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Intra-Plant Variability for Heat Tolerance Related Attributes in Upland Cotton

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Abstract: Abiotic stress, particularly heat stress, affects various parts of the cotton plant and ultimately impacts the seed cotton yield. Different portions of a single cotton plant of a cultivar exhibit variable responses to stress during reproductive and vegetative phases. To test this hypothesis, physiological and morphological traits related to heat stress were observed for two flowering positions in 13 genotypes of upland cotton. These genotypes were sown in field conditions in triplicate following a randomized complete block design. Data were collected for pollen germination, pollen viability, cell membrane thermostability, chlorophyll content, boll weight, and boll retention for both the top and bottom branches of each genotype. The collected data were analyzed for the identification of variability within and between genotypes for these two flowering positions. Tukey's test was applied to estimate the significance of differences between genotypes and positions within each genotype. Results showed that the two positions within the same plant statistically varied from each other. The bottom branches of the genotypes performed significantly better for all traits measured except boll weight. The genotype AA-933 performed best for pollen germination and boll retention, while CYTO-608 exhibited maximum pollen viability in both the bottom and top flower positions compared with other genotypes. Overall, MNH-1016 and CIM-602 showed better cell membrane thermostability and chlorophyll content, respectively. This intra-plant variability can be further exploited in breeding programs to enhance the stress tolerance capabilities of the resulting varieties.

Keywords: genetic variability; *Gossypium hirsutum*; intra-plant variation; heat tolerance

1. Introduction

Cotton is a Kharif season crop grown mainly for feed, fiber, and oil in the Punjab and Sindh regions of Pakistan. These are considered hot regions since the temperature reaches 47 °C during the growing season. Environmental stresses such as heat, and drought affect cotton plants by impeding normal physiological processes which lead to morphological abnormalities and yield reduction [1]. Plants mostly invest their defense in the most valuable sections, such as reproductive parts under various stress conditions. Cotton production is vulnerable to abiotic stresses, particularly during the growth stages of

blooming and boll formation, which have become more frequent as our climate changes [2]. Any stress during this stage abruptly reduces the yield. Numerous efforts have been made to understand the physiological, molecular, and genetic pathways of the cotton plant related to sustaining yield under stress conditions [3,4].

The reproductive efficiency of the cotton crop is negatively impacted by temperatures above 32 °C in a variety of ways, including reduced metabolism as well as suppression of photosynthesis, pollination, fertilization, and crop growth rate [5]. Heat and drought stress causes male gametes to undergo metabolic and structural changes that result in meiotic abnormalities or premature spore abortion [6]. It also results in poor pollen germination and short pollen tube growth in cotton [7,8]. It was reported that pollen germination is better in flowers that have been pollinated under the canopy of the plant as compared to flowers that are directly exposed to sunlight and pollinated during high-temperature stress [9].

Yield reduction is also associated with certain changes in metabolic and biochemical pathways in plant cells, i.e., excessive accumulation of reactive oxygen species (ROS) such as H_2O_2, singlet oxygen, hydroxyl ions, etc., during stress conditions [10]. As a result of a dramatic accumulation of ROS during stress, programmed cell death has been observed in developing pollen grains [11]. Hence, ROS scavenging through the action of antioxidants in anthers has a role in maintaining pollen viability under abiotic stress [12]. Under high temperature or water deficit conditions, the role of the cell membrane in maintaining cell osmotic balance may be impeded due to leakage of electrolytes [13]. In cotton, temperatures over 35 °C increased membrane leakage and reduced leaf size [14]. High canopy temperature adversely affects the chlorophyll content in leaf tissues and lowers the rate of photosynthesis and carbohydrate production [15]. Reduction in carbohydrate content is also associated with decreased lint yield [16]. Cotton plants will shed bolls when they are stressed, thus boll retention drops significantly under harsh environmental conditions [17,18].

It was noticed that different portions of a single cotton plant of a cultivar exhibit variable responses to stress during reproductive and vegetative phases. Although every cell in a plant has the same genetic material, the different behavior might be due to epigenetic [19,20] or other effects. Every cell expresses itself according to the stimulus received from the environment. Young leaves are more resistant to insect damage compared to old ones [21]. So, every part of the plant faces a different environment. As a result, these positions phenotypically behave differently. Cultivars also differ in canopy shape and intra-plant morphological features. Moreover, cultivars are grown in the same region exhibit variation among them. Environmental and genotypic effects both contribute to the phenotype. Therefore, the objective of this study was to identify inter- and intra-cultivar variability for physiological as well as morphological attributes associated with the yield of seed cotton under heat stress conditions.

2. Materials and Methods

2.1. Genotypes and Experimental Design

This experiment was performed in the field area of the Department of Plant Breeding and Genetics, the University of Agriculture, Faisalabad located at 31.4504° N, 73.1350° E, Pakistan. Thirteen genotypes of cotton were collected from the germplasm units of the Central Cotton Research Institute (CCRI), Multan; Cotton Research Institute (CRI), Multan; Cotton Research Station (CRS), Faisalabad; and other institutes in Pakistan listed in Table 1. These genotypes have different genetic backgrounds, have genetic variability, and grow well in the ecological niche present in the field area for this experiment. Cotton genotypes were sown on 16 May 2019 in three replications under a randomized complete block design (RCBD). Plots were single rows, 10 feet (3.1 m) long with a plant-to-plant distance of 12 inches (30 cm). Distance between rows was 30 inches (76 cm). All agronomic practices, including thinning, irrigation, weeding, and plant protection measures were performed at the appropriate crop growth stage according to cotton production technology

approved for the Punjab province by the Directorate of Agriculture to maintain a healthy plant population.

Table 1. List of 13 cotton genotypes of *G. hirsutum* L. evaluated for heat tolerance.

