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Evaluation of Cimmyt's Biofortified Elite Wheat (Triticum Aestivum L.) Genotypes for Heat Stress at Different Reproductive Stages

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Title of Manuscript

EVALUATION OF CIMMYT'S BIOFORTIFIED ELITE WHEAT (*Triticum aestivum* L.) GENOTYPES FOR HEAT STRESS AT DIFFERENT REPRODUCTIVE STAGES

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Abstract

Wheat (*Triticum aestivum* L.) is a global cereal crop and a primary food source, but its yield and nutritional value of the grain is getting vulnerable to changing climate patterns especially heat stress during reproductive development. The present study was designed to screen forty nine elite wheat genotypes from CIMMYT's 13th High Zinc Wheat Trial (13th HZWYT) alongside a local check (Akbar-19) for agro-morphological traits, disease resistance, yield parameters and grain Zn and Fe concentrations under normal field conditions during 2022–23, followed by targeted heat stress evaluation at anthesis (T1) and grain filling (T2) stages using polythene tunnels during 2023–24. For all the traits evaluated, highly significant genotypic variation ($P \leq 0.001$) was observed during both the cropping seasons. Resistance to moderately resistant responses against yellow and stem rust were observed for the majority of CIMMYT genotypes, with Akbar-19 being highly susceptible. Ten superior genotypes along with check out of fifty were selected based on principal component analysis and Pearson correlation for heat stress study. Heat stress significantly reduced spike length, spikelets per spike, thousand grain weight, and grain yield per plant, with anthesis-stage stress causing greater yield reductions than grain filling stress. The Zn concentration in grain increased in a progressive manner during both stress treatments, whereas the Fe concentration decreased during the heat stress at grain filling stage indicating differential heat sensitivity of the micronutrient loading pathway. Regression analysis between control and stress environments revealed that grain Fe and Zn were the most genotypically stable traits, while TGW showed the greatest stress timing-dependent rank instability. Genotypes G-445, G-439, G-434, G-419, G-443 and G-405 were high yielding with high micronutrient concentrations and were consistently found to be heat stress resistant in both anthesis and grain-filling stages. These findings provide a reliable basis for selection of genotypes for further screening, genotyping, and multi-location testing in heat-resilient wheat biofortification breeding programs.

Keywords: wheat, zinc concentration, iron concentration, heat stress, anthesis stage

1 Introduction:

Wheat (*Triticum aestivum* L.) is one of the first domesticated cereals [32] and is a staple food of about 35% of the world's population [26]. The extensive genetic diversity of wheat enables it to survive across various weather patterns [8], making it the leading cereal crop globally [19, 26]. It provides 70–75% calories and 8–15% proteins in a routine diet [27]. Wheat maintains a central position when striving it comes to the United Nations Sustainable Development Goals (SDGs), primarily through efforts in reducing global food scarcity [44]. Unfortunately, global climate change (extreme weather, especially temperature fluctuation

and irregular rainfall) presents a critical challenge for sustainable wheat production. Abiotic stresses, especially heat stress is among the most prevalent and detrimental. This phenomenon is anticipated to intensify the frequency and severity, particularly in major wheat-producing areas, posing significant threats to global food security [40].

The worldwide area under wheat cultivation was about 219.52 million hectares and reached an average production of 3.64 tons per hectare, resulting in a total output of 798.48 million tons [11]. Wheat occupies the position of third most-produced cereal after maize and rice because of its elevated production levels [11]. The global human population is anticipated to reach 9.8 billion by 2050, requiring an additional 198 million tons of wheat to fulfill the dietary needs of this growing population sustainably [52]. Pakistan stands as the eighth largest wheat producer [45], with the production of 28.98 million tons annually across 9.1 million hectares during 2024-25. Wheat contributes 8.2 percent to the agricultural sector value while making up 1.9 percent of the GDP [17]. Climate change presents a substantial risk to global food [30] and nutritional security [14].

Earth has experienced a 1.1°C temperature increase compared to pre-industrial levels [23], due to elevated greenhouse gas concentrations. The temperature increase resulting from industrialization and deforestation presents substantial challenges to worldwide agriculture [16], particularly winter crops, because it impacts the grain size and grain weight at critical developmental stages [12]. As crops face high vulnerability to climate change, developing heat-resilient crop varieties stands as a top priority [14]. The International Maize and Wheat Improvement Center (CIMMYT) has made breeding efforts to develop stress-tolerant germplasm that shows high grain yield and disease resistance, benefiting all stakeholders in the wheat value chain [15].

Wheat crop is susceptible to heat stress during its reproductive and grain-filling stages, leading to significant yield reductions [42]. Grain yield exists as a polygenic inherited character that shows a substantial environmental impact, causing severe economic losses [48]. Different phenophases (plant's life cycle phases) have their own genetics to tolerate heat-stress effects depending upon the period and frequency of heat waves [40]. Globally, wheat yield generally decreases by 6% for each 1°C increase in ambient temperature [25]. Studies showed that temperatures above the optimum trigger a 2.5% to 10% yield decline in key growth stages, but the reproductive stage experiences the maximum reduction of 21% [47]. Heat stress shortens the days to anthesis, decreases grains per spike, damages pollen viability and tube development, and prevents proper ovary development, which causes impaired seed formation. Sustained heat stress (33-40°C for three days) during anthesis and grain filling drastically reduces grain size, number, and weight, often leading to develop malformed grains [3]. Even a single hot day can cause irreversible

harm. Heat stress also shortens key phases, including flowering and grain filling, with the latter shrinking of grain by 45–60% [40]. The crucial physiological processes, such as photosynthesis, are disrupted by heat stress [47] while the rooting system becomes dysfunctional, resulting in deficient water, Zn, and Fe absorption [14].

The diet provides the vital nutrients Zn and Fe, which serve an essential role in human metabolism [5]. However, calorie-rich staple grains such as wheat are often nutrient-deficient, contributing significantly to malnutrition affecting more than one-third of the global population (Over 3 billion people globally are Zn deficient) [28]. The occurrence of micronutrient deficiencies is growing vigorously among populations that rely on cereals as their sole source of nutrition [5]. Iron (Fe) deficiency is the most common nutritional cause of anemia (a significant health concern), affecting 40% of children and 37% of pregnant women [51]. Malnutrition is also a primary health concern for almost 50% of the population of Pakistan. Zn is essential for the regulation of gene expression, enzyme function, and cellular development in plants. At the same time, Fe is essential for the synthesis of chlorophyll in plants and the formation of hemoglobin in humans. Zn and Fe serve as a vital cofactor for numerous antioxidant enzymes [28].

It is crucial to develop heat-tolerant wheat varieties to reduce the negative influence of heat stress on wheat productivity [14, 20]. To develop or improve wheat genotypes, the first step is the evaluation of the cultivated germplasm's genetic diversity for heat tolerance and selection of heat-tolerant genotypes [40]. In this study, the morphological screening of CIMMYT-based wheat germplasm was conducted to identify climate-resilient genotypes with high yield potential. Biplot and correlation analysis categorized wheat genotypes and determined their relationship with yield-associated traits through mean performance under both normal and heat stress conditions. The genotypes that demonstrated superior tolerance to heat stress and had high micronutrient (Zn and Fe) concentrations were screened. This germplasm will later be used to improve local cultivars to develop region-specific, high-yielding wheat varieties to ensure an adequate food supply and availability of micronutrient-enriched wheat grains to the human population.

