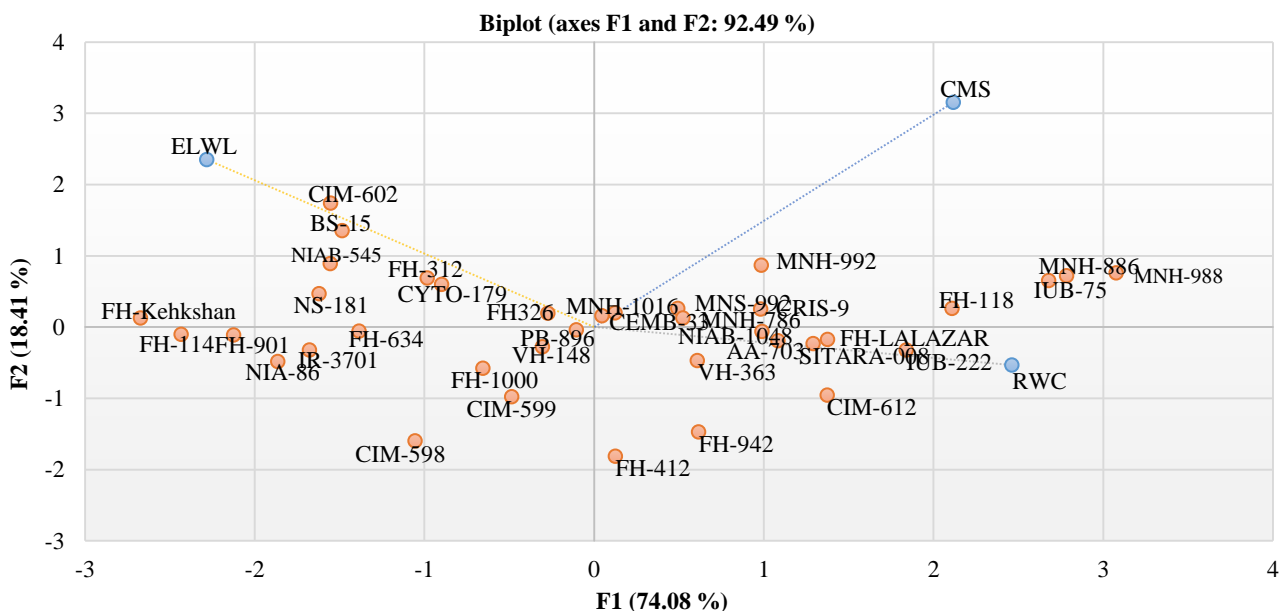


Fig. 3. Biplot analysis of 37 cotton genotypes on the base of relative water content (RWC), excised leaf water loss (ELWL) and cell membrane stability (CMS) under drought condition.