Sr #	Genotype Name	Origin	Prominent Characteristics
1	CRS-2	Advance strain	Spreading growth habit, creamy yellow pollens, heat tolerant.
2	VH-377	CRS Vehari	Medium leaf pubescence, creamy pollen color, Good fiber quality.
3	FH-215	CRS Faisalabad	Resistant to CLCuV and pink bollworm, moderate pubescence on leaves, semi-erect branches.
4	CIM-343	CCRI Multan	Heat and drought tolerant, high yielding Bt-variety [22]
5	CIM-602	CCRI Multan	Early maturity, high lint percentage, and heat tolerant Bt-variety [23]
6	MNH-1016	CRI Multan	Semi erect branches, stem pigmentation, creamy pollen color, round shape boll, tolerant to CLCuV, high yielding Bt variety
7	MNH-1026	CRI Multan	Medium compact growth habit, semi-erect branches, creamy pollen, oblong boll shape, CLCuV tolerant, white fiber color, high yielding Bt variety
8	NIBGE-2	NIBGE Faisalabad	Resistant to Multan and Burewala strain of CLCuV, Drought resistant, Spreading growth habit. [24]
9	N-777	NIAB Faisalabad	High-density planting cotton, tolerant to heat and CLCuV-B strain. [25]
10	N-1048	NIAB Faisalabad	Tolerant to CLCuV, Spreading growth habit.
11	CYTO-124	CCRI Multan	Highly CLCuV tolerant, Non-Bt interspecific variety [23]
12	CYTO-608	CCRI Multan	Non-Bt interspecific variety [23]
13	AA-933	Ali Akbar group, Multan	Heat tolerant, good fiber quality, resistant to CLCuV, yellow pollen color. Spreading growth habit.

CRS = Cotton Research Station, CCRI = Central Cotton Research Institute, CRI = Cotton Research Institute, NIBGE = National Institute for Biotechnology and Genetic Engineering, NIAB = Nuclear Institute for Agriculture and Biology.

2.2. Data Collection

Heat tolerance measurements were taken during the growing season when 50% of the crop was flowering. Each plant was divided into two equal parts by measuring plant height in such a way that the bottom portion was under the shade of the plant canopy while the upper portion was exposed to direct sunlight. Flowers in the top part of the plant were exposed to direct sunlight while flowers on the bottom part of the plant were under the leaf canopy and received indirect sunlight. Heat tolerance-related parameters including pollen germination (PG), pollen viability (PV), and cell membrane thermostability (CMT) were assessed under in vitro conditions while boll retention and boll weight were measured in vivo.

Flowers that showed dehiscence of anthers were collected from the field and immediately transported to the laboratory where pollen grains were deposited on pollen germination media. The media was prepared following the method explained by Burke et al. [9] with little modifications. The solid germination medium consisted of 2% (*w*/*v*) agarose (Product no. A4718, Sigma Aldrich, Merck, Darmstadt Germany), 25% (*w*/*v*) sucrose (Product no. S0389, Sigma Aldrich, Merck, Germany), 0.52 mM KNO_3 (Product no. P8291, Sigma Aldrich, Merck, Darmstadt Germany), 3.06 mM $MnSO_4$ (Product no. M7899, Sigma Aldrich, Merck, Darmstadt Germany), 1.66 mM H_3BO_3 (Product no. B6768, Sigma Aldrich, Merck, Darmstadt Germany), 0.42 mM $MgSO_4 \cdot 7H_2O$ (Product no. M2643, Sigma Aldrich, Merck, Darmstadt Germany) and 1.0 µM A_3 gibberellic acid (Product no. G7645, Sigma Aldrich, Merck, Darmstadt Germany). The pH of the germination medium was brought to 7.6 before adding sucrose and agarose. The medium was autoclaved and poured into Petri plates (100 × 15 mm, Product no. P5856, Sigma Aldrich, Merck, Darmstadt Germany) under a laminar flow hood to avoid contamination. Plates were wrapped with cling

film tape then placed in a refrigerator until used. Pollen grains with pollen tube lengths greater than the diameter of the pollen grains themselves were considered to be germinated (Figure 1). Percent pollen germination was estimated using the following equation:

$$\text{Pollen germination } (\%) = \frac{\text{Number of germinated pollen grains}}{\text{Total number of pollen grains}} \times 100$$

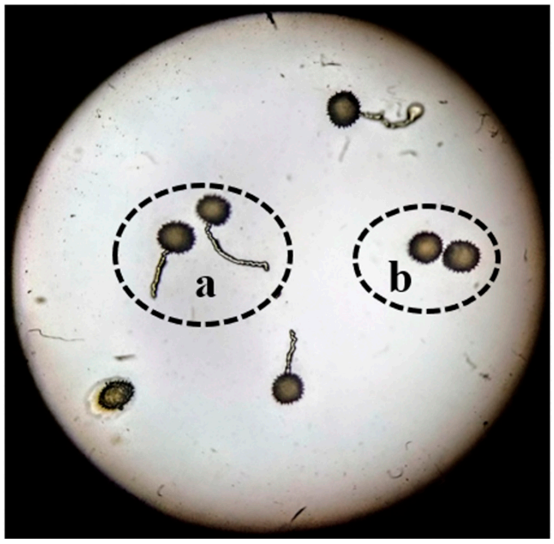

Figure 1. An example of pollen tube growth. A pollen grain that has germinated its pollen tube is labeled as 'a' while non-germinating pollen grains are labeled as 'b'.

