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BIOCHEMICAL MARKERS ASSISTED SCREENING OF PAKISTANI WHEAT (*Triticum aestivum* L.) CULTIVARS FOR TERMINAL HEAT STRESS TOLERANCE

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Heat stress is an important abiotic constraint limiting crop productivity. An experiment was conducted to elucidate the impacts of heat induced biochemical transformations on morphological attributes of Pakistani wheat genotypes. Experiment was laid out in randomized complete block design (RCBD) with the split plot arrangement during the winter season 2014-15. Main plot treatments were comprised of H_0 (No heat imposition) and H_1 [(Heat imposition from spike emergence to grain filling initiation (Feekes Scale = 10.50 to 11.00)]. Subplots treatments were comprised of eleven wheat genotypes (Punjab-2011, AARI-2011, Galaxy-2013, Millat-2011, Aas-2011, Fareed-2006, Chakwal-50, Mairaj-2008, Pakistan-2013, NIBGE-NIAB-1 and Kohistan-97). Under heat over control, lesser decrease in chlorophyll contents and enhancement in antioxidant activities were depicted by Aas-2011, Chakwal-50, and Mairaj-2008. Higher chlorophyll degradation and diminishment in antioxidant activities under heat stress over control was observed for all other cultivars. Comparatively lower grain filling rate, higher duration, number of grains per spike and grain yield was observed for cultivars Aas-2011, Chakwal-50, Mairaj-2008 and Punjab-2011 under heat stress over control. Conclusively, based on biochemical response and morphological markers genotypes Aas-2011, Chakwal-50, and Mairaj-2008 manifested heat tolerance. Genotypes Fareed-2006 and Punjab-2011 manifested medium heat tolerance. Whereas, genotypes AARI-2011, Galaxy-2013, Millat-2011, Pakistan-2013, NIBGE-NIAB-1 and Kohistan-97 depicted susceptibility to terminal heat stress.

Keywords: Antioxidant defense system, grain yield, morphogenesis, stay green, thermotolerance

INTRODUCTION

Wheat is the largest growing cereal around the globe. It is grown on an area of 222.24 million hectares with production of 737.83 million metric tons in world (USDA, 2017). Food security in Pakistan is affiliated with wheat production and consumption. In Pakistan, share of wheat in gross domestic product is 2.0% and in value addition 9.9%. It is cultivated on an area of 9.260 million hectares (Govt. of Pakistan, 2016).

The increasing prevalence of intense temperature is becoming a limiting factor for crop production specifically for wheat crop. Over the years, wheat productivity is threatened by increasing climatic skepticism (Wang *et al*., 2015). Wheat production under changing climate has been an arduous task. Hence, it warrants the prerequisite of boosting its yield on per unit land area basis and negotiates wheat production under high-temperature environment (Trnka *et al*., 2014). As a temperate climate crop, wheat crop prefers to grow in cool temperature (Asseng *et al*., 2015). The temperature optima for terminal spikelet, anthesis, and grain filling for wheat are 12, 23 and 21ºC, respectively (Innes *et al*., 2015). According to an assessment, every 1ºC rise of temperature declines grain yield by 3-17% in Pakistan and India (Mondal *et al*., 2013).

The higher temperature is foretold to rise further in future and terminal heat stress (>35ºC) deleteriously impacting grain yield in wheat (Wang *et al*., 2012). Exposure of reproductive stages to the higher temperature is known as terminal heat stress. Heat stress is of "heat shock" and "chronic heat" types. Heat shock is the abrupt and utmost increment in temperature above 35°C for duration of 4-5 days. Whereas, chronic heat stress is occurrence of moderately high temperature (25-30°C) for the relatively longer duration (Li *et al*., 2013).

Negative impacts of heat stress include photorespiration, pollen infertility, cellular dehydration, rapid phenology, declining availability of assimilates for grain filling, chlorophyll degradation, decreasing number and size of grains and eventually decline in grain yield (Ghaffari *et al*., 2015). The most important adverse effect of heat stress is generation of excessive reactive oxygen species (ROS) that leads to oxidation of lipids of cellular membranes (Hasanuzzaman *et al*., 2013). Consequently, plant synthesizes antioxidants to scavenge ROS. Plants also accumulate compatible solutes and osmo-protectants as a defensive mechanism to regain cellular redox balance and homeostasis. Antioxidants and compatible solutes develop heat tolerance and maintain growth (Kamal *et al*., 2017).

Different wheat cultivars depict assortment and heterogeneity in response to high temperature (Siebert and Ewert, 2014). Furthermore, numerous quantitative trait loci exist for a single targeted trait having complex inheritance pattern (Mwadzingeni *et al*., 2016). Therefore, selection of polygenic target traits can be accomplished indirectly employing biochemical markers closely related to heat tolerance (Sadat *et al*., 2013). Likewise, diversity among wheat cultivars combined with polyploidy and genes profusion makes it challenge to select a suitable genotype using morphological trait under high-temperature environment (Dube *et al*., 2016). Hence, selection of wheat genotypes merely since response of morphological traits often leads to faulty inferences (Reynolds and Langridge, 2016). Moreover, physiochemical marker assisted selection of genotypes depicts higher efficacy of selection than mere morphological markers based selection for polygenic traits (Sadat *et al*., 2013). Therefore, phenological and biochemical markers assisted screening of wheat cultivars enhances efficacy of cultivar selection (Nawaz *et al*., 2015).