2 Materials and Methods:

2.1 Plant material and experimental site

The germplasm of 13th High Zinc Wheat Trial (13th HZWYT) was received from International Center for Wheat and Maize Improvement Center (CIMMYT) comprising 49 elite biofortified wheat genotypes.

The present study was carried out during two consecutive growing seasons 2022-23 and 2023-24 at the research farm of the Department of Plant Breeding and Genetics, University of Agriculture, Faisalabad which is geographically located at 30º N and 70º E. The soil analysis revealed that soil was slightly alkaline (pH 7.97), with low organic matter (0.603 %) and low Zn and Fe concentrations (0.54 and 1.19 mg/kg

130 respectively). This field trial conducted with fifty wheat genotypes, including a locally approved variety,
131 i.e., Akbar-19, as a control, and forty-nine 13th HZWYT genotypes sourced from CIMMYT, Mexico.

132 **2.2 Screening experiment (2022-23)**

133 The experiment was conducted following randomized complete block design (RCBD) with two
134 replications. During 2022-23, all the fifty genotypes were sown as per standard agronomic practices
135 including recommended fertilizer application (except Zn and Fe) and irrigation schedules. Plantation was
136 done by the hand drill, by maintaining the plant-to-plant and row-to-row distances at 15 cm and 30 cm,
137 respectively. At the maturity stage three competitive plants from each replication were selected randomly
138 to record data of plant height (cm), peduncle length (cm), spike length (cm), number of spikelets per plant
139 (SPS), number of tillers per plant (NoT), thousand grain weight (g), grain yield per plant (g), days to
140 heading, and days to anthesis. In wheat genotypes, the disease severity scoring ran two to three times in
141 each experiment using the modified Cobb's scale [38], with the host response to infection determined
142 according to [41].

143 **2.3 Heat stress experiment (2023-24)**

144 Out of fifty genotypes, on the base of recorded data, ten high yielding genotypes were selected and
145 further evaluated with check for heat stress in the year 2023-24. This experiment includes two stress
146 conditions i.e. heat stress at anthesis (T1), heat stress at grain filling stage (T2) and a control treatment (T0)
147 to compare. The plots underwent heat stress stimulation by enclosing them with polythene sheet tunnels.
148 The constructed polythene sheet tunnels raised the interior temperature, which simulated a heat-stress
149 environment. The anthesis and grain filling stages of wheat were exposed to heat treatment to evaluate the
150 effect of heat stress on grain yield and yield-related traits and grain Zn and Fe concentrations.

151 For the heat stress at anthesis stage (T1), the tunnels were erected when the plants started extrusion
152 of anthers from the spikelets. The 12-day stress period started from 8 March 2024, when the genotypes had
153 begun flowering, corresponding to the growth scale 61 on the BBCH growth scale [31]. For the heat stress
154 at grain filling stage (T2), the tunnels were set up when the plants started the grain filling phase. This 12-
155 day stress period started on 3 April 2024, when all the genotypes had begun grain filling, corresponding to
156 the growth scale 73 on the BBCH growth scale [31]. The polythene sheet tunnel was detached from both
157 T1 and T2 blocks following the termination of the heat stress treatment, thereby restoring the environmental
158 conditions to ambient zone. The control block was left uncovered to maintain optimum temperature
159 conditions throughout the experiment.

160 **2.4 Meteorological measurements**

161 The ambient air temperature in the polythene sheet tunnels and the non-stressed control was measured using
162 a digital temperature and humidity meter (Model: HTC-1, ElectRobes, Islamabad, Pakistan). The weather
163 data recorded reveals that the average minimum and maximum temperatures at the anthesis stage were

21°C and 26.5°C, respectively, and the average minimum and maximum temperatures at the grain filling stage were 25°C and 33.6°C, respectively (Figure 1.).

2.5 Data recording and Grain quality analysis

At the maturity stage three competitive plants from each replication were selected randomly to record data of plant height (cm), peduncle length (cm), spike length (cm), number of spikelets per plant (SPS), number of tillers per plant (NoT), thousand grain weight (g), grain yield per plant (g), days to heading, and days to anthesis.

All genotypes were harvested when the moisture content of grains reached 8-10%. The grain samples of each genotype were cleaned to eliminate contaminants and broken grains and were thereafter utilized for micronutrient analysis. The wet digestion method was employed to digest 0.5 g of each sample in a 3:1 ratio of Nitric Acid (HNO_3): Per chloric Acid ($HClO_4$). The concentrations of grain Zn and Fe were determined in mg/kg using an atomic absorption spectrophotometer (AAS). Zn and Fe contents per plant were determined by following formula

$$\text{Zn Contents (µg/Plant)} = \text{Zn Concentration (mg/Kg) x grain yield per plant (g)}$$

$$\text{Fe Contents (µg/Plant)} = \text{Fe Concentration (mg/Kg) x grain yield per plant (g)}$$

2.6 Statistical analysis

Data for morphological, yield related and micronutrient parameters were analyzed using analysis of variance (ANOVA). Microsoft Excel® 2016 was used to draw graphs. ANOVA and mean comparison were calculated by Statistix 8.1 while PCA analysis was conducted in XLSTAT. Correlation and scatterplot were computed using R-Studio 4.5.

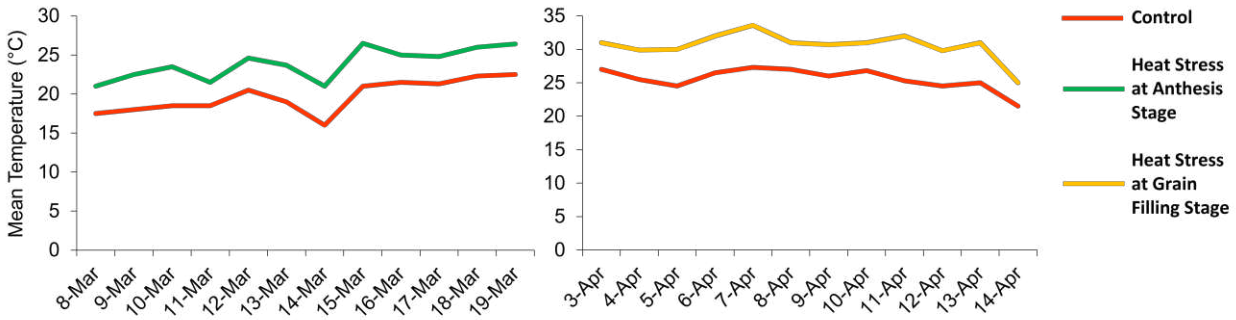


Figure 1. Mean temperature comparison for heat stress at anthesis and grain filling stage respective to control

3 Results:

3.1 Screening of germplasm (Cropping season 2022-23)

The genotypic variation for all the evaluated traits of 50 genotypes, such as PH, PL, SL, SPS, NoT, DH, DA, TGW, GYPP, and grain Zn and Fe concentrations (Table 1), Zn and Fe contents (Sup. Table 1) was highly significant ($P \leq 0.001$), and indicating that there was significant phenotypic variation among the germplasm evaluated. Non-significant replication mean squares verified uniformity and reliability of RCBD.