The triphenyl-tetrazolium chloride (TTC) test was used to test the viability of pollen grains [26]. Flowers that showed dehiscence of anthers were taken into the laboratory to test pollen viability. Fresh pollen grains were sprinkled on a glass slide (76 × 26 mm) by gently tapping the flower. Two to three drops of 0.5% 2,3,5-triphenyl tetrazolium chloride (Product no. 17779, Millipore, Merck, Germany) were added in a 15% sucrose solution (Product no. S0389, Sigma Aldrich, Merck, Germany). The slide was covered with a coverslip (20 × 20 mm) to prevent desiccation and then placed under sunlight for 60 min at 30–37 °C. After this exposure, slides were observed under a light microscope (Model XSZ 107BN, Manufacturer: Zenith Lab Inc., Zhejiang China). The pollen grains that changed to red color after exposure to the TTC solution were considered viable while non-viable pollen remained yellowish in color (Figure 2). Pollen viability percentage was estimated using the following equation:

$$\text{Pollen viability } (\%) = \frac{\text{Number of viable pollen grains}}{\text{Total number of pollen grains}} \times 100$$

Two leaves from the top of the plant and two leaves from the bottom of the plant were selected for measuring CMT following the protocol of Sullivan [27] and using the following equation:

$$\text{Cell membrane thermostability } (\%) = \left[\frac{1 - T_1/T_2}{1 - C_1/C_2}\right] \times 100$$

where, the subscripts 1 and 2 refer to the 1st and 2nd electrical conductivity (EC) readings, respectively, and T and C refer to the EC of heat-treated (T) and control (C) sets of test tubes. The EC value was measured by a portable EC meter (FieldScout EC 110 Meter).

Boll weight, boll retention percentage, chlorophyll content, and canopy temperature were measured at harvest. For boll weight, all bolls from plants within the plot were harvested and weighed using an analytical balance (least count = 0.01 g). Total boll weight

was divided by the total number of selected bolls to get the average weight of an individual boll. Boll retention percentage was estimated as the number of fruiting positions on the plant that had bolls divided by the total number of fruiting positions. To measure boll retention, all fruiting squares were labeled 60 days after sowing (DAS). One hundred days after sowing, the number of labeled bolls was counted. Boll retention was calculated as follows:

$$\text{Boll retention} = \frac{\text{Number of labeled bolls 100 DAS}}{\text{Number of labeled fruiting squares 60 DAS}} \times 100$$

The leaf chlorophyll content was measured using a "SPAD 502 Plus" (Konica Minolta, Japan) chlorophyll meter which works on the principle of red and blue light absorption (therefore, the SPAD measurement has no units). Top and bottom canopy temperatures of upland cotton genotypes was measured using an infrared crop temperature meter (Model: 2956, Spectrum technologies, Inc., Plainfield, NJ, USA) at crop maturity (Table 2).

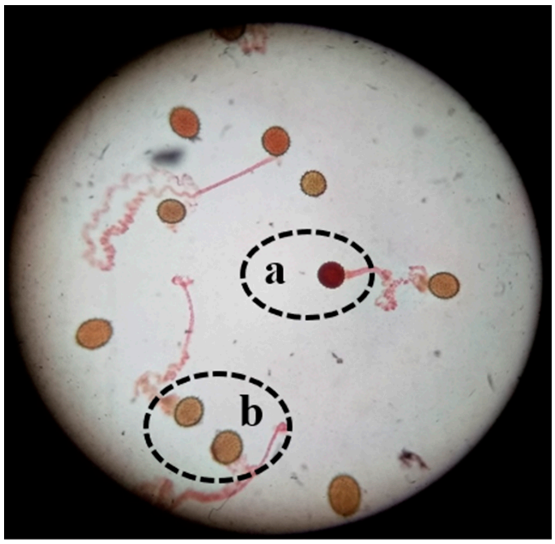

Figure 2. An example of results from the triphenyl-tetrazolium chloride (TTC) test. Viable pollen has changed to a red color (labeled as 'a') while non-viable pollen does not change color (labeled as 'b').

Table 2. Top and bottom canopy temperature in 13 cotton genotypes grown under field conditions in 2019 in Faisalabad, Pakistan.

Genotypes	Top Temp. (°C)	Bottom Temp. (°C)
CRS-2	35	33
VH-377	37	36
FH-215	35	33
CIM-343	37	35
CIM-602	35	34
MNH-1016	34	32
MNH-1026	35	33
NIBGE-2	36	34
N-777	37	35
N-1048	35	33
CYTO-124	35	33
CYTO-608	36	34
AA-933	36	35

2.3. Data Analysis

Analysis of variance was conducted with replication, genotype, and position as main effects. The interaction effect of genotype and position was also analyzed to identify sources

of variation [28]. Statistix 8.1 (An Software, 2003) was used to calculate ANOVA and Tukey's test [29]. Tukey's test was applied to test the significant difference of variation between the cotton genotypes and variation between the two positions for selected traits [30]. Cluster analysis was carried out using the statistical software package of Minitab ver.17.

3. Results

Genotypes were significantly different for all recorded parameters, and plant position was also significantly different for all parameters except boll weight (Table 3). Of the sources of variation, plant position had the largest effect on PV, PG, chlorophyll content, and boll retention. The effect of genotype was largest for the cell membrane thermostability and boll weight parameters. The mean values for each measure of heat tolerance in the top and bottom plant positions of the genotypes are provided in Table 4.

Table 3. Mean squares for six measures of heat tolerance in cotton grown under field conditions.

Source of Variation	DF	PV	PG	Chl. Cont.	CMT	Boll Wt.	Boll Ret.
Replication	2	166.88 **	51.50 ^{NS}	46.67 ^{NS}	10.14 ^{NS}	0.13 ^{NS}	2.79 ^{NS}
Genotype	12	199.58 **	173.82 **	1218.46 **	3142.60 **	0.80 **	18.10 **
Position	1	1456.01 **	873.35 **	1813.86 **	2807.12 **	0.28 ^{NS}	304.88 **
Genotype × Position	12	19.37 ^{NS}	6.18 ^{NS}	65.06 ^{NS}	91.64 **	0.10 ^{NS}	6.41 ^{NS}
Error	50	21.02	26.83	41.93	30.79	0.09	6.27
Total	77						

** $p < 0.01$ and NS = Nonsignificant; DF = Degree of freedom; PV = Pollen viability; PG = Pollen germination; Chl. Cont. = Chlorophyll content; CMT = Cell membrane thermostability; Boll Wt. = Boll weight; Boll Ret. = Boll retention.