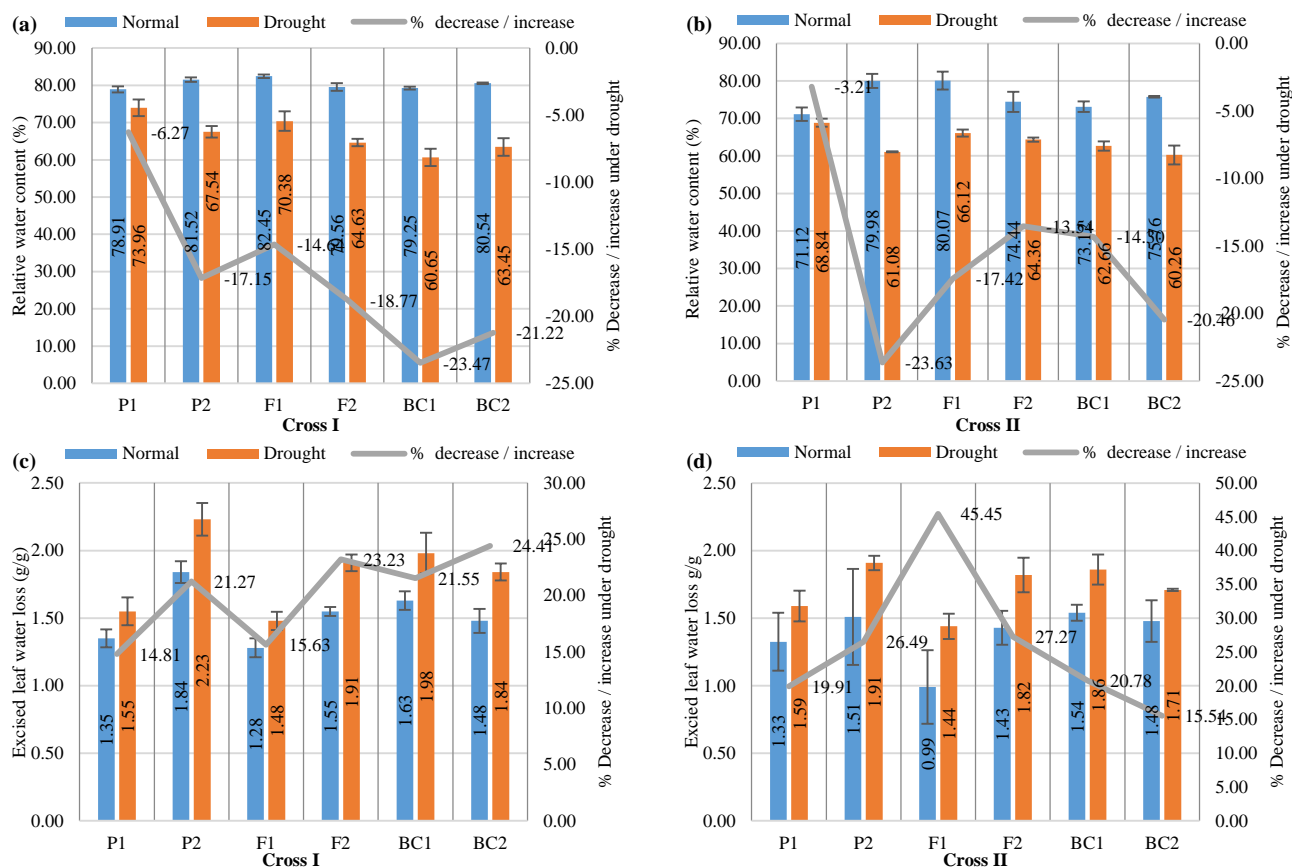


Fig. 4. Relative water content (a and b) and excised leaf water loss (c and d) of six generations of Cross I (MNH-886 × FH-114) and Cross II (MNH-988 × FH-Kehkshan) under normal and drought environments.