Disease evaluation revealed different resistance profiles in the two rusts. The resistance reactions to yellow rust were quite consistent, with 21 genotypes (42%) being resistant, 14 (28%) moderately resistant, 10 (20%) moderately susceptible and only 5 (10%) susceptible, while there was no MR or susceptible reaction to stem rust, indicating a significantly better resistance reaction with 42 genotypes (84%) being resistant (Figure 2a). Resistance to the two rusts was mostly independent, and there was little overlap in reaction categories (Figure 2b). Only 14 of 42 genotypes were resistant to both stem and yellow rust. All 5 yellow rust susceptible genotypes i.e. Akbar-19 (90S), G-424, G-441, G-444, and G-450 (60S) were fully resistant to stem rust. Genotypes G-403, G-405, G-406, G-416, G-419, G-425, G-426, G-434, G-439, G-443, G-445, G-446, G-447, and G-449 exhibited complete resistance to both rusts, identifying them as priority multi-rust resistant candidates.

PCA of all 11 traits across 50 genotypes explained 37.91% of total variance (F1 = 24.83%, F2 = 13.08%) (Figure 3). In PC1 ten traits showed positive correlation and one (DH) showed negative correlation. In PC2 five traits showed positive correlation including SPS, TGW and GYPP while six traits exhibited negative correlation. G-443 and G-426 were the elite yield performers (GYPP) and G-445, G-416, G-406 and G-439 grouped around Fe and Zn vectors, representing the elite grain micronutrient accumulators (SPS). Akbar-19, G-424 and G-428 were on the negative F1 side indicating low yield and micronutrient performance, compared to other elite genotypes.

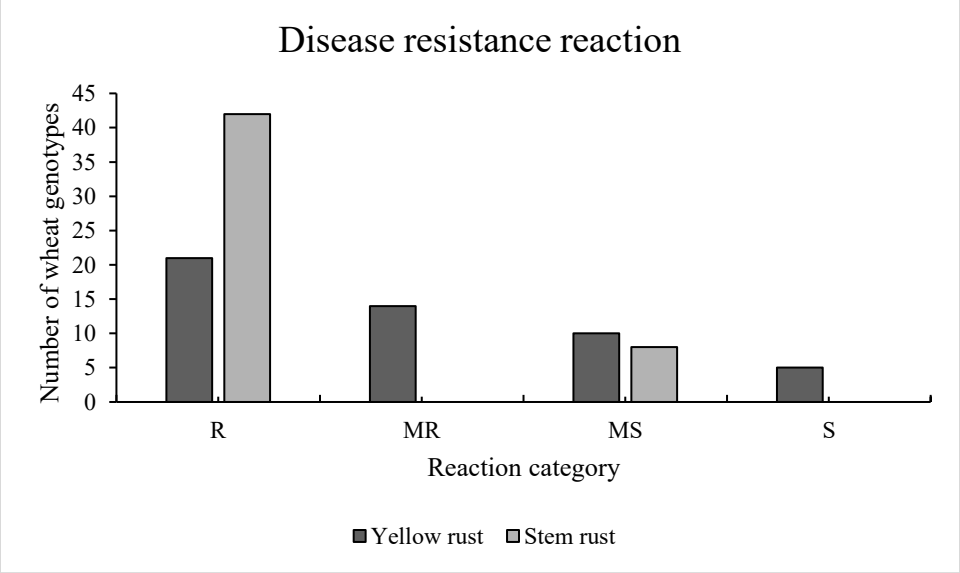
Pearson correlation analysis among all the 50 genotypes revealed a strong positive correlation between SPS and GYPP ($r = 0.77$, $P \leq 0.001$) and hence spikelet productivity was the main factor of influencing GYPP (Figure 4). TGW was positively correlated with SPS and GYPP, and DH and DA were weakly negatively correlated. Across the germplasm, a positive association was observed between grain Zn and Fe levels, while the other yield-related traits were only moderately associated with either of the micronutrients, indicating that the genetic regulation of micronutrient accumulation is largely independent of the regulation of yield. Ten superior multi-trait performers (G-443, G-426, G-405, G-434, G-416, G-445, G-419, G-425, G-406 and G-439) with high GYPP and elevated grain Zn and Fe concentrations coupled with broad rust resistance were selected for further evaluation under heat stress conditions based on combined proximity with respect to the yield and micronutrient trait vectors in the PCA biplot along with check for comparison (Table 2).

Table 1. Analysis of variance (RCBD) for all the traits for 13th HZWYT and local check wheat genotypes

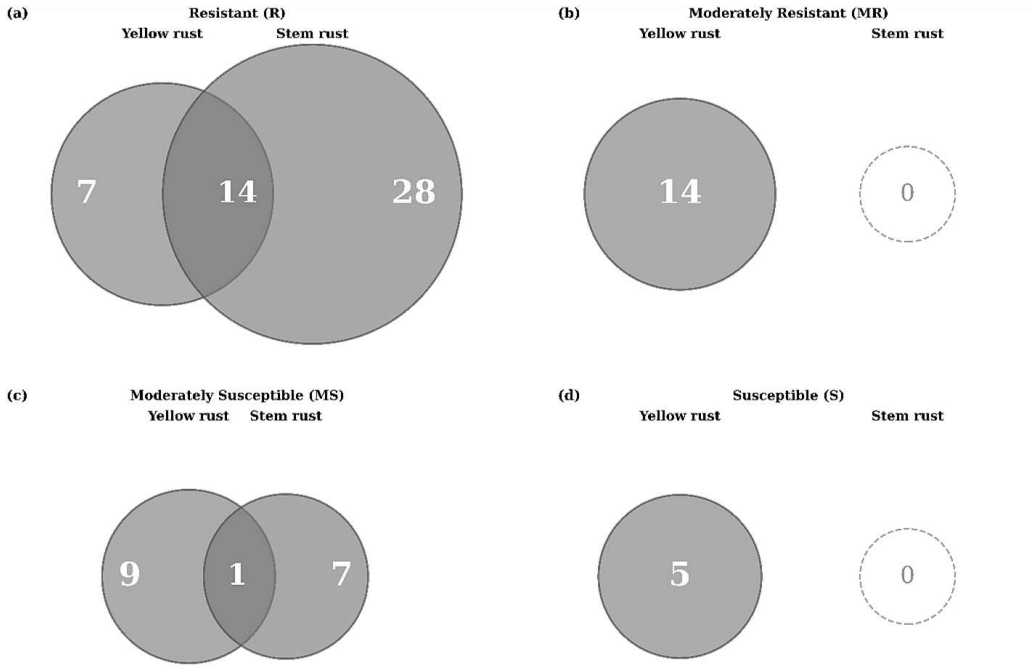
SOV	df	PH	PL	SL	SPS	NOT	DH	DA	TGW	GYPP	Zn CNC	Fe CNC
Rep	1	26.94	12.89	0.23	1.70	0.25	0.09	0.09	4.26	0.004	0.0008	0.007
G	49	193.14***	30.90***	2.59***	7.70***	0.47***	12.14***	21.13***	25.35***	11.13***	0.19***	8.11***
Error	49	51.21	3.88	0.15	0.69	0.09	0.11	0.11	1.61	0.11	0.002	1.19

PH = plant height (cm), PL = peduncle length (cm), SL = spike length (cm), SPS = spikelets per spike, NoT= Number of Tillers, DH = days to heading, DA= Days to Anthesis, TGW=Thousand Grain weight (g), GYPP = grain yield per plant (g), Zn CNC = grain zinc concentration (mg kg^{-1}), Fe CNC= grain iron concentration (mg kg^{-1})

*, ** and *** indicate significance at $P \leq 0.05$, $P \leq 0.01$ and $P \leq 0.001$, respectively.