Table 4. Mean values with standard errors for six measures of heat tolerance in the top and bottom positions of 13 cotton genotypes grown in 2019 in Faisalabad, Pakistan.

Genotypes	Positions	PV (%)	PG (%)	Chl. Cont.	CMT (%)	Boll Wt. (g)	Boll Ret. (%)
CRS-2	Top	30.33 ± 2.03	24.00 ± 2.31	47.70 ± 3.65	73.80 ± 4.91	3.14 ± 0.09	29.11 ± 2.73
	Bottom	34.33 ± 2.33	28.67 ± 2.96	50.87 ± 3.88	88.75 ± 5.95	3.30 ± 0.17	36.45 ± 0.75
VH-377	Top	25.33 ± 2.91	14.67 ± 3.18	62.20 ± 2.11	78.68 ± 2.52	2.80 ± 0.20	30.51 ± 0.26
	Bottom	33.00 ± 4.36	21.00 ± 1.73	72.37 ± 3.46	86.61 ± 1.61	3.30 ± 0.07	32.48 ± 0.85
FH-215	Top	26.67 ± 1.45	17.67 ± 3.18	48.80 ± 2.93	72.81 ± 0.94	2.61 ± 0.15	28.97 ± 2.20
	Bottom	31.33 ± 2.03	25.67 ± 2.85	65.40 ± 2.95	75.95 ± 2.21	2.84 ± 0.06	32.88 ± 2.73
CIM-343	Top	23.33 ± 0.88	15.00 ± 2.65	51.20 ± 1.95	72.53 ± 4.36	3.26 ± 0.08	24.85 ± 1.19
	Bottom	32.00 ± 1.15	23.00 ± 4.16	60.23 ± 2.45	79.39 ± 1.79	3.40 ± 0.11	30.79 ± 1.27
CIM-602	Top	21.00 ± 1.73	17.00 ± 1.15	96.90 ± 4.39	81.68 ± 3.48	2.19 ± 0.06	26.79 ± 1.92
	Bottom	31.00 ± 4.16	22.33 ± 1.20	103.8 ± 2.39	91.31 ± 1.19	2.36 ± 0.16	31.34 ± 0.28
MNH-1016	Top	21.00 ± 1.73	10.33 ± 1.86	50.10 ± 4.72	88.67 ± 2.56	2.45 ± 0.19	30.53 ± 2.24
	Bottom	28.67 ± 2.03	15.67 ± 2.03	56.37 ± 2.86	92.50 ± 0.74	2.71 ± 0.08	32.01 ± 2.11
MNH-1026	Top	29.67 ± 1.67	19.00 ± 3.06	55.20 ± 3.02	83.19 ± 0.50	2.50 ± 0.13	30.21 ± 1.27
	Bottom	43.00 ± 3.61	25.33 ± 5.78	60.25 ± 3.01	88.82 ± 1.98	2.36 ± 0.20	31.95 ± 0.29
NIBGE-2	Top	19.00 ± 2.08	10.00 ± 1.53	51.27 ± 2.59	18.24 ± 2.98	2.58 ± 0.24	27.07 ± 1.32
	Bottom	31.67 ± 3.48	16.00 ± 2.08	57.63 ± 1.87	32.54 ± 3.51	2.42 ± 0.24	32.17 ± 0.66
N-777	Top	30.33 ± 3.18	17.67 ± 3.93	55.80 ± 5.42	78.88 ± 4.63	2.77 ± 0.33	29.16 ± 1.43
	Bottom	38.33 ± 3.28	23.33 ± 3.18	61.50 ± 4.85	88.16 ± 0.65	2.63 ± 0.09	34.42 ± 0.20
N-1048	Top	36.00 ± 2.89	9.00 ± 2.08	51.87 ± 1.30	41.47 ± 3.46	2.22 ± 0.26	24.08 ± 1.47
	Bottom	39.67 ± 2.91	12.67 ± 2.40	74.67 ± 4.64	70.03 ± 2.81	1.83 ± 0.05	30.35 ± 0.91
CYTO-124	Top	26.67 ± 4.26	17.67 ± 1.76	45.70 ± 5.23	22.95 ± 2.78	2.49 ± 0.16	31.71 ± 0.87
	Bottom	42.00 ± 5.51	29.00 ± 5.03	51.99 ± 4.56	36.99 ± 2.66	2.76 ± 0.09	33.72 ± 0.91
CYTO-608	Top	40.33 ± 4.26	22.00 ± 2.65	70.93 ± 2.86	83.47 ± 2.90	2.34 ± 0.13	28.35 ± 1.11
	Bottom	47.00 ± 4.04	31.00 ± 3.79	87.13 ±7.27	92.35 ± 1.47	2.58 ± 0.22	30.69 ± 0.90
AA-933	Top	32.67 ± 1.76	24.00 ± 3.06	68.00 ± 2.09	83.88 ± 2.50	2.38 ± 0.11	30.17 ± 1.72
	Bottom	42.67 ± 1.45	31.33 ± 4.06	78.83 ± 4.42	92.87 ± 2.70	2.77 ± 0.40	33.64 ± 0.56

PV = Pollen viability; PG = Pollen germination; Chl. Cont. = Chlorophyll content; CMT = Cell membrane thermostability; Boll Wt. = Boll weight; Boll Ret. = Boll retention.