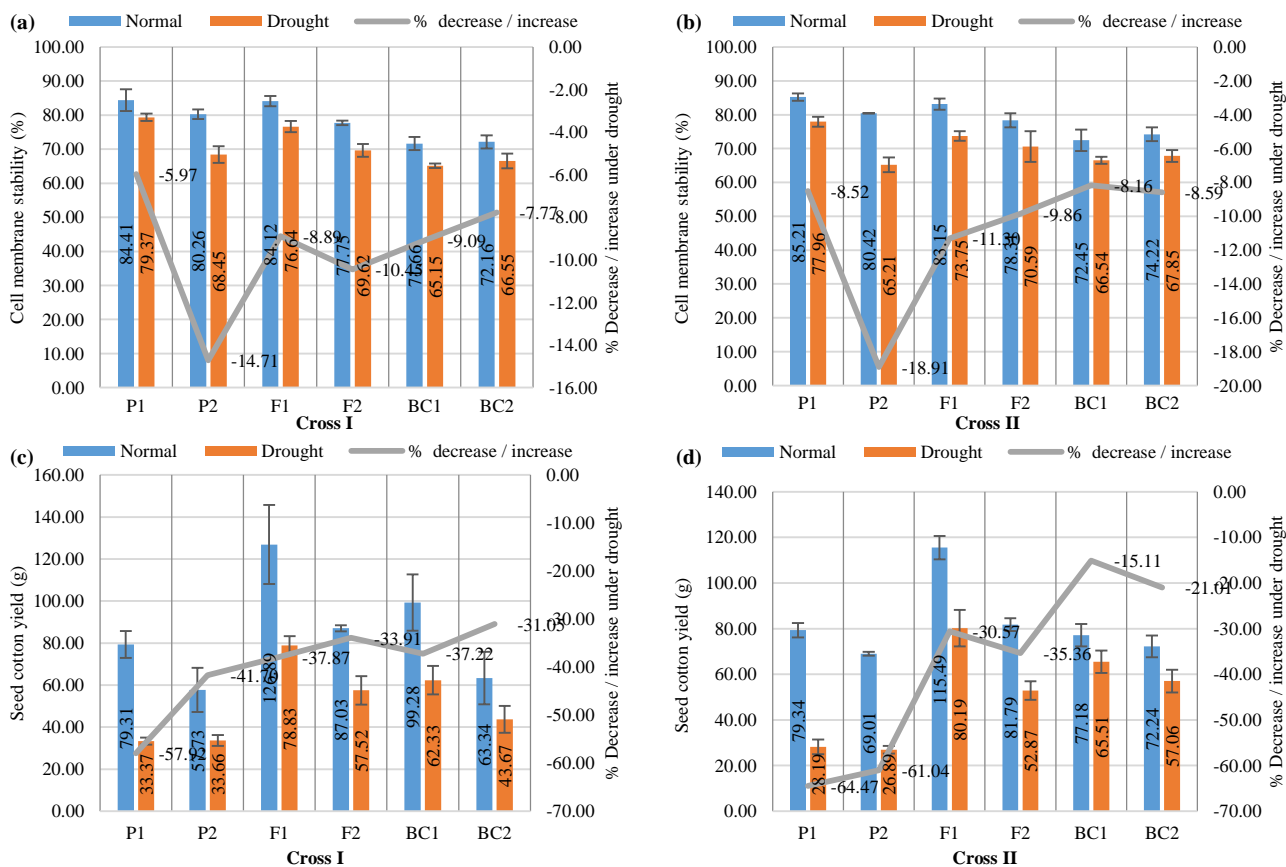


Fig. 5. Cell membrane stability (a and b) and Seed cotton yield (c and d) of six generations of Cross I (MNH-886 × FH-114) and Cross II (MNH-988 × FH-Kehkshan) under normal and drought environments.

Table 4. Mean squares of six generations of Cross I (MNH-886 × FH-114) and Cross II (MNH-988 × FH-Kehkshan) for relative water content (RWC), excised leaf water loss (ELWL), cell membrane stability (CMS) and seed cotton yield (SCY) under normal and drought conditions.

Traits	Normal			Drought		
	Replication	Generations	Error	Replication	Generations	Error
	Cross I					
RWC	0.535	5.821*	1.462	12.52	71.05*	13.30
ELWL	0.018	0.148**	0.013	0.107	0.205**	0.014
CMS	16.81	94.22**	10.07	5.73	98.57**	10.02
SCY	185.12	1923.68*	469.70	18.20	965.26**	10.39
Cross II						
RWC	13.41	39.96*	10.21	1.209	31.35*	5.877
ELWL	0.022	0.130 ^{NS}	0.169	0.018	0.098*	0.028
CMS	22.19	74.40**	9.05	65.56	69.98**	6.988
SCY	20.35	848.54**	50.91	10.78	1320.5**	83.01

*, $p < (0.05)$; **, $p < (0.01)$

Table 5. Estimates of the best fit model for generation means parameters by weighted least squares analysis in respect of various traits of Cross I (MNH-886 × FH-114) and Cross II (MNH-988 × FH-Kehkshan) under normal and drought conditions in the field.