(a)



(b)

Figure 2. Disease reaction of 50 wheat genotypes (13th HZWYT + Akbar-19) to yellow rust and stem rust under field conditions. (a) The number of genotypes in each category of reactions (R; moderately resistant (MR); moderately susceptible (MS); susceptible (S)) for yellow rust and stem rust. (b) Venn diagrams showing the overlap in reaction category between yellow rust and stem rust for each category; numbers in non-overlapping regions indicate genotypes with that reaction to one rust only, and numbers in overlapping regions indicate genotypes sharing the same reaction to both rusts.

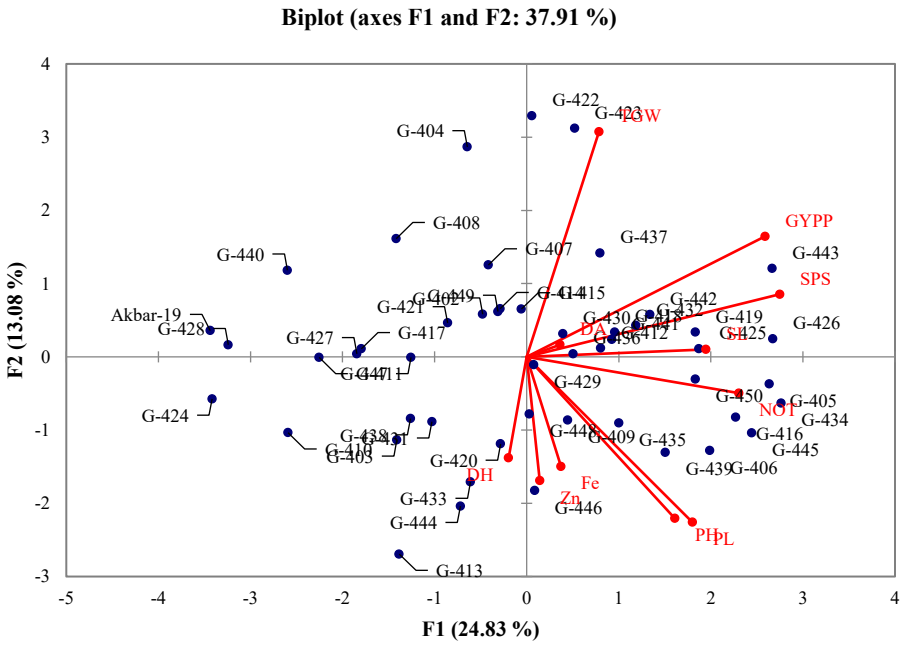


Figure 3. Biplot of all the studied traits of 50 wheat genotypes. PH = plant height (cm), PL = peduncle length (cm), SL = spike length (cm), SPS = spikelets per spike, NoT= Number of Tillers, DH = days to heading, DA= Days to Anthesis, TGW=Thousand Grain weight (g), GYPP = grain yield per plant (g), Zn = grain zinc concentration (mg kg^{-1}), Fe = grain iron concentration (mg kg^{-1})

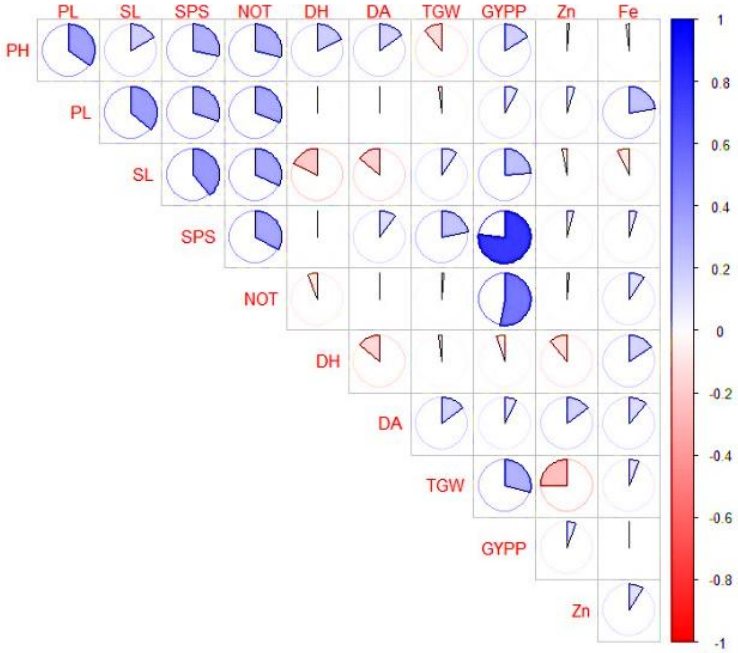


Figure 4. Correlation analysis for all the studied traits. PH = plant height (cm), PL = peduncle length (cm), SL = spike length (cm), SPS = spikelets per spike, NoT= Number of Tillers, DH = days to heading, DA= Days to Anthesis, TGW=Thousand Grain weight (g), GYPP = grain yield per plant (g), Zn = grain zinc concentration (mg kg^{-1}), Fe = grain iron concentration (mg kg^{-1})

Table 2. List of germplasm selected for heat stress experiment

Entry Name	Pedigree
G-401 Local check (Akbar-19)	CMSS07B00230S-099M-099NJ-099NJ-23WGY-0B
G-405	CMSS17Y01162T-099TOPM-099Y-099M-6Y-0WGY
G-406	CMSS17Y01164T-099TOPM-099Y-099M-8Y-0WGY
G-416	CMSS17Y01201T-099TOPM-099Y-099M-9Y-0WGY
G-419	CMSS17Y01219T-099TOPM-099Y-099M-20Y-0WGY
G-425	CMSS17Y01255T-099TOPM-099Y-099M-2Y-0WGY
G-426	CMSS17Y01271T-099TOPM-099Y-099M-6Y-0WGY
G-434	CMSS17Y01335T-099TOPM-099Y-099M-12Y-0WGY
G-439	CMSS17Y01372T-099TOPM-099Y-099M-15Y-0WGY
G-443	CMSS17Y01384T-099TOPM-099Y-099M-17Y-0WGY
G-445	CMSS17Y01395T-099TOPM-099Y-099M-12Y-0WGY

3.2 Germplasm evaluation for heat stress at anthesis (T1) and grain filling (T2) stages

A total of 10 wheat genotypes along with check, derived from CIMMYT, were selected from the 49 genotypes of 13th HZWYT that were screened in nursery for further advancement to heat stress evaluation, as they were resistant to yellow and leaf rusts and had high grain yield potential with high grain Fe and Zn concentrations. The genotypic variation for all evaluated traits was highly significant ($P \leq 0.001$) under both control and both heat stress treatments (Table 3). The heat stress had a significant effect on all yield components ($P \leq 0.001$), with anthesis stage stress (T1) producing larger reductions in GYPP and SPS than grain filling stage stress (T2). T2 also imposed a greater reduction in TGW and DA, indicating stage-specific sensitivity of yield components to heat stress. There were no significant treatment effects for PH, PL, and NoT, which further demonstrated their stress resistance when exposed to heat stress at the reproductive stage. The genotype x treatment interaction is significant for DH ($P \leq 0.05$) and grain Zn concentration ($P \leq 0.01$) (Table 3).