3.1. Physiological Traits

The viability and germination of pollen grains were higher in the bottom part of the plant as compared to pollen that developed in flowers on the top part of the plant (Figure 3). The maximum viability of pollen grains in the bottom position was seen in CYTO-608 (47%) followed by MNH-1026 (43%), and the lowest value was observed in MNH-1016 (28.67%). The top flowers of CYTO-608 shown in Figure 4 and N-1048 showed the highest pollen viability measures of 40.33% and 36%, respectively, while MNH-1016 and CIM-602 had the lowest pollen viability of 21% for both genotypes (Table 4). Tukey's mean comparison test for pollen viability revealed non-significant differences for the genotypes CYTO-608, N-1048, and AA-933. On average, these genotypes performed well in both top and bottom positions (Table 5). Mean values of pollen viability across all genotypes to compare top and bottom positions of plants revealed a significant difference between top and bottom positions. Pollen viability at the bottom position exhibited more value compared to the top position (Figure 3).

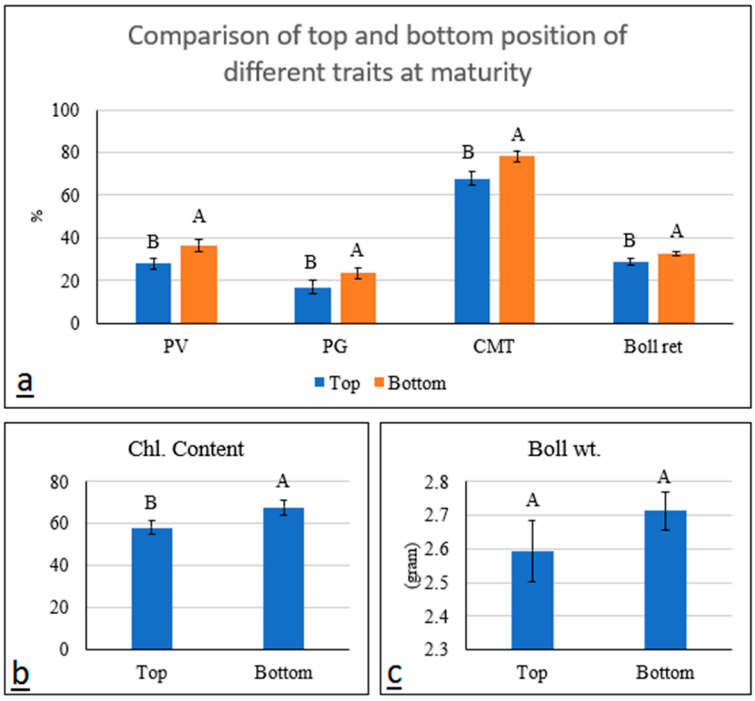

Figure 3. Means averaged across all genotypes to compare top and bottom positions of plants for different traits at maturity. The error bars are standard errors. The letters show Tukey's mean comparison where different letters show significant differences between top and bottom positions for each trait. (**a**) PV (Pollen viability), PG (Pollen germination), CMT (Cell membrane thermostability) and boll ret (boll retention), (**b**) Chl. Content (Chlorophyll content), (**c**) Boll wt (Boll weight).

The highest pollen germination from flowers at the bottom position was observed in AA-933 (31.33%) while in the top position, both AA-933 and CRS-2 showed 24% pollen germination. The lowest value for this parameter was observed in N-1048 with 12.67% and 9% germination in the bottom and top positions, respectively (Table 4). Overall, pollen from flowers that bloomed on top parts of the plant showed less germination when compared to pollen from bottom flowers (Figure 3). Pollen germination estimates were also lower than pollen viability estimates. Tukey's mean test revealed a non-significant difference between CRS-2, Cyto-608, and AA-933. It was observed that these genotypes performed well in both viability and germination tests, except CRS-2 which showed good pollen germination (Table 5). This indicates that most of the viable pollen of CRS-2 did germinate. Variation in pollen tube length was also observed as shown in Figure 5.

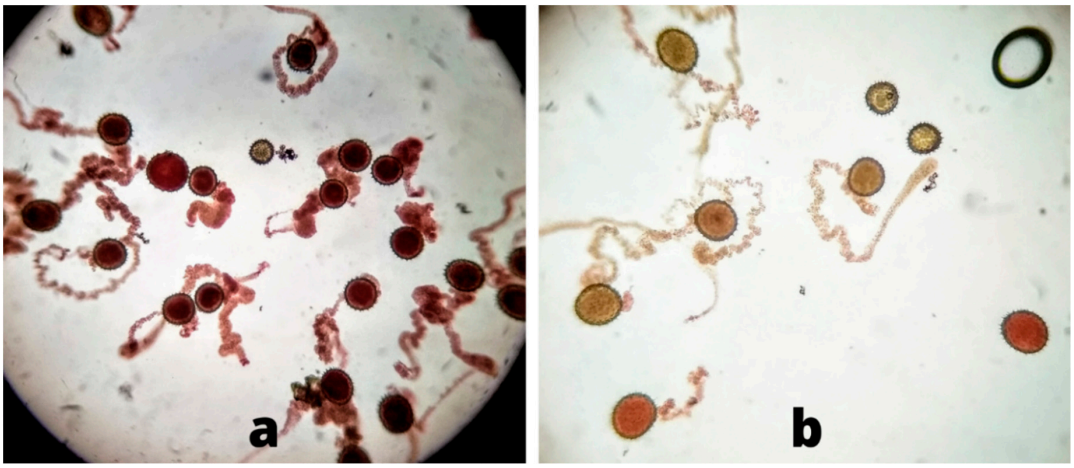

Figure 4. Results from staining pollen with triphenyl-tetrazolium chloride (TTC). (**a**) Pollen from flowers from the bottom positions of CYTO-608 shows the highest pollen viability. (**b**) Pollen from flowers from the top positions of NIBGE-2 shows the lowest pollen viability.

Table 5. Mean values for six measures of heat tolerance in 13 cotton genotypes grown in 2019 in Faisalabad, Pakistan.