Previous experiments were mainly comprised of heat imposition under controlled environments of greenhouse. Although, studies regarding manipulation of sowing dates are abundantly available to observe adverse effects of high temperature. Whereas, relatively a little information is available regarding the imposition of heat stress under field conditions. Moreover, studying biochemical mediated transformations in correlation with morphological traits might prove advantageous for breeding for heat tolerance. Whereas, information regarding the correlation of antioxidant enzymes, chlorophyll contents and total soluble proteins with growth and yield attributes at terminal stages predisposed to heat are scarce. Most of previous studies quantified biochemical attributes only at seedling stages without considering yield and other phenotypic traits at seedling stage. Thus, study was conducted with hypotheses that antioxidant enzymatic activities, stay greenness, total soluble proteins, grain filling rate and duration tend to be highly correlated with agronomic traits. Objectives of study were (i) to investigate enzymatic activities and stay green trait as potential regulators of morphological markers in Pakistani wheat genotypes under terminal heat (ii) to examine either morphological attributes are reliant on physio-chemical processes and can be manipulated for improving heat tolerance.

MATERIALS AND METHODS

***Experimental site*:** An experiment was conducted to observe negative implications of terminal heat stress on different cultivars of wheat. The experiment was carried out at the Agronomic Research Area, University of Agriculture Faisalabad, Pakistan during winter season 2014-15. Experimental site is located at 73˚ east longitude, 31˚ north latitude, and at an altitude of 184.4 meters.

***Plant material*:** The seed of varieties Punjab-2011, AARI-2011, Galaxy-2013, Mairaj-2008, and Millat-2011 was procured from Ayub Agriculture Research Institute (AARI) Faisalabad, Pakistan. The seed of genotypes Aas-2011 and Fareed-2006 was obtained from Adaptive Research Farm, Bahawalpur while seed of Pakistan-2013 was obtained from National Agricultural Research Centre (NARC), Islamabad. Plant material of genotypes Chakwal-50 and Kohistan-97 was procured from University of Agriculture Faisalabad (UAF), Pakistan. Seed of genotype NIBGE-NIAB-1 was obtained from Nuclear Institute for Agriculture and Biology *(NIAB*) Faisalabad, Pakistan.

***Treatments and agronomic practices*:** The experiment comprised of two heat stress treatments [H_0= No heat stress (plots without polythene sheet) and H_1= Heat stress (plots covered with polythene sheet from the complete emergence of spike to grain filling initiation, Feekes Scale= 10.50 to 11.00)] and eleven varieties (Punjab-2011, AARI-2011, Galaxy-2013, Millat-2011, Aas-2011, Fareed-2006, Chakwal-50, Mairaj-2008, Pakistan-2013, NIBGE-NIAB-1, Kohistan-97)**.**

Sowing was done on 17th of November 2014 with the help of single row hand drill with R × R of 22.5 cm. Seed rate used during sowing was 100 kg ha^{-1}. Gross plot size used was 3.0 m × 0.90 m having four rows of wheat in each plot with row × row distance of 22.50 cm. Fertilizer was applied at the rate of 120:75:60 kg NPK ha^{-1}. Half of nitrogen fertilizer (urea) and all the phosphorus (SSP) and potash fertilizers (SOP) were applied as basal dose. Remaining half nitrogen fertilizer was applied with first irrigation at crown root initiation. Irrigations were applied at four critical growth stages viz. crown root initiation, tillering, spike initiation and flowering. The crop was harvested on 2nd May 2015. Two hoeings were done in all treatments to control weeds; first after 40 days of sowing and second after 60 days of sowing.

***Imposition of heat stress*:** Heat stress was imposed at heading stage by covering main plots with polythene sheet while control plot was left in the ambient environment. Heat stress was imposed from heading to grain filling stage (Feekes Scale= 10.50 to 11.0), during this duration temperature was recorded in the morning, noon and in evening with the help of digital temperature and humidity probe (Digital Multimeter-50302). The mean daily temperature in control and heat stressed main plots are shown graphically in Figure 1.

***Experimental design*:** Randomized complete block design (RCBD) with split plot arrangement having four replications was used to conduct the experiment. Heat stress treatments were randomized in main plots while varieties in subplots. The data were analyzed statistically ($p \leq 0.05$) using the Fisher's analysis of variance technique (Steel *et al*., 1997) and treatments' means were compared by using Tukey's Honestly Significant Difference (Tukey's HSD) test at 5% probability level. Moreover, strength of association among recorded

attributes under heat and no heat was determined using correlation analysis (Gomez and Gomez, 1984).