The means of the GYPP (across the genotypes) showed that it was decreased by 13.4% and 7.9% under (T1) and (T2) respectively as compared to control (T0) (Table 4a). The concentration effect of heat stress was observed as the grain Zn concentration increased gradually from T0 to T1 and T2 for all genotypes (overall means: 26.82, 32.03, and 34.24 mg kg^{-1}; $P \leq 0.001$. In contrast, under both stress treatments, grain Fe concentration decreased, with T2 showing the largest decrease (overall means: 32.11, 31.48, and 30.49 mg kg^{-1}; $P \leq 0.001$) (Tables 4b, 4c). G-445 recorded the highest Zn (43.26, 54.89, and 56.32 mg kg^{-1}) and Fe (51.26, 49.51, and 44.95 mg kg^{-1}) concentrations across all treatments, followed by G-439 for both traits, while G-416 and G-425 consistently recorded the lowest values. G-445 and G-439 also showed best performance in terms of Zn and Fe content per plant under both stress timings, indicating high micronutrient stability (Sup. Table 2a, 2b).

The cumulative variance explained by PCA showed a progressive decrease from control (74.07%) to T2 (69.83%) to T1 (64.74%) (Figure 5). Under control, GYPP, TGW, SL, and NoT dominated PC1 while Fe and Zn loaded on PC2. Under T1, genotype discrimination was highest for GYPP, SPS, and Zn, with G-419, G-434, G-439, and G-445 positioned closest to yield and micronutrient vectors. Under T2, G-445 and G-439 clustered near TGW, Fe, and Zn vectors, while G-419, G-434, G-443, and G-405 grouped near GYPP and SPS vectors, reflecting the shift in dominant trait axes between the two stress stages. Akbar-19, G-406, G-416, G-426 and G-432 consistently had negative F1 values across all three treatments and were poor performers in both normal and heat-stressed conditions.

The correlation analysis showed that the associations were higher and positive overall during the grain filling heat stress than during the anthesis heat stress. Positive strong correlations were seen between TGW with GYPP and SPS with GYPP and DH with GYPP and DA with GYPP under the control. Zn and Fe were positively correlated under the control. With regard to overall trait correlations, T1 resulted in reduced positive correlation between GYPP and SPS, with TGW–GYPP also becoming less positive. In T2, there were strong positive GYPP–DH and GYPP–DA and Zn-Fe correlation along with moderate GYPP–SPS and GYPP–NoT associations were observed (Figure 6).

Scatter plot analysis established a consistent hierarchy of genotypic rank stability between T0 and stress treatment: Fe ($R^2 = 0.99$, T1; $R^2 = 0.97$, T2) ≥ Zn ($R^2 = 0.98$, T1; $R^2 = 0.99$, T2) > TGW under T1 ($R^2 = 0.93$) > SPS ($R^2 = 0.65$, T1; $R^2 = 0.73$, T2) > GYPP ($R^2 = 0.67$, T1; $R^2 = 0.51$, T2) > TGW under T2 ($R^2 = 0.24$) > NoT ($R^2 = 0.17$, T1; $R^2 = 0.12$, T2) (Figure 7). Grain Fe and Zn were most genotypically stable across both stress timings and had regression trajectories parallel or higher than the 1:1 isoline. TGW had the lowest rank predictability with T2 (R2 = 0.24) compared to T1 (R2 = 0.93). Under grain filling heat stress, regression lines for TGW and GYPP continued to drop further below the 1:1 isoline from T1 to T2, and NoT consistently showed the lowest and most variable genotypic responses under both treatments of heat stress.

Table 3. ANOVA for all the traits under heat stress

SOV	DF	PH	PL	SL	SPS	NoT	TGW	GYPP	DH	DA	Zn CNC	Fe CNC
Rep	1	116.54	11.46	1.46	0.06	3.41	1.94	29.87	2.18	1.52	0.61	0.23
G	10	147.59***	28.36**	2.57**	10.14**	3.47**	21.26***	54.07**	36.45**	34.91**	663.73**	627.95**
T	2	14.90^{NS}	0.79^{NS}	3.60**	31.58**	0.65^{NS}	182.92**	40.48**	94.61**	10.92**	319.85**	14.66**
G*T	20	8.61^{NS}	3.66^{NS}	0.03^{NS}	0.50^{NS}	0.80^{NS}	3.93^{NS}	4.73^{NS}	1.84*	0.61^{NS}	5.47**	2.70^{NS}
Error	32	11.37	2.12	0.04	0.38	0.57	2.09	3.77	0.74	0.48	1.69	1.65

PH = plant height (cm), PL = peduncle length (cm), SL = spike length (cm), SPS = spikelets per spike, NoT= Number of Tillers, DH = days to heading, DA= Days to Anthesis, TGW=Thousand Grain weight (g), GYPP = grain yield per plant (g), Zn CNC = grain zinc concentration (mg kg^{-1}), Fe CNC= grain iron concentration (mg kg^{-1}),

*, ** and *** indicate significance at $P \leq 0.05$, $P \leq 0.01$ and $P \leq 0.001$, respectively.

Table 4a. Mean values of grain yield per plant under control (T0), heat stress at anthesis (T1) and Heat stress at grain filling stage (T2)

Genotypes	Grain Yield per Plant (g)		
	T0	**T1**	**T2**
Akbar-19	17.24 ± 2.13 a-f	13.61 ± 1.92 ef	16.29 ± 2.01 a-f
G-405	21.67 ± 1.61 a-d	18.32 ± 0.08 a-f	16.80 ± 4.90 a-f
G-406	18.92 ± 1.11 a-f	14.18 ± 1.28 d-f	17.97 ± 0.53 a-f
G-416	14.16 ± 1.20 d-f	12.53 ± 1.03 f	12.72 ± 1.35 f
G-419	23.74 ± 0.37 a	21.14 ± 3.45 a-e	22.00 ± 2.82 a-d
G-425	20.09 ± 1.95 a-f	16.28 ± 1.35 a-f	14.63 ± 3.01 c-f
G-426	18.52 ± 0.13 a-f	19.75 ± 2.24 a-f	19.90 ± 1.26 a-f
G-434	24.00 ± 0.00 a	21.28 ± 2.67 a-e	22.76 ± 1.76 ab
G-439	22.46 ± 2.18 a-c	21.98 ± 0.06 a-d	21.23 ± 3.91 a-e
G-443	17.49 ± 3.59 a-f	15.49 ± 3.50 b-f	20.20 ± 2.33 a-f
G-445	24.00 ± 0.00 a	18.07 ± 1.22 a-f	20.18 ± 0.69 a-f
Total Mean	**20.21 A**	**17.51 B**	**18.61 B**

p- Value	P_{G}*** P_{T}*** $P_{G \times T}$**

310

311 **Table 4b. Mean values of grain Zn concentrations under control (T0), heat stress at anthesis (T1) and**