Genotypes	PV (%)	PG (%)	Chl. Cont.	CMT (%)	Boll Wt. (g)	Boll Ret. (%)
CRS-2	32.33 ^{BCDE}	26.33 ^{A}	49.28 ^{F}	81.28 ^{ABC}	3.21 ^{AB}	32.78 ^{A}
VH-377	29.17 ^{BCDE}	17.83 ^{ABC}	67.28 ^{BCD}	82.64 ^{ABC}	3.05 ^{ABC}	31.50 ^{AB}
FH-215	29.00 ^{BCDE}	21.67 ^{AB}	57.10 ^{DEF}	74.38 ^{C}	2.73 ^{ABCD}	30.92 ^{AB}
CIM-343	27.67 ^{CDE}	19.00 ^{ABC}	55.72 ^{DEF}	75.96 ^{BC}	3.33 ^{A}	27.82 ^{AB}
CIM-602	26.00 ^{DE}	19.67 ^{ABC}	100.35 ^{A}	86.64 ^{A}	2.27 ^{DE}	28.57 ^{AB}
MNH-1016	24.83 ^{E}	13.00 ^{BC}	53.23 ^{EF}	90.59 ^{A}	2.58 ^{CDE}	31.27 ^{AB}
MNH-1026	36.33 ^{ABC}	22.17 ^{AB}	57.73 ^{DEF}	86.01 ^{AB}	2.43 ^{DE}	31.08 ^{AB}
NIBGE-2	25.33 ^{DE}	13.00 ^{BC}	54.45 ^{DEF}	25.39 ^{E}	2.50 ^{CDE}	29.62 ^{AB}
N-777	34.33 ^{BCD}	20.50 ^{ABC}	58.65 ^{CDEF}	85.61 ^{AB}	2.69 ^{BCD}	31.79 ^{AB}
N-1048	37.83 ^{AB}	10.83 ^{C}	65.35 ^{CDE}	55.75 ^{D}	2.03 ^{E}	31.96 ^{AB}
CYTO-124	34.33 ^{BCD}	23.33 ^{AB}	48.85 ^{F}	29.97 ^{E}	2.62 ^{BCDE}	32.67 ^{AB}
CYTO-608	43.67 ^{A}	26.50 ^{A}	79.03 ^{B}	85.81 ^{AB}	2.46 ^{CDE}	29.52 ^{AB}
AA-933	37.67 ^{AB}	27.67 ^{A}	71.33 ^{BC}	88.38 ^{A}	2.58 ^{CDE}	27.71 ^{B}

PV = Pollen viability; PG = Pollen germination; Chl. Cont. = Chlorophyll content; CMT = Cell membrane thermostability; Boll Wt. = Boll weight; Boll Ret. = Boll retention. Means with the same letters in each column are not significantly different according to Tukey's test.

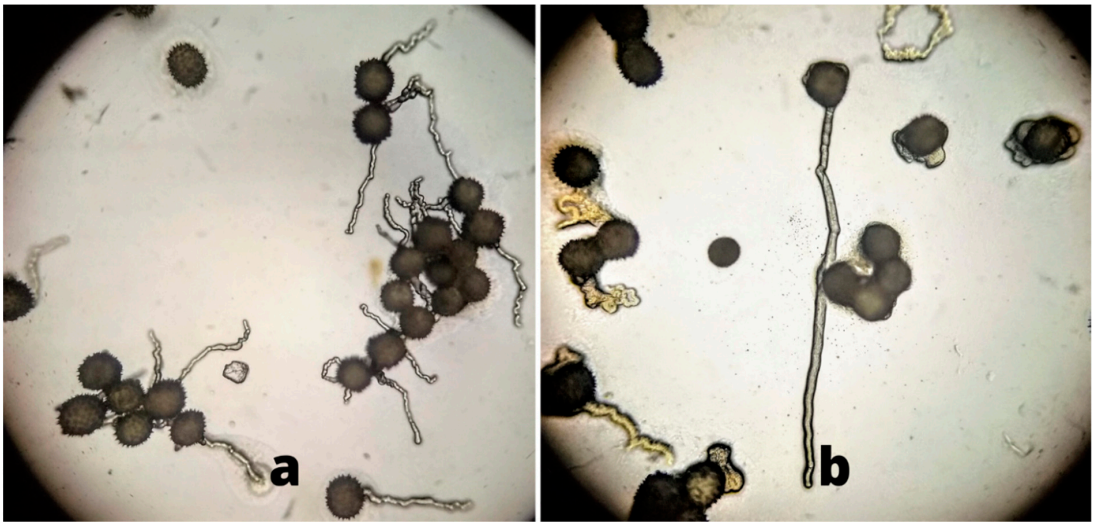

Figure 5. The observed variation in pollen tube germination where (**a**) Bottom flowers from AA-933 showed maximum pollen germination (**b**) Top flowers from N-1048 showed lowest pollen germination.

Cell membrane thermostability (CMT) and chlorophyll content were also significantly different for genotypes and plant positions (Table 3). The CMT values for top leaves (67.96%) were lower than values for bottom leaves (78.17%) (Figure 3). At the bottom of the plant, the maximum value for CMT was recorded for the genotype AA-933 (92.87%) followed by MNH-1016, CYTO-608, and CIM-602 which presented 92.50%, 92.35%, and 92.31% CMT, respectively (Table 4). Leaves of the bottom branches had more chlorophyll content as compared to leaves from the top branches (Figure 3). The genotypes CIM-602, CYTO-608, and AA-933 showed the highest chlorophyll contents in bottom branches (103.8, 87.13, and 78.83, respectively) while CIM-602 also had the highest chlorophyll content in leaves of top branches (96.9) (Table 4).