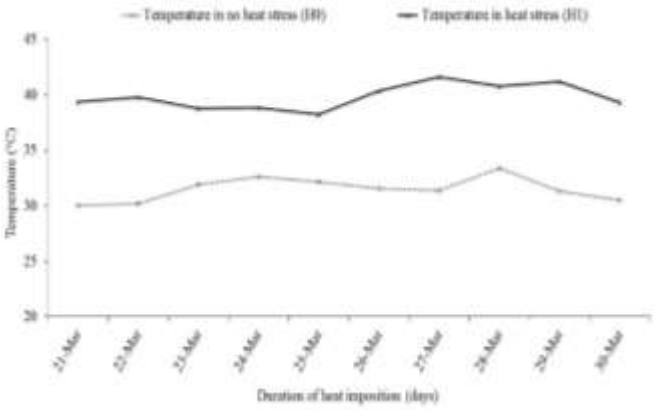

Figure 1. Mean daily temperature in no heat and heat stressed main plots.

***Observations recorded*:** Chlorophyll *a* and *b* contents were recorded by collecting samples from each treatment. The sub samples of 0.5 g from each plot were soaked overnight in 80% acetone and recorded absorbance at 663 and 645 nm using ELISA plate. Chlorophyll *a* and *b* contents (mg g^{-1}) were determined using formulae given by Arnon (1949):

$$\text{Chl } a = [12.7 \times A663 - 2.69 \times A645] \times \frac{V}{1000} \times W$$

$$\text{Chl } b = [22.9 \times A645 - 4.68 \times A663] \times \frac{V}{1000} \times W$$

Where "A" indicates absorbance (nm), "V" volume of the extract (mL) and "W" weight of the fresh leaf tissue (g).

Total soluble proteins (TSP) were quantified by preparing enzyme extract of leaves samples stored at -80°C in a freezer. Enzyme extract was prepared in potassium phosphate buffer (pH 4), vortex and centrifuged. Added Bradford Reagent and recorded absorbance using ELISA plate at 595 nm (Bradford, 1976).

Superoxide dismutase (SOD) activity was measured as an amount of enzyme that inhibited photochemical reduction of nitro blue tetrazolium (NBT). Reaction mixture used was 100 µL enzyme extract prepared same as for TSP + 500 µL potassium phosphate buffer (pH 5) + 200 µL methionine + 200 µL triton X + 100 µL NBT + 800 µL distilled water. Placed under ultraviolet light for 15 minutes, added 100 µL riboflavin and noted absorbance at 560 nm with the help of ELISA plate (Giannopolitis and Ries, 1977). Peroxidase (POD) activity was recorded as an amount of enzyme required for guaiacol oxidation. Enzyme extract used for TSP was also used to measure POD activity. Reaction mixture comprised of 800 µL potassium phosphate buffer (pH 5) + 100 µL H_2O_2 (40 mM) + 100 µL guaiacol (20 mM). Added 100 µL enzyme extract, 100 µL reaction mixture, and recorded absorbance at 470 nm using ELISA plate (Liu *et al*., 2009). The activity of catalase (CAT) was determined as the amount of H_2O_2 consumed by the enzyme and converted to H_2O and O_2. Same enzyme extract as for TSP determination was used for measurement of CAT activity. Enzyme extract 100 µL was taken in well plate, added 100 µL H_2O_2 (5.9 mM) and recorded absorbance at 240 nm wavelength on ELISA plate (Liu *et al*., 2009).

Grain filling rate (GFR) was recorded by randomly selecting 5 spikes in each treatment. Spikes were harvested at 5 days' interval and oven dried to record dry weight. GFR was determined using formula by Hunt (1978).

$$\text{GFR} = \frac{W_2 - W_1}{t_2 - t_1}$$

Where W_1 and W_2 represent the dry weight of spikes at first harvest and second harvest, respectively. To determine grain filling duration (GFD) five plants in each plot were tagged. The number of days from heading to physiological maturity was taken as grain filling duration (Hunt, 1978). Number of grains per spike was determined by taking the average of 10 spikes harvested randomly from each treatment. For grain yield, each plot was harvested and threshed and converted to tons per hectare.

RESULTS

Heat stress had an overall deleterious effect at reproductive stages of wheat. However, cultivars specific response was evident on different growth, yield, and biochemical attributes. The varied response of genotypes under heat and control resulted in significant heat × genotypes effect for various parameters.

Different wheat genotypes manifested significant difference for chlorophyll (CHL) contents (Table 1). High-temperature stress had the negative impact on chlorophyll pigment. Heat stress diminished CHL *a* (33%) and CHL *b* (38%) contents of control. All cultivars showed similar trend under heat and no heat-induced conditions to depict non-significant interaction. Cultivars varied response was apparent as Aas-2011 recorded maximum CHL contents (2.08 mg g^{-1}) and it was statistically alike to the cultivars Chakwal-50 and Mairaj-2008. Similarly, the wheat cultivar Aas-2011 recorded highest CHL *b* contents (0.58 mg g^{-1}) and was statistically like to cultivar Chakwal-50. Conversely, minimum CHL contents were observed for cultivar NIBGE-NIAB-1 (CHL *a* 1.01 mg g^{-1}) (CHL *b* 0.15 mg g^{-1}). Wheat cultivar NIBGE-NIAB-1 depicted greater reduction than other genotypes in CHL *a* (52%) and CHL *b* (53%) contents. Contrarily, genotypes Chakwal-50, Mairaj-2008, Aas-2011, and Fareed-2006 recorded almost similar response and lesser decline in CHL *a* (15-22%) and CHL *b* (27-35%) contents than other genotypes were observed (Table 2).