Traits	Cross #	Genetic effects						χ^2 (df)	h^2_{ns}
		[m] ± S.E.	[d] ± S.E.	[h] ± S.E.	[i] ± S.E.	[j] ± S.E.	[l] ± S.E.		
RWC	N1	76.65±1.63	-	5.32±2.52	4.01±1.84	3.23±2.13	-	2.45(2)	0.28
	D1	62.35±0.56	2.74±0.79	-	6.81±0.96	10.43±2.89	6.75±1.68	1.92(1)	0.31
	N2	69.94±1.68	2.45±0.71	8.62±3.00	4.94±1.80	-	-	2.47(2)	0.27
	D2	64.10±0.28	3.72±0.51	-	0.41±0.59	6.39±2.65	-	2.01(2)	0.30
ELWL	N1	1.62±0.027	0.28±0.03	-	0.09±0.04	0.27±0.12	0.56±0.08	0.98(1)	0.25
	D1	2.05±0.019	0.30±0.04	-	-	0.32±0.14	0.32±0.05	3.39(2)	0.24
	D2	1.94±0.12	-	1.30±0.33	0.32±0.12	0.12±0.09	1.45±0.23	2.22(1)	0.27
CMS	N1	76.21±1.25	-	0.58±2.17	1.83±1.37	3.60±1.85	-	1.25(2)	0.30
	D1	69.02±0.25	6.16±0.73	-	2.33±0.77	20.82±2.21	-	3.83(2)	0.31
	N2	74.41±1.32	2.60±0.75	5.33±2.16	6.52±1.50	6.32±2.96	-	2.33(1)	0.30
	D2	69.95±0.48	6.59±1.06	-	0.19±1.19	17.60±3.64	-	4.39(2)	0.31
SCY	N1	66.44±2.31	11.72±2.41	41.11±4.6	-	49.89±11.2	-	2.48(2)	0.28
	D1	33.44±0.80	0.149±0.85	45.33±1.7	-	37.70±5.42	-	3.65(2)	0.20
	N2	70.16±0.94	4.60±0.91	-	2.45±1.36	-	43.04±2.9	4.55(2)	0.29
	D2	44.08±1.33	1.16±1.00	-	14.47±1.6	15.96±4.84	43.18±4.3	2.98(1)	0.19

[m]; mean, [d]; additive effects, [h]; dominance effects, [i]; additive × additive effects, [j]; additive × dominance effects, [l]; dominance × dominance effects, S.E; standard error, χ^2 ; chi square, d.f; degree of freedom, h^2_{ns} ; narrow sense heritability, N1; cross I under normal condition, D1; cross I under drought condition, N2; cross II under normal condition, D2; cross II under drought condition, RWC; relative water content (%), ELWL; excised leaf water loss (g/g), CMS; cell membrane stability (%) and SCY; seed cotton yield (g)

Table 6. Correlation coefficient analysis of physiological traits and seed cotton yield of Cross I (MNH-886 × FH-114) and Cross II (MNH-988 × FH-Kehkshan) under normal and drought conditions in the field.

Traits	Cross #	RWC	ELWL	CMS
ELWL	N1	-0.120		
	D1	-0.483*		
	N2	-		
	D2	-0.533*		
CMS	N1	0.175	-0.416	
	D1	0.776**	-0.624**	
	N2	0.259	-	
	D2	0.730**	-0.468*	
SCY	N1	0.163	-0.478*	0.053
	D1	-0.066	-0.258	-0.038
	N2	0.313	-	0.386
	D2	-0.038	-0.315	-0.020

* = Significant ($p < 0.05$), ** = Highly significant ($p < 0.01$)

N1; cross I under normal condition, D1; cross I under drought condition, N2; cross II under normal condition, D2; cross II under drought condition, RWC; relative water content (%), ELWL; excised leaf water loss (g/g), CMS; cell membrane stability (%) and SCY; seed cotton yield (g)