3.2. Morphological Traits

Genotypes varied significantly for boll weight, but non-significant differences were observed between the top and bottom plant portions for this trait (Table 3). On average, the largest boll weight at the bottom position was observed for CIM-343 (3.40 g) followed by CRS-2 and VH-377 (3.30 g for each) while N-1048 exhibited the lowest boll weight (Table 4). Tukey's test revealed non-significant differences among CIM-343, CRS-2, and VH-377 genotypes for boll weight (Table 5). Boll retention percentage was significantly different for genotypes and positions (Table 3). Lower boll retention was observed in the top position branches as compared to bottom branches (Figure 3). In the bottom branches, the genotype CRS-2 had maximum boll retention (36.45%) followed by N-777 (34.42%) and CYTO-124 (33.72%). The minimum boll retention was observed in N-1048 for both portions of the plant. Boll retention was also low in the top branches of CIM-343 (Table 4). It was noted that genotypes with high pollen germination retained more bolls.

3.3. Cluster Analysis

All the genotypes were clustered using pollen germination, pollen viability, cell membrane thermostability, chlorophyll content, boll weight, and boll retention at high-temperature stress as variables. The dendrogram showed three clusters with a minimum of 33.33% similarity level. The highest Euclidean distance was found between clusters 2 and 3 (57.76) and lowest between clusters 1 and 2 (27.95) as presented in Table 6.

Table 6. The distance among the various cluster centroid of cotton genotypes under high temperature.

	Cluster 1	**Cluster 2**	**Cluster 3**
Cluster 1	0	27.9541	45.5783
Cluster 2		0	57.7641
Cluster 3			0

The clusters were divided into two groups Group Y and Group Z. Group Y included two clusters named cluster 1 and cluster 2 while Group Z included only one cluster named cluster 3. Cluster 1 included seven genotypes named CRS-2, MNH-1026, N-777, MNH-1016, VH-377, FH-215, and CIM-343 which represent 53.85% of total genotypes. Cluster 2 includes three genotypes named CIM-602, Cyto-608 and AA-933 represent 23.07% of total genotypes. In cluster 3, three genotypes are included named NIBGE-2, Cyto-124, and N-1048 representing 23.07% of the total genotype (Figure 6). The genotypes within each cluster exhibited similar behavior based on six traits used in this study. The genotypes in cluster 1 showed good performance based on boll weight. The genotypes grouped in cluster 2 are characterized by high pollen germination, pollen viability, chlorophyll content, and CMT. This indicated that the genotypes grouped in cluster 2 could be selected for the breeding program. The genotypes of cluster 3 were grouped by the lowest value of pollen germination, pollen viability, CMT, and boll weight (Table 7).

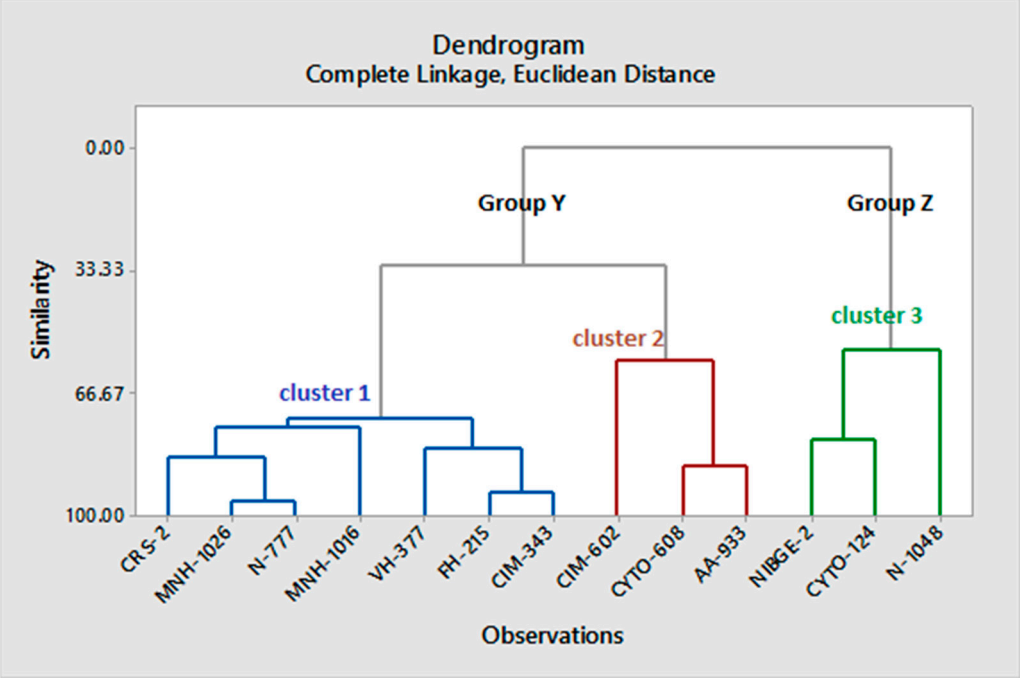

Figure 6. Cluster analysis of thirteen accessions of upland cotton evaluated for high-temperature regimes.

Table 7. Means of clusters of 13 cotton genotypes of all observed tr under high-temperature stress.

Variable	Cluster 1	Cluster 2	Cluster 3	Grand Centroid
No. of genotypes	7	3	3	13
PV (%)	30.5229	35.78	32.4967	32.1915
PG (%)	20.0714	24.6133	15.72	20.1154
Chl. Cont.	56.9986	83.57	56.2167	62.95
CMT (%)	82.3529	86.9433	37.0367	72.9546
Boll Wt. (g)	2.86	2.4367	2.3833	2.6523
Boll Ret. (%)	31.0229	28.6	31.4167	30.5546

4. Discussion

Higher pollen germination and viability percentages from flowers under the canopy of the plant as compared to flowers in direct sunlight were observed in this study. As the temperature in the experimental region in Pakistan rises to 47 °C during the time of cotton flowering, this damages the lipid as well as protein parts of the pollen membrane, thus resulting in decreases in pollen viability [12]. Pollen viability was determined by analyzing dehydrogenase enzyme activity in the pollen grains—if the enzyme is active, viable pollen grains change to a red color after TTC staining. However, there may be damage to the pollen grains that reduces germination despite this enzyme activity. It has been reported that the distribution of cell organelles such as mitochondria, vacuoles, and endoplasmic reticulum of pollen cells become disturbed under high temperatures. Lipid and starch granules are also reduced in pollen cells during heat stress [8].