Table 1. Analysis of variance for effect of heat stress on biochemical, growth and yield parameters of wheat varieties. A) Mean sum of squares.

SOV	DF	Parameters									
		CHL *a*	CHL *b*	TSP	SOD	POD	CAT	GFR	GFD	GPS	GY
Blocks	3	7.76	0.117	0.027	15226.7	55.72	224.34	0.00038	7.14	483.10	21.31
Heat (H)	1	7.35**	0.482**	0.130*	7103.0**	128.87**	375.80*	0.05600**	2132.53**	1071.01**	60.40**
Error 1	3	0.19	0.009	0.004	142.3	3.74	23.27	0.00038	7.99	8.28	0.17
Varieties (V)	10	1.10**	0.212**	0.304**	15882.3**	342.18**	1170.58**	0.00519**	178.51**	247.07**	2.49**
H × V	10	0.04^{NS}	0.002^{NS}	0.026*	1673.9**	47.09**	143.30**	0.00035^{NS}	4.34^{NS}	9.51*	0.26**
Error 2	60	0.04	0.002	0.003	35.9	1.13	2.55	0.00030	4.60	4.45	0.09

SOV = Source of variation; DF = Degree of freedom; * = Significant ($p \leq 0.05$); ** = Highly significant ($p \leq 0.01$); NS = Non-significant; CHL *a* = Chlorophyll *a* contents (mg g^{-1}); CHL *b* = Chlorophyll *b* contents (mg g^{-1}); TSP = Total soluble proteins (mg g^{-1}); SOD = Super oxide dismutase (U per mg protein); POD = Peroxidase (U per mg protein); CAT = Catalase (U per mg protein); GFR = Grain filling rate (g per day); GFD = Grain filling duration (days); GPS = Number of grains per spike; GY = Grain yield (t ha^{-1})

Table 2. Effect of heat stress on biochemical and growth parameters of wheat varieties.

Treatments	Parameters			
	CHL *a*	CHL *b*	GFR	GFD
Heat stress				
No heat stress (H_0)	1.75 A	0.39 A	0.10 B	35.86 A
Heat stress (H_1)	1.18 B	0.24 B	0.15 A	26.01 B
Tukey's HSD ($p \leq 0.05$)	0.298	0.064	0.013	1.918
Varieties				
Punjab-2011	1.53 CDE	0.32 C	0.15 AB	32.29 CD
AARI-2011	1.28 EFG	0.24 DE	0.14 AB	29.11 DE
Galaxy-2013	1.40 DEF	0.28 CD	0.16 A	31.68 CD
Millat-2011	1.18 FG	0.19 EF	0.12 B	27.37 EF
Aas-2011	2.08 A	0.58 A	0.08 C	37.59 A
Fareed-2006	1.71 BCD	0.40 B	0.12 B	32.89 BC
Chakwal-50	1.92 AB	0.57 A	0.08 C	36.69 A
Mairaj-2008	1.82 ABC	0.46 B	0.13 B	36.47 AB
Pakistan-2013	1.15 FG	0.15 F	0.14 AB	25.23 F
NIBGE-NIAB-1	1.01 G	0.15 F	0.14 AB	24.72 F
Kohistan-97	1.07 G	0.16 F	0.14 AB	26.29 EF
Tukey's HSD ($p \leq 0.05$)	0.320	0.072	0.029	3.586

Any two means not sharing a letter in common differ significantly at $p \leq 0.05$; CHL *a* = Chlorophyll *a* contents (mg g^{-1}); CHL *b* = Chlorophyll *b* contents (mg g^{-1}); GFR = Grain filling rate (g per day); GFD = Grain filling duration (days)

Dissimilar performance of different wheat genotypes for antioxidant activities was observed in main plots and resulted in significant interaction of genotypes and heat stress (Table 1). Superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) activity of Aas-2011, Chakwal-50 and Mairaj-2008 was enhanced under heat over ambient conditions. Whereas, all other cultivars recorded diminishing trend in enzymatic activity under stressed environment over control (Table 3).

Regarding SOD activity, Aas-2011, Chakwal-50 and Mairaj-2008 depicted an increase of 12, 14 and 14%, respectively under heat stress over control. In control Aas-2011 and Chakwal-50 were statistically alike while in heat stress Aas-2011, Chakwal-50 and Mairaj-2008 remained at par. Galaxy-2013 and Millat-2011 depicted maximum decline (38 and 40%, respectively) in SOD activity under heat compared to no heat environment (Table 3). The cultivars Aas-2011, Chakwal-50 and Mairaj-2008 exhibited 17, 15 and 24% enhancement in POD activity, respectively under heat-induced conditions over no heat imposition. Under high-temperature maximum diminishment (52%) in POD activity was observed for Punjab-2011 and Galaxy-2013. Under high-temperature stress, Aas-2011, Chakwal-50 and Mairaj-2008 recorded significantly higher POD activity than all other genotypes (Table 3).