312 **Heat stress at grain filling stage (T2)**

Genotypes	**Zn Concentration (mg/Kg)**		
	T0	**T1**	**T2**
Akbar-19	23.52 ± 1.27 m-p	27.13 ± 0.84 j-m	30.31 ± 0.87 i-k
G-405	29.20 ± 2.11 i-l	33.20 ± 1.43 f-i	35.88 ± 0.27 e-h
G-406	19.73 ± 1.83 p-r	21.58 ± 0.63 n-q	24.06 ± 1.49 l-p
G-416	14.77 ± 1.02 r	17.74 ± 0.77 qr	19.96 ± 1.15 p-r
G-419	26.85 ± 0.89 j-o	30.92 ± 0.18 h-j	33.30 ± 0.33 f-i
G-425	16.77 ± 0.50 qr	20.43 ± 0.20 pq	21.49 ± 0.29 o-q
G-426	19.84 ± 0.49 p-r	25.01 ± 1.66 k-p	26.97 ± 1.29 j-n
G-434	31.81 ± 2.43 g-j	37.07 ± 0.30 d-g	39.17 ± 0.81 c-e
G-439	36.33 ± 0.90 e-g	46.12 ± 2.39 b	46.99 ± 2.92 b
G-443	32.90 ± 1.60 f-h	38.22 ± 0.98 c-f	42.23 ± 0.20 b-d
G-445	43.26 ± 1.54 bc	54.89 ± 1.44 a	56.32 ± 0.65 a
Total Mean	**26.82 C**	**32.03 B**	**34.24 A**
p- Value	P_{G}*** P_{T}*** $P_{G \times T}$**		

313

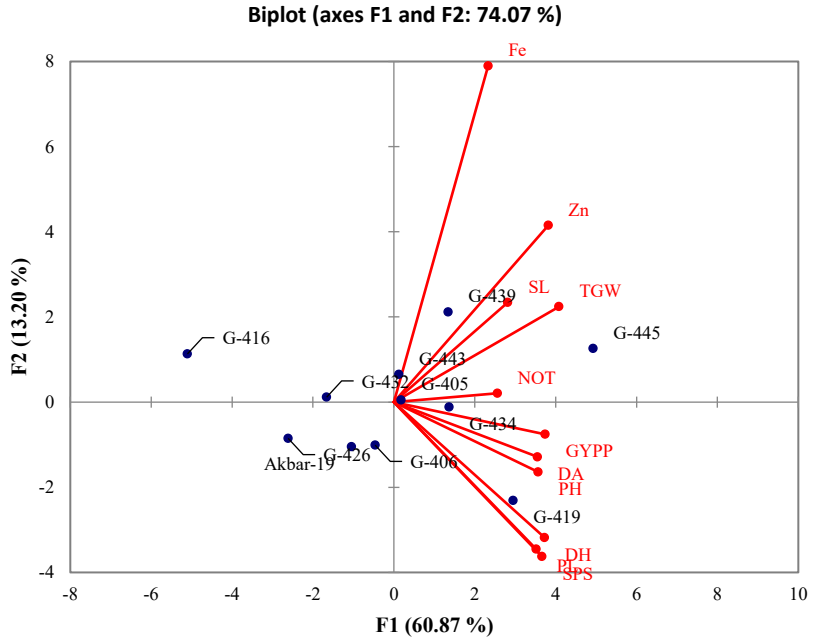
314 **Table 4c. Mean values of grain Fe concentrations under control (T0), heat stress at anthesis (T1) and**

315 **Heat stress at grain filling stage (T2)**

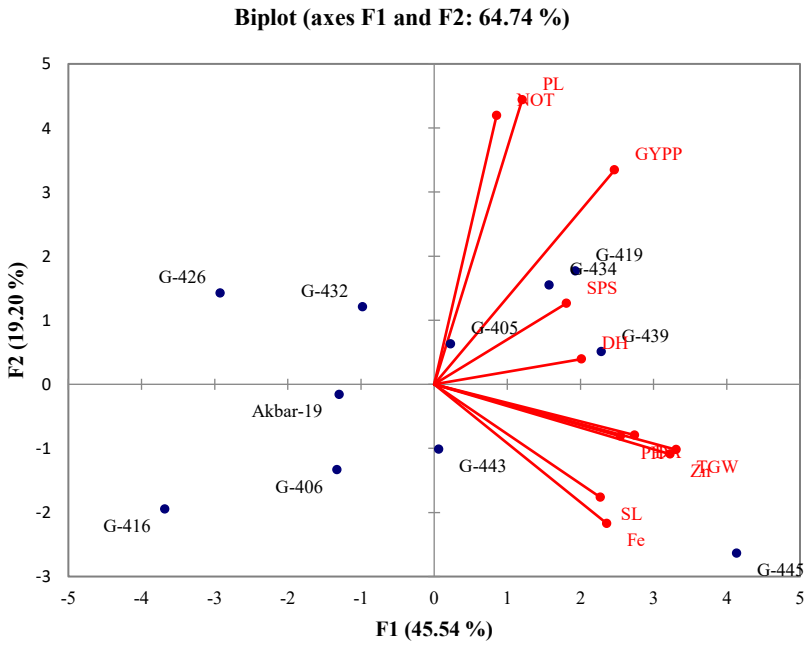
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Genotypes	**Fe Concentration (mg/Kg)**		
	T0	**T1**	**T2**
Akbar-19	25.49 ± 1.79 g-j	25.71 ± 0.58 g-i	25.06 ± 0.27 h-j
G-405	34.64 ± 1.50 c-e	35.63 ± 1.23 cd	35.22 ± 1.77 c-e
G-406	22.11 ± 0.97 i-k	22.36 ± 1.57 i-k	21.56 ± 0.15 i-k
G-416	28.71 ± 0.95 f-h	28.12 ± 0.03 f-h	26.52 ± 1.30 f-i

G-419	20.36 ± 0.31 jk	20.29 ± 0.01 jk	19.29 ± 0.65 k
G-425	18.84 ± 0.36 k	18.28 ± 1.17 k	18.30 ± 1.06 k
G-426	29.92 ± 0.37 e-h	30.75 ± 1.13 d-g	28.88 ± 0.74 f-h
G-434	35.35 ± 0A.68 cd	31.64 ± 0.21 d-f	31.52 ± 1.91 d-f
G-439	48.04 ± 1.25 ab	46.81 ± 1.97 ab	48.24 ± 1.35 ab
G-443	38.48 ± 0.41 c	37.15 ± 0.10 c	35.85 ± 1.73 cd
G-445	51.26 ± 0.83 a	49.51 ± 0.30 ab	44.95 ± 2.35 b
Total Mean	**32.11 A**	**31.48 A**	**30.49 B**
p- Value	P_{G} *** P_{T} *** $P_{G \times T}$ NS		