In our study, lower pollen germination was observed as compared to pollen viability. Most pollen could not develop the pollen tube required for germination, likely due to metabolic or structural abnormalities of pollen grains [31]. Drought or heat stress significantly lowers carbohydrate metabolism in the pistil, resulting in a lower energy supply to the pollen tube in the style, thus leading to a failure of fertilization [32]. Under excessively high temperatures, heat shock proteins (HSPs) work to stabilize proteins that were damaged when exposed to stressful conditions. As the expression of HSPs varies between genotypes, some genotypes showed good pollen germination even in the top portion of the plant [33].

Genotypic variability for CMT has been previously reported [3,34]. Here, we have observed CMT differences between plant positions. The significant differences among cultivars are due to several factors including cuticle thickness, secondary metabolites, and heat shock proteins [35–37]. Lower CMT estimates in top leaves were due to sunlight exposure. The membranes of leaves facing direct sunlight in high-temperature conditions were more prone to damage. Sun rays cause oxidative damages to both lipid and protein parts of the cellular membranes and cause the leakage of electrolytes [38]. UV radiation from the sun causes irreversible damage to plant pigments [39]. It causes conformational changes in the structure of nucleic acids, proteins, and macromolecules in the cell and degrades the chlorophyll pigment [40,41]. Heat stress directly affects the flow of fluid through the cell membrane as relative electrical conductivity increases with temperature [42,43].

Since the chlorophyll contents under the canopy were higher as compared to the top position, it has been assumed that chlorophyll loses its integrity under direct sunlight. In addition to direct sunlight, the higher temperature in the top portion of the plant also causes chlorophyll damage [44]. Heat stress that denatures thylakoid membranes results in a loss of chlorophyll [45]. Moreover, the enzymes required for the synthesis of chlorophyll and its normal activity were also denatured under high-temperature conditions [46]. As a result, photosynthetic activity was reduced in the top portion of plants. On average, the genotypes AA-933 and CYTO-608 had good heat tolerance features in bottom positions; therefore, these genotypes would be useful as parents in a breeding program

Ascorbic acid has the potential to mitigate the negative effects of stress. It acts in ROS scavenging and maintains the integrity of membranes, including the thylakoid membrane [47]. So, ascorbic acid could be used to overcome the heat stress problem. The cell membrane thermostability of cotton crops can be improved significantly by applying the foliar application of 40 mg L^{-1} ascorbic acid [48].

Boll weight is positively associated with seed cotton yield. It is a complex polygenic trait that depends upon numerous factors namely, the weight of seed, seed size, protein and oil content within the seed, and cellulose deposition during fiber development and maturity [49]. It is one of the most important characters linked to improved yield, and significant variation for this trait has been reported in germplasm [50,51]. Although the genotypes used in this experiment were significantly different for boll weight, no significant differences for this trait were recorded between the top and bottom portions of the same genotypes. Retention of bolls during the developmental period varied significantly between the top and bottom branches. The bottom branches tend to hold more bolls as compared to the top branches. It was noted that the genotypes with higher pollen viability and germination also retained more bolls. This study revealed that the heat tolerance ability of the genotypes was associated with boll retention while heat stress has been considered one of the major factors in bolls dropping before maturity [52,53]. Thus, high temperature in the top portion of a plant due to direct exposure to sunlight can explain retaining a lower number of bolls in this portion of the plant.

The variability was found between the genotypes as shown in Table 3. Cluster analysis has revealed that CIM-602, Cyto-608, and AA-933 grouped in cluster 2 performed well and these genotypes could be used further in any breeding program. Since all genotypes are grown in the Punjab region of Pakistan, these are therefore acclimatized to this environment. These genotypes share some common, as well as different phenotypes, which showed variability based on six traits used in this study. The variability was also observed in the cotton genotypes cultivated in the Punjab region of Pakistan by khan [54].

This study provides an understanding of the role of flowering in the top and bottom portions of the cotton plant in response to high-temperature stress because high temperature is a major factor in reducing yield. It is assumed that by increasing the vegetative growth and leaf surface area, the shading effect can be increased. The spreading-type behavior of the cotton plant could be able to produce more shading. The shading effect will reduce canopy temperature and hence yield could be increased. Likewise, screening for early maturing cultivars and for having more branches on the bottom part of the plant

could be beneficial because the bottom branches have shown more productivity than top branches. Keeping in view the importance of the study, another study may be conducted to assess the temperature of the microenvironment i.e., the temperature of leaf, bud, and/or boll at the top and bottom regions of each genotype, followed by correlation analysis with each trait to understand the relationship of various traits during heat stress.

5. Conclusions

Both top and bottom branches of the cotton plant exhibited variable responses for physiological and morphological traits. Significant genotypic variability for these attributes was also observed. The bottom branches of the genotypes performed better for all the recorded parameters except boll weight which was non-significant for both positions. The high temperature was found to disrupt plant physiology and morphology more on top position flowers. Further study of the shading effect is an objective for future breeding programs. A focus on increasing vegetative growth, leaf surface area, and a more spreading growth pattern would increase the canopy size and allow for shading to improve pollen germination and pollen tube growth. Further, research focusing on increased resilience to high temperature itself would allow top portions of a cotton plant to deliver a higher yield thus increasing overall yields.

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