Cultivars Aas-2011, Chakwal-50, and Mairaj-2008 depicted an increase of 19, 15 and 16% respectively in CAT activity under high-temperature stress environment over control. The highest reduction in CAT activity was recorded for genotypes Punjab-2011 (50%) and Galaxy-2013 (46%). Aas-2011, Chakwal-50, and Miaraj-2008 manifested significantly higher SOD, POD and CAT activity than all other cultivars in heat stressed main plots. Likewise, almost similar trend was observed in control (Table 3). Differential response of wheat

Table 3. Effect of heat stress on antioxidant activity and grain yield of wheat varieties.

Treatments	TSP	SOD	POD	CAT	GPS	GY
No heat stress (H_0)						
Punjab-2011	0.569 cd	143.85 b	17.00 c	34.41 c	55.75 a	5.09 ab
AARI-2011	0.466 de	108.47 d	11.54 ef	24.63 e	41.75 cde	4.22 c
Galaxy-2013	0.388 e	124.38 c	13.89 de	29.69 d	42.50 cde	4.15 c
Millat-2011	0.410 e	95.47 de	10.22 fg	20.64 f	43.50 cd	4.06 c
Aas-2011	0.752 a	168.90 a	21.28 a	38.58 ab	53.50 ab	5.22 a
Fareed-2006	0.620 bc	144.43 b	15.51 cd	35.14 bc	44.00 c	4.19 c
Chakwal-50	0.737 ab	165.98 a	19.84 ab	38.98 a	49.25 b	4.95 ab
Mairaj-2008	0.689 abc	155.42 ab	17.79 bc	37.66 abc	52.25 ab	4.46 bc
Pakistan-2013	0.384 e	83.97 ef	8.02 gh	15.94 g	39.00 de	4.10 c
NIBGE-NIAB-1	0.371 e	79.20 f	7.03 h	14.13 g	38.50 e	3.35 d
Kohistan-97	0.375 e	81.40 ef	8.09 gh	15.56 g	44.25 c	3.31 d
Heat stress (H_1)						
Punjab-2011	0.418 cd	96.50 c	8.09 bc	18.94 c	45.00 ab	2.71 bcd
AARI-2011	0.313 de	69.15 de	6.70 cd	15.62 cd	37.00 d	2.20 d
Galaxy-2013	0.333 de	76.80 d	6.67 cd	16.13 c	37.00 d	2.26 d
Millat-2011	0.274 e	57.33 ef	5.32 d	11.90 de	38.25 cd	2.40 cd
Aas-2011	0.894 a	192.07 a	25.77 a	47.11 a	48.00 a	3.71 a
Fareed-2006	0.493 bc	112.92 b	10.10 b	26.02 b	38.00 cd	2.73 bcd
Chakwal-50	0.888 a	192.85 a	23.28 a	46.45 a	44.25 ab	3.36 ab
Mairaj-2008	0.555 b	179.93 a	23.49 a	45.08 a	42.00 bc	3.04 abc
Pakistan-2013	0.261 e	62.20 ef	4.63 d	11.31 e	32.00 e	2.21 d
NIBGE-NIAB-1	0.235 e	54.30 f	4.25 d	10.28 e	31.00 e	2.03 d
Kohistan-97	0.250 e	59.77 ef	5.27 d	11.05 e	35.00 de	2.24 d
Tukey's HSD ($p \leq 0.05$)	0.1295	14.170	2.514	3.777	4.989	0.709

Any two means not sharing a letter in common differ significantly at $p \leq 0.05$; TSP = Total soluble proteins (mg g^{-1}); SOD = Super oxide dismutase (U per mg protein); POD = Peroxidase (U per mg protein); CAT = Catalase (U per mg protein); GPS = Number of grains per spike; GY = Grain yield (t ha^{-1})

genotypes resulted in significant heat × varieties effect for total soluble proteins (TSP). Higher temperature enhanced accumulation of TSP in cultivars Aas-2011 (16%) and Chakwal-50 (17%) and both cultivars were statistically similar. In control Aas-2011 and Chakwal-50 were also statistically resembling to Mairaj-2008 for TSP. The highest diminishment in TSP under heat over control was observed for cultivars AARI-2011 (40%) and Millat-2011 (64%) (Table 3).

Heat stress (H) and varied performance of genotypes (V) significantly affected grain filling rate (GFR) and grain filling duration (GFD). Whereas, same trend amongst all cultivars was observed in both main plots resulting in non-significant interaction (H × V) for GFR and GFD (Table 1). Heat stress accelerated GFR by 33% while diminished GFD by 27%. Concerning GFR, Galaxy-2013 recorded highest GFR (0.16 g per day) and it was statistically similar to Punjab-2011, AARI-2011, Pakistan-2013, NIBGE-NIAB-1 and Kohistan-97. Significantly lowest GFR was noted for cultivars Aas-2011 and Chakwal-50. Regarding GFD, the cultivar Aas-2011 recorded highest value (37.59 days). Aas-2011 was statistically comparable to Chakwal-50 and Mairaj-2008. The genotype NIBGE-NIAB-1 recorded lowest GFD (24.72 days) and it was statistically alike to Millat-2011, Pakistan-2013 and Kohistan-97. High-temperature environment caused rapid grain filling rate (GFR) and diminished grain filling duration (GFD). Even though, genotypes Aas-2011 and Chakwal-50 maintained significantly lower GFR than all other cultivars. Genotypes Aas-2011 and Chakwal-50 manifested significantly lowest decline in GFD against maximum in cultivars Punjab-2011 and AARI-2011 (Table 3).