Mean comparison among generations of cross combinations: Fig. 4a and 4b presented RWC of six generations of Cross I and II under both conditions along with % decrease in mean value due to drought stress. Among six generations of Cross I, maximum % decline in mean value was observed in BC₁ (-23.47%) due to drought stress followed by BC₂ (-21.22%) whereas minimum percentage was noted in P₁ generation (-6.27%). F₁ (82.45%) and P₁ (73.96%) populations showed highest mean performance while lowest RWC under water stress environment was observed in BC₁ generation (60.65%). In Cross II, P₂ (-23.63%) and BC₂ (-20.46%) populations showed maximum % decline due to water deficiency. Minimum % decline was noted in P₁ population (-3.21%) with highest RWC (68.84%) under drought condition followed by F₁ generation (66.12%). BC₂ (60.26%) and P₂ (61.08%) generations exhibited lowest RWC under drought environment. For ELWL, maximum increase in percentage due to water stress was noted in BC₂ generation (24.41%) followed by F₂ (23.23%), BC₁ (21.55%) and P₂ (21.27%) in Cross I. Highest ELWL was exhibited by P₂ generation (2.23g/g) under drought condition. Lowest mean value is desirable to indicate drought tolerance in plants which was observed in F₁ generation (1.48g/g) under water deficient environment (Fig. 4c). ELWL in Cross II increased in all generations due to drought stress and maximum increase in percentage was noted in F₁ generation (45.45%) achieving lowest ELWL under normal (0.99g/g) and drought (1.44g/g) conditions. Minimum % increase was observed in BC₂ (15.54%) and P₁ (19.91%) populations. Highest mean value was exhibited by P₂ generation (1.91g/g) under drought condition (Fig. 4d). In Cross I, maximum % decline in CMS was noted in P₂ generation (-14.71%) while minimum % decline was exhibited in P₁ generation (-5.97%) which also showed highest mean value (79.37%) under drought condition followed by F₁ generation (76.64%). Backcrosses exhibited lowest CMS among six generations under both water regimes but there was minimum % decline due to moisture stress (Fig. 5a). CMS in Cross II declined in all generations due to drought stress and maximum decline was noted in P₂ generation (-18.91%). P₁ generation and backcrosses (BC₁ and BC₂) showed minimum percent decline ranging from -8.59% to -8.16%. Also, highest mean value for CMS was exhibited by P₁ generation (77.96%) under drought condition followed by F₁ generation (73.75%). Backcrosses exhibited lower mean values among six generations under both treatments (Fig. 5b). For seed cotton yield (SCY), all the six generations were badly effected due to drought stress in Cross I. Maximum decline in percentage was noted in P₁ (-57.92%) generation followed by P₂ (-41.70%), F₁ (-37.87%), BC₁ (-37.22%), F₂ (-33.91%) and BC₂ (-31.05%) generations. F₁ generation (78.83g) showed highest mean value for SCY under drought condition. P₁ generation showed lowest mean value of 33.37g under drought condition (Fig. 5c). SCY in Cross II declined considerably under drought condition and maximum decline in percentage was noted in P₁ (-64.47%) and P₂ (-61.04%) generation. Minimum % decrease in yield was observed in backcrosses (-15.11% in BC₁ and -21.01% in BC₂). F₁ (-30.57%) and F₂ (-35.36%) populations showed moderate decline in yield. Moreover, F₁ generation showed highest

SCY under drought condition (80.19g). P₂ generation (26.89g) showed minimum yield under water deficit environment. P₁ generation (28.19g) performed almost similar to the P₂ generation under drought condition but it performed well under normal condition (79.34g) as compared to P₂ generation (Fig. 5d).

Generation mean analysis and heritability estimation: Genetic effects of both cross combinations for physiological parameters and seed cotton yield (SCY) under both water treatments *i.e.*, normal and drought were estimated (Table 5). The significance of mean effect [m] reflected towards the quantitative type of genetic nature of the traits. Additive main effect [d] was observed in all the traits except for RWC and CMS in N1 and ELWL in D2. Considerable magnitude of dominance main effect [h] in traits viz., RWC (N1 and N2), ELWL (D2), CMS (N1 and N2), and SCY (N1 and D1) explained the existence of non-fixable genes. Among epistatic effects, additive × additive [i] effect was shown by most of the traits except for ELWL in D1 and SCY in Cross 1. [d] and [i] effects expressed the occurrence of fixable genes in traits under study. However, the presence of additive × dominance [j] effect in most of the traits and dominance × dominance [l] effect in RWC (N1), ELWL and SCY (Cross II) with larger magnitude explicated that the traits were under influence of non-additive type of genetic variability. Estimation of narrow sense heritability ranged from 0.19 to 0.31 among traits. All the traits showed moderate heritability among generations of both cross combinations under both water regimes except seed cotton yield in cross combination II under drought condition which has exhibited low narrow sense heritability (0.19).