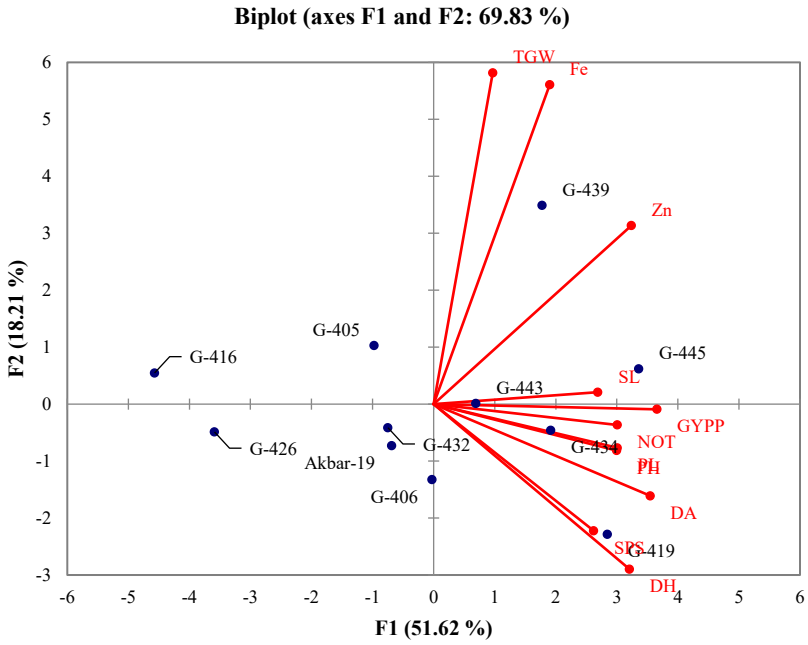


(a)



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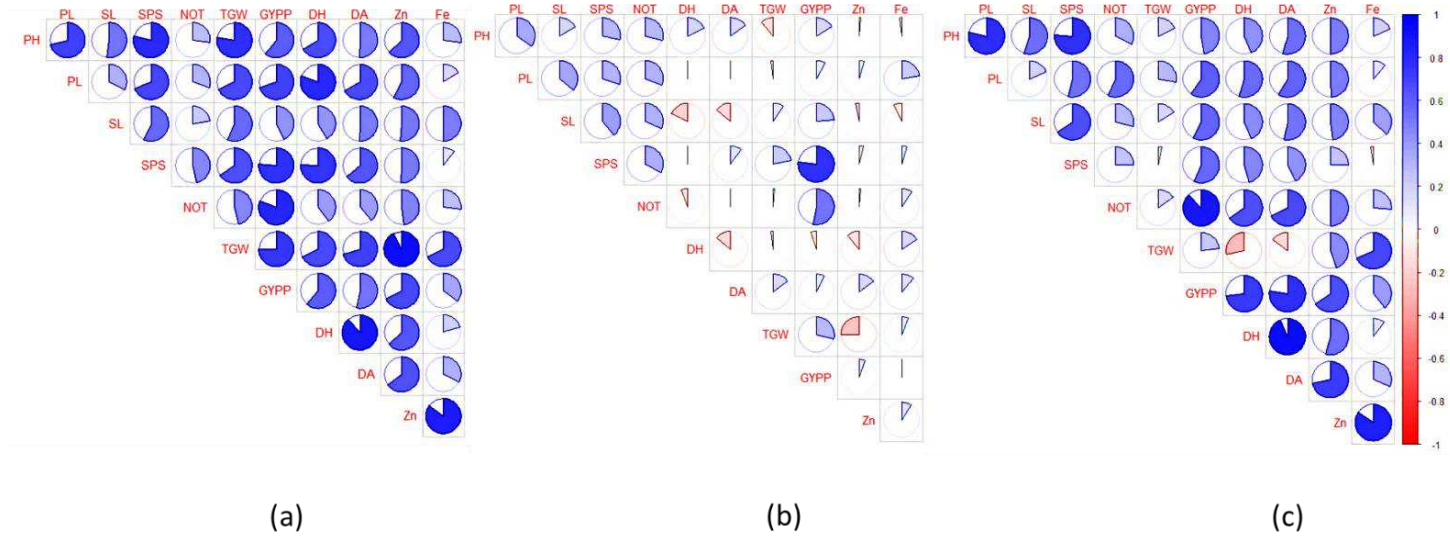
320 (b)



321

322 (c)

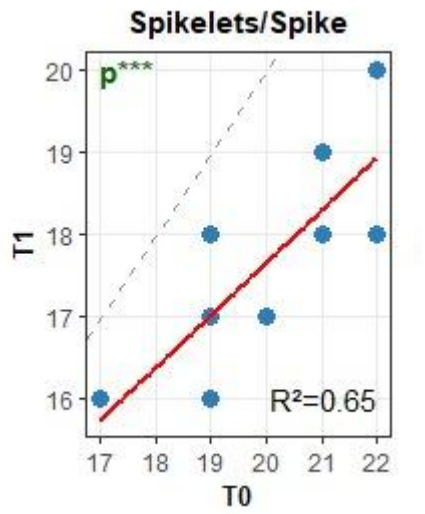
323 **Figure 5. Biplot of all the studied traits of 11 wheat genotypes under (a) Control (T0), (b) Heat stress**
324 **at anthesis (T1) and (c) Heat stress at grain filling stage (T2)**

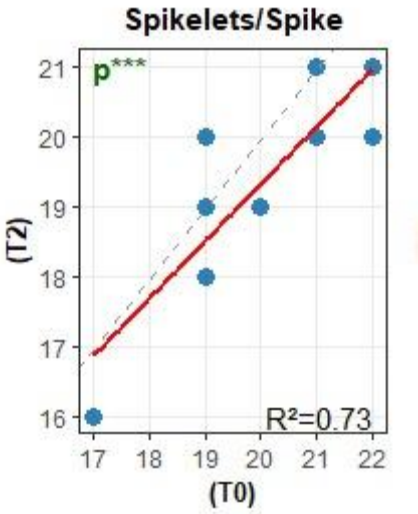


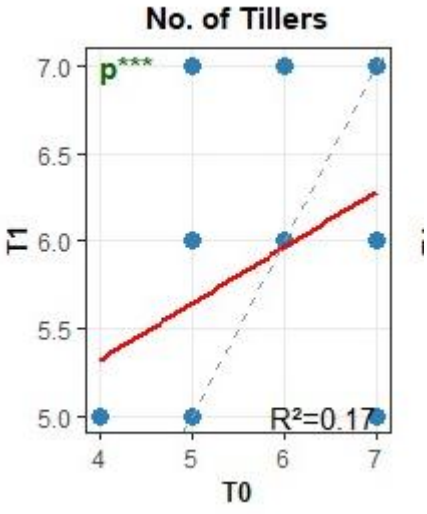
(a) (b) (c)