Varied response of genotypes was obvious under heat and control to manifest significant heat × genotypes effect (Table 1). More heat triggered diminishment in number of grains per spike over control was observed for genotype Mairaj-2008 (20%), NIBGE-NIAB-1 (19%) and Kohistan-97 (21%), whereas, AARI-2011, AAS-2011 and Chakwal-50 exhibited smaller decline (<11%) in this regard. Grain yield (GY) was significantly decreased due to bad implications of heat. The distinct genetic makeup of genotypes was statistically apparent indicating their varied competency to produce yield under different environments. Nevertheless, all cultivars responded differently in control and heat stressed environment to produce significant interaction of varieties and heat stress. While comparing control with heat stress

Table 4. Correlation analyses showing strength of association among recorded attributes of genotypes under no heat and heat stress.

Parameter	Treatment	TSP	SOD	POD	CAT	CHL *a*	CHL *b*	GFR	GFD	GPS	GY
TSP	H_0	1.00									
	H_1	1.00									
SOD	H_0	0.99**	1.00								
	H_1	0.95**	1.00								
POD	H_0	0.98**	0.99**	1.00							
	H_1	0.93**	0.99**	1.00							
CAT	H_0	0.97**	0.99**	0.98**	1.00						
	H_1	0.94**	1.00**	0.99**	1.00						
CHL *a*	H_0	0.98**	0.97**	0.98**	0.95**	1.00					
	H_1	0.94**	0.96**	0.92**	0.95**	1.00					
CHL *b*	H_0	0.99**	0.97**	0.96**	0.94**	0.97**	1.00				
	H_1	0.97**	0.98**	0.95**	0.98**	0.98**	1.00				
GFR	H_0	- 0.66*	- 0.58^{NS}	- 0.62*	- 0.51^{NS}	- 0.67*	- 0.73*	1.00			
	H_1	- 0.90**	- 0.80**	- 0.80**	0.81**	- 0.76**	- 0.82**	1.00			
GFD	H_0	0.97**	0.98**	0.98**	0.98**	0.96**	0.94**	- 0.50^{NS}	1.00		
	H_1	0.91**	0.96**	0.93**	0.96**	0.98**	0.98**	- 0.71*	1.00		
GPS	H_0	0.77**	0.80**	0.84**	0.79**	0.77**	0.74**	- 0.55^{NS}	0.80**	1.00	
	H_1	0.83**	0.81**	0.79**	0.79**	0.85**	0.83**	-0.60^{NS}	0.86**	1.00	
GY	H_0	0.82**	0.86**	0.89**	0.84**	0.86**	0.81**	- 0.54^{NS}	0.85**	0.80**	1.00
	H_1	0.97**	0.95**	0.94**	0.94**	0.95**	0.96**	- 0.82**	0.93**	0.90**	1.00

*= Significant ($p \leq 0.05$); **= Highly significant ($p \leq 0.01$); NS = Non-significant; CHL *a* = Chlorophyll *a* contents (mg g^{-1}); CHL *b* = Chlorophyll *b* contents (mg g^{-1}); TSP = Total soluble proteins (mg g^{-1}); SOD = Super oxide dismutase (U per mg protein); POD = Peroxidase (U per mg protein); CAT = Catalase (U per mg protein); GFR = Grain filling rate (g per day); GFD = Grain filling duration (days); GPS = Number of grains per spike; GY = Grain yield (t ha^{-1})

main plots, Aas-2011, Chakwal-50, Mairaj-2008 and Kohistan-97 recorded 29, 32, 32% decrement in grain yield, respectively. Maximum decline in GY due to adverse effects of heat stress was observed for cultivar AARI-2011 (48%), Galaxy-2013 and Punjab-2011 (46%) (Table 3).

Stronger correlation among all attributes under heat stress over control depicted heat stress adversities. Biochemical parameters showed a strong positive correlation with all attributes except GFR which was negatively correlated with all these parameters (Table 4).

DISCUSSION

Reduction in chlorophyll contents under the elevated temperature can be explained in the context of increased rate of chlorophyll degradation over its biosynthesis. High-temperature stress induced chlorophyll degradation might be an outcome of impaired biosynthesis of total soluble proteins and antioxidants. Similar performance of cultivars Chakwal-50, Aas-2011 and Mairaj-2008 might be related to staying green trait under heat. Higher chlorophyll *a* and *b* established staying green trait. Capability to maintain chlorophyll structure and function might improve heat tolerance of cultivars. Strong positive correlation of chlorophyll *a* and *b* contents with number of grains per spike, grain yield and other biochemical attributes further accomplished role of stay green in enhancing heat tolerance (Table 4). Declining protochlorophyllide reduced the rate of chlorophyll biosynthesis over degradation because of depressed TSP and antioxidant under heat (Hemantaranjan *et al*., 2014). Decline in chlorophyll contents under stress might be due to the accelerated production of reactive oxygen species (ROS) that increased thylakoid membrane leakiness of photosystem-II and disrupted chlorophyll structure (Raza *et al*., 2015). Prolonged high temperature suppressed chloroplast enzymatic activity and restricted rate of chlorophyll de novo synthesis (Wang *et al*., 2015).