Correlation studies of traits: Correlation coefficient analysis for traits under study was also observed (Table 6). RWC showed highly significant positive association with CMS in both crosses under drought condition. ELWL was significantly in negative correlation with RWC and CMS in both crosses under drought environment. SCY only showed significant negative correlation with ELWL under normal condition but non-significant negative association under drought condition. SCY showed no considerable association with RWC and CMS.

Discussion

It is a challenge to detect stress tolerant lines in a crop. Though, it is possible to develop stress tolerance in plants by selecting and assembling traits contributing towards stress tolerance (Soomro *et al.*, 2011). RWC (Shi *et al.*, 2013; Abdel-Kader *et al.*, 2015), ELWL (Rahman *et al.*, 2000; Paloti *et al.*, 2017) and CMS (Brito *et al.*, 2011) were selected as most important indicators for selecting drought tolerance in plants. Reduction in RWC under drought condition was observed due to the affected plant vigor (Liu *et al.*, 2002), increased penetrability and decreased sustainability of cell membrane (Blokina *et al.*, 2003). Moreover, cytoplasmic contents sedimentation and membrane cleavage added to the cause of reduction in RWC (Blackman *et al.*, 1995). Genotypes performed with high RWC and CMS would be considered for high

resistance to water deficiency. However, genotypes with low RWC and CMS might reflect to drought sensitive (Hassanzadeh *et al.*, 2009). Under water deficiency, maintaining structural integrity and stability of plant's cellular membrane has vital importance to survive (Martinez *et al.*, 2004) and considered to be tolerating component against drought stress (Bajji *et al.*, 2002). For screening drought tolerant genotypes, the degree of CMS reflected as good indicator for selection of genotypes (Kocheva *et al.*, 2004). Decrease in cellular integrity and stability or increase in percent cell injury due to drought stress would reflect to decrease in CMS that ultimately increases electrolyte leakage from cells. Therefore, plants maintaining high CMS under water deficient environment would be considered as drought tolerant plants and vice versa (Chowdhury *et al.*, 2017). ELWL is the measure of loss of water from epidermal layer of leaf as the stomatal closure delays two minutes after excision and considered as indirect cuticular thickness measurement (Ahmad *et al.*, 2009). Moreover, the difference of thickness would reflect to variability in ELWL (Malik *et al.*, 2006). Therefore, genotypes exhibited low ELWL would be considered as drought tolerant genotypes.

K-means cluster analysis is a biometric approach to form groups of similar characteristics from the data set (Simic *et al.*, 2016). The technique made clusters on the basis of partitioning pattern and divides the data set variables into non-overlapping clusters (Morissette & Chartier, 2013). Principal component analysis (PCA) also validated results obtained from k-means cluster analysis. This technique was helpful in distribution of total variability of data into components on the basis of mean values of traits. The level of variation among physiological traits was identified using first and second component as most important for estimating variability (Javed *et al.*, 2017). K-means cluster analysis and principal component analysis were also studied by various researchers for assessment or screening of cotton germplasm for various biotic or abiotic stresses (Javed *et al.*, 2017; Majeed *et al.*, 2019; Chaudhary *et al.*, 2020; Jie *et al.*, 2020).

In a breeding program, variability among populations and within segregating populations for characteristics under study is related to the different allelic combinations (Ngangkham *et al.*, 2018). The knowledge about genetic effects of traits and availability of genetic variability is considered to be essential for a successful breeding approach, otherwise results may not be improved significantly (Munir *et al.*, 2007). The mean performance among generations in both cross combinations indicated highest performance of F₁ population for seed cotton yield while segregating populations had mostly shown lowest percent decline due to drought stress. Hence, it reflected that the highest performance of F₁ hybrids in two cross combinations for yield related traits could be used for hybrid production (Ketageri *et al.*, 1992; Iqbal & Nadeem, 2003) but the minimum % reduction in segregating populations reflected the presence of complex genetic makeup of the plants which had introduced drought tolerance mechanism from superior parents to the segregating generations. Highest relative water content with lowest excised leaf water loss was experienced in F₁