Antioxidant defense system of varieties Aas-2011, Chakwal-50, and Mairaj-2008 manifested improvement against decline for other cultivars. Heat stress might have aggravated oxidative stress and thus antioxidants activities were improved in Aas-2011, Chakwal-50, and Mairaj-2008 as an adaptation to detoxify excessive reactive oxygen species (ROS). Contrarily, antioxidant activities were not strong enough to scavenge excessive ROS in all other cultivars. Consequently, increased biosynthesis of ROS overcame defensive mechanism and thus heat impacts were more pronounced. High temperature might increase production of superoxide ($O_2^{\bullet -}$) radical. Superoxide radical might act as the substrate for SOD. Moreover, strong association of SOD and POD (0.99) under high temperature environment confirmed accelerated synthesis of $O_2^{\bullet -}$ (Table 4). The increment in SOD activity in tolerant cultivars might be an adaptive response to high temperature. For susceptible cultivars, decrement in SOD activity under high temperature can be attributed to lower efficacy of scavenging mechanism of susceptible wheat

cultivars. Decline in SOD activity might be due to declined chlorophyll *a* and *b* contents. Thus, diminished chlorophyll *a* and *b* contents confirmed the diminished SOD activity. While, Aas-2011, Chakwal-50, and Mairaj-2008 still maintained higher chlorophyll contents than other cultivars.

Enhancement in POD and CAT activity under high temperature over control was recorded for genotypes Aas-2011, Chakwal-50 and Mairaj-2008. It can be attributed to enhanced SOD activities of genotypes Aas-2011, Chakwal-50 and Mairaj-2008. Enhanced generation of $O_2^{\bullet-}$ might have enhanced H_2O_2 level in leaves under high-temperature environment. Subsequently, detoxification of H_2O_2 to H_2O and O_2 in plants was mediated by POD and CAT. Greater H_2O_2 production in heat stress might have resulted in the increment of POD and CAT activity as the defensive mechanism against stress in tolerant cultivars. Strong positive and highly significant association (0.99) between POD and CAT under stressed conditions confirmed the escalated generation of $O_2^{\bullet-}$ and H_2O_2 (Table 4). The varied response of susceptible genotypes under high-temperature stress might be due to the dissimilar genetic capability for heat tolerance. Decline in SOD, POD and CAT activity in cultivars Punjab-2011, AARI-2011, Galaxy-2013, Millat-2011, Fareed-2006, Pakistan-2013, NIBGE-NIAB-1, and Kohistan-97 can be defined in the context of poor antioxidant defense system due to excessive generation of reactive oxygen species (ROS). Cultivars Galaxy-2013, Punjab-2011, and Millat-2011 depicted the highest decline in antioxidant activities under stressed environment over non-stressed environment. It can be interrelated to the inability of these cultivars to counteract production of superoxide ($O_2^{\bullet-}$), hydroxyl ($OH^{\bullet-}$), singlet oxygen ($^1O_2^*$) and hydrogen peroxide (H_2O_2) under high-temperature environment. Moreover, higher SOD activity for Aas-2011, Chakwal-50, and Mairaj-2008 and decreased SOD for other cultivars confirmed excessive generation of $O_2^{\bullet-}$. It can also be elucidated in terms of autocatalytic peroxidation of membrane lipid and degradation of chlorophyll together with other pigments resulting into significant cell damage. Declined CHL *a* and *b* contents under heat have confirmed enhanced lipid peroxidation. Furthermore, chlorophyll mediated boost in SOD activity under heat was further confirmed from strong association of CHL *a* (0.96) and *b* (0.97) with SOD activities (Table 4).

Naderi *et al*. (2014) observed that heat tolerant cultivars recorded enhancement while susceptible cultivars depicted decline in SOD activity. Tolerant spring wheat genotypes depicted increasing while susceptible recorded diminishing trends in antioxidant activities under heat over ambient conditions (Iqbal *et al*., 2015). These results are also analogous to Khaliq *et al*. (2015), they observed increase in POD and CAT activity in salt stress environment for tolerant cultivars of wheat. However, decline or no change in CAT activity was also recorded for stress tolerant cultivars (Wang *et al*., 2014).

Interactive effect of heat and varieties was significant for total soluble proteins (TSP). Cultivars Aas-2011 and Chakwal-50 manifested increment while other cultivars recorded decrement in TSP under heat stress over control. Augmented production of TSP for genotypes Aas-2011 and Chakwal-50 might be due to enhancement of antioxidant enzymes. Stronger correlation between TSP and antioxidants under heat and control further accomplished antioxidant mediated augmentation in TSP (Table 4). Augmentation in antioxidants might slow down the degradation of chlorophyll. Higher CHL *a* and *b* contents further confirmed role of antioxidants in chlorophyll activity. Higher TSP might be a consequence of chlorophyll mediated carbohydrate biosynthesis. Higher grain yield under heat stress for Aas-2011 and Chakwal-50 was observed. Therefore, contribution of TSP and chlorophyll for maintaining assimilate partitioning towards grains under heat stress was confirmed in form of higher grain yield. The decrease of TSP in cultivars Punjab-2011, AARI-2011, Galaxy-2013, Millat-2011, Fareed-2006, Mairaj-2008, Pakistan-2013, NIBGE-NIAB-1, and Kohistan-97 might be due to denaturation of proteins and inability of these cultivars to enhance HSPs under heat stress. Declined chlorophyll contents and antioxidant activities established escalation in protein denaturation. Our results are like those of Li *et al*. (2013), under high-temperature environment, glutenin biosynthesis declined, gliadin remained stable resulting in deteriorated grain proteins and quality. Wheat genotypes with higher antioxidants depicted heat tolerance at physiological maturity that consequently enhanced TSPs (Sharma *et al*., 2014).

Minimum GFR and maximum GFD in Aas-2011 and Chakwal-50 can be explained in the context of high chlorophyll contents and enhancement of antioxidant defense system under heat. Higher chlorophyll contents might maintain assimilate partitioning for the longer duration of time. Declined chlorophyll degradation might be a consequence of detoxification of ROS in cultivars AAS-2011 and Chakwal-50. Highest grain yields in AAS-2011 and Chakwal-50 further confirmed the contribution of chlorophyll contents in maintaining GFR and GFD. Strong positive correlation (0.93) between GFD and grain yield under heat established contribution of longer GFD in grain yield (Table 4). Furthermore, Enhanced antioxidants and TSPs was observed for AAS-2011 and Chakwal-50 under heat over control. Thus, improved defense might have maintained growth rate of grains and consequence in higher grain yield. Under heat stress, higher flux intensity and greater difference in maximum day temperature and minimum night temperature caused rapid morphogenesis. The increment in GFR can also be considered an adaptive behavior to complete growing cycle rapidly and produce seed for the upcoming generation. Each 5ºC rise of temperature above 20ºC resulted in increased GFR and reduced GFD by 12 days in wheat. Moreover, every 1ºC increase in temperature declined GFD

by 2.8 days, enhanced ROS, lipid peroxidation and decreased chlorophyll contents (Talukder *et al*., 2014).

Enhanced GFR and diminished GFD might decline grain yield in susceptible varieties. Thus, enhanced GFR could not compensate for diminished phenology. Rapid GFR and declined GFD might have adversely affected grain yield. Negative correlation of grain yield with GFR (- 0.82) under high temperature environment further accomplished the adverse effects of rapid grain filling (Table 4). Reduction in phenology might have reduced assimilates partitioning towards grains. Pollen grains in wheat were not able to produce heat shock proteins and thus highly sensitive to rise in temperature. Consequently, decreased grain setting negatively affected grain yield under high temperature environment (Hasanuzzaman *et al*., 2013; Shahid *et al*., 2015).

Degradation of chlorophyll under high-temperature environment might reduce yield by decreasing assimilate availability for grain filling. The decrement in antioxidant activity under heat stress might be related to enhanced oxidative stress causing more reduction in yield in cultivars AARI-2011, Galaxy-2013 and Punjab-2011 than tolerant genotypes Aas-2011, Chakwal-50 and Mairaj-2008. Less heat mediated reduction in yield for cultivars Aas-2011, Chakwal-50, and Mairaj-2008 might be due to their capability to maintain higher CHL *a* and *b* contents under heat than other cultivars. Moreover, higher yield of AAS-2011 and Chakwal-50 than other cultivars under heat over control can be attributed to number of grains per spike. Capability of AAS-2011 and Chakwal-50 to maintain higher number of grains might have enhanced grain yield. Moreover, strong positive correlation of number of grains per spike and grain yield under heat imposed conditions was confirmed from correlation (Table 4). Our results correspond to those of Innes *et al*. (2015), they recorded 15% reduction in GY per annum due to the higher temperature. Every 1ºC rise in temperature declined GY by 5.3%. The decrement in GY is also alike to findings of Tao *et al*. (2015); the three-decade experiment was conducted. Wheat varieties were exposed to the temperature of above 34ºC during booting to maturity. High temperature decreased GY and reduced growing period in all wheat genotypes.

***Conclusion*:** Strong dependence of morphological markers on biochemical activities intimated importance of biochemical responses in improving heat tolerance. On basis of biochemical, growth and yield attributes genotypes Aas-2011, Chakwal-50 and Mairaj-2008 depicted tolerance while Fareed-2006 and Punjab-2011 were medium tolerant to terminal heat. Whereas, cultivars Millat-2011, AARI-2011, Galaxy-2013, NIBGE-NIAB-1, NIBGE-NIAB-2 and Pakistan-2013 were susceptible to heat stress. Heat stress tolerance in tolerant genotypes was correlated with enhanced antioxidant enzymatic activities under high-temperature environment over control. Higher enzymatic activities promoted scavenging of ROS, maintained higher chlorophyll contents, slowed grain filling rate and maintained longer grain filling duration.

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