hybrids and P₁ superior parents. It reflected the presence of dominant genes governing the physiological parameters. Highest cell membrane stability was also noted in P₁ generation with lowest % decline in BC₁ and P₁ generations. Hussain & Qayyum (2017) observed similar mean performance of generations of physiological traits in cotton under heat stress. Majeed (2021) also reported potential of backcrosses and F₂ populations for selection on the basis of pollen viability in upland cotton under heat stress. Therefore, the variable and unpredicted pattern of mean performance of aforementioned traits appeared to be inherited in complex manner under drought stress condition. The understanding of genetic effect is important for their improvement through proper breeding approach. The estimation of genetic effects is appropriate for traits related to drought tolerance and yield (Khalaf *et al.*, 2017). Generation mean analysis consists of six generations considered to be a useful method for estimation of genes average effect like epistasis, additive and dominance controlling the expression of quantitative traits (Mather & Jinks, 1982). Various workers (Singh & Sandhu, 1985; Kalsy and Garg, 1988) in cotton and (Malik *et al.*, 1999; Munir *et al.*, 2007) in other crops had previously studied the genetic effects of different traits using generation mean analysis. The present study revealed variable genetic effects controlling genetic variability of the traits among populations of two cross combinations under drought stress. Higher magnitude of dominance effect and epistatic effects like additive × dominance and dominance × dominance reflected towards the non-additive genetic variability. Therefore, selection for the traits under study in early segregating populations would be restricted. The selection based on performance of progeny only feats for additive type of variability. Non-additive and non-allelic variability would be exploited through desirable segregates intermating. Subsequently, selection of desirable intermating populations or early segregates biparental mating or multiple crossing would lead to improvement in yield related traits (Joshi, 1979; Singh *et al.*, 2008). This type of complex inheritance of yield related traits was also reported by Iqbal & Nadeem (2003), Hussain *et al.*, (2009), Sarwar *et al.*, (2012) and Yadav *et al.*, (2020). Epistatic effects like additive × dominance and dominance × dominance type of gene actions also prevailed in RWC and ELWL. Additive × dominance type of non-allelic interaction in CMS also contributed to overall non-additive genetic variability suggesting delayed selection in later segregating generations (Hussain *et al.*, 2009; Hussain & Qayyum, 2017). Negative association of ELWL with SCY indicated that the selection of plants with lower ELWL would give high yield which is desirable for developing drought tolerant varieties. The non-significant association of RWC and CMS with SCY was not desirable. Although, desirable association of RWC and CMS with yield related traits (Saleem *et al.*, 2015) have explained the indirect relation to SCY. Hence, selection would be delayed for later segregating generations as to break undesired

linkages among traits or to develop desirable association for better selection considering physiological indicators. Heritability was divided in three groups, *i.e.*, high heritability > 0.50, medium heritability = 0.20 to 0.50 and low heritability < 0.20 (Rehman *et al.*, 2020). Low heritability reflects toward the presence of non-additive effects in large magnitude whereas high heritability appears because of the larger additive effect controlling genetic variability. Aforementioned facts, led this research to selection in later segregating generations for attaining drought tolerant plants and to use the selected material for further breeding programs for drought tolerance. Delayed selection of traits under study has also reported by various researchers' viz., Lin & Zhao (1988), Murtaza *et al.*, (2004) and Rahman *et al.*, (2005).

Conclusion

Relative water content, excised leaf water loss and cell membrane stability were exposed as reliable indicators for drought tolerance under hydroponic culture. In response, two highly drought tolerant cotton genotypes (MNH-886 and MNH-988) and two highly drought sensitive ones (FH-114 and FH-Kehkshan) were identified using k-means cluster analysis and principal component biplot analysis. In field trial, the inheritance of physiological traits and seed cotton yield revealed complex genetic architecture of the material under study and suggested for delayed selection in segregating populations to improve drought tolerance in current plant material. The genetic architecture of cotton attributes needs an inclusive understanding for improvement of drought tolerance in cotton plants as provided in current article based on concepts of classical breeding. Hence, these results and findings would be helpful for future prospects and breeding programs to develop drought tolerant varieties.

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(Received for publication 22 November 2021)