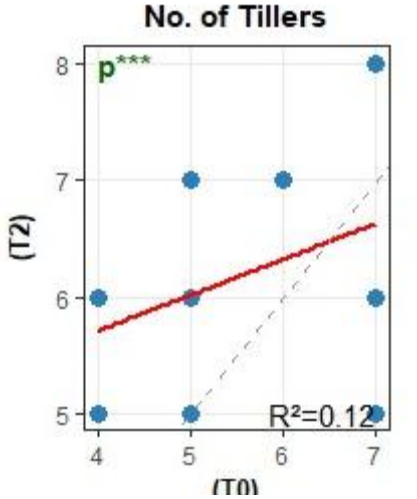
Figure 6. Correlation analysis of all the studied traits under (a) Control (T0), (b) Heat stress at anthesis (T1) and (c) Heat stress at grain filling stage (T2)

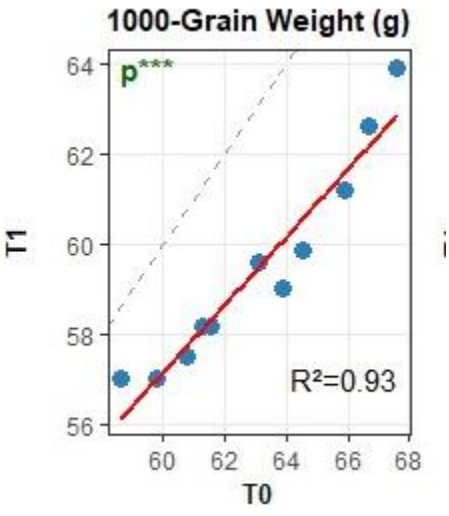
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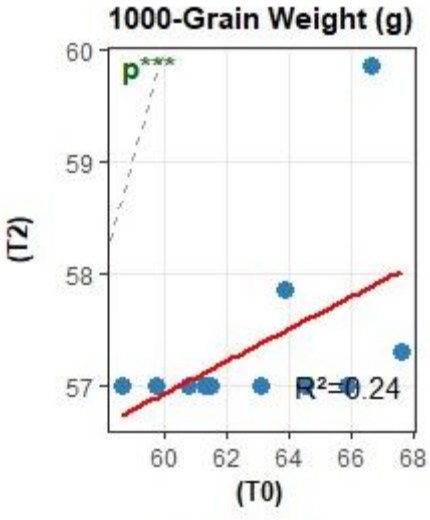

Spikelets/Spike
p***
R²=0.65
T1
T0

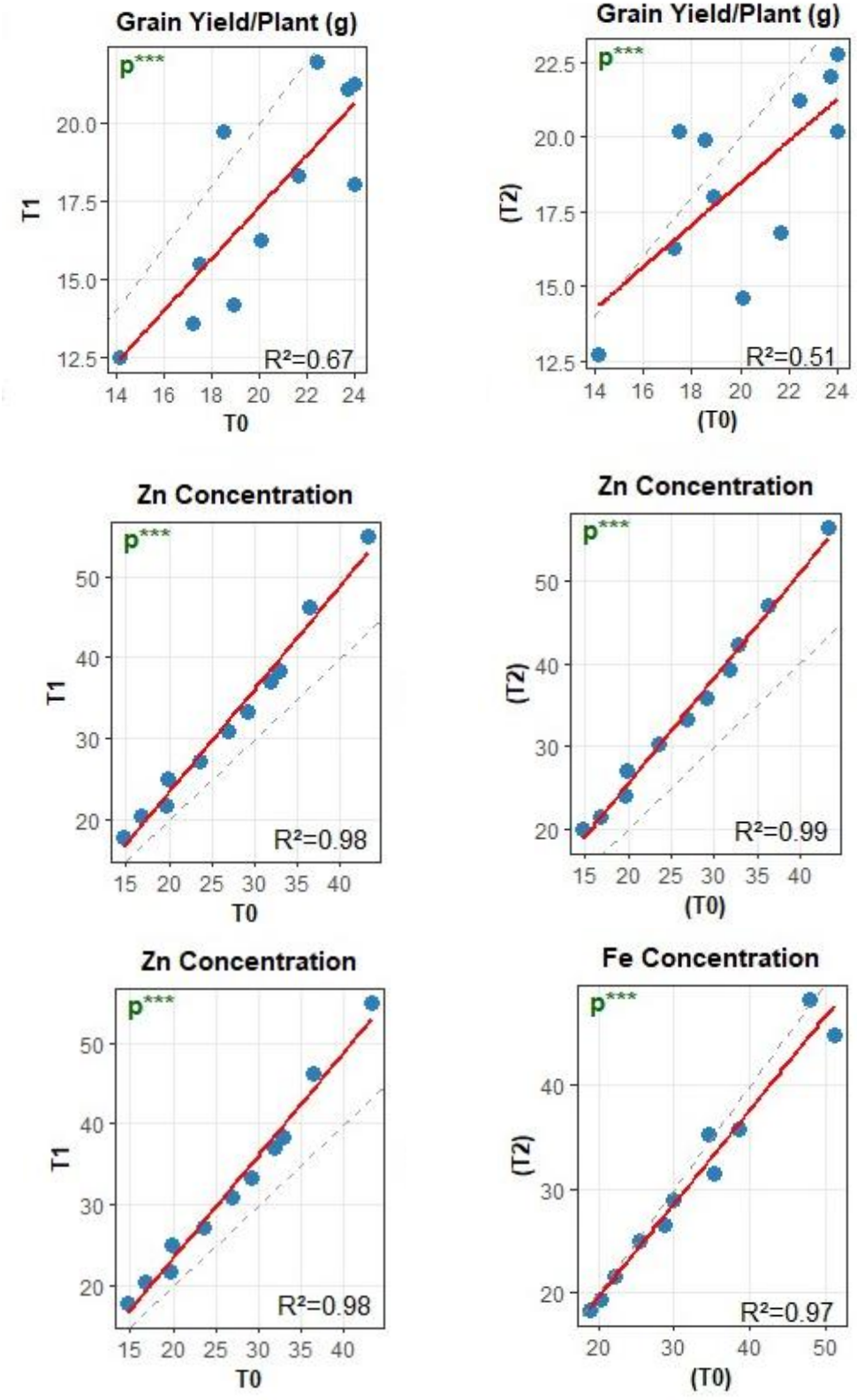

Spikelets/Spike
p***
R²=0.73
(T2)
(T0)


No. of Tillers
p***
R²=0.17
T1
T0


No. of Tillers
p***
R²=0.12
(T2)


1000-Grain Weight (g)
p***
R²=0.93
T1
T0


1000-Grain Weight (g)
p***
R²=0.24
(T2)
(T0)


Grain Yield/Plant (g)
p***
R²=0.67
T1
T0
Grain Yield/Plant (g)
p***
R²=0.51
(T2)
(T0)
Zn Concentration
p***
R²=0.98
T1
T0
Zn Concentration
p***
R²=0.99
(T2)
(T0)
Zn Concentration
p***
R²=0.98
T1
T0
Fe Concentration
p***
R²=0.97
(T2)
(T0)

330

Figure 7. Scatterplot of genotypic mean performance under (a) control (T0) versus heat stress at anthesis (T1) and (b) control (T0) versus heat stress at grain filling stage

4 Discussion

Highly significant genotypic variation ($P \leq 0.001$) for all traits evaluated confirmed high genetic diversity among the 13th HZWYT germplasm, similar to the reports on international wheat nurseries evaluated under normal field conditions [43, 50]. The high percentage of resistant to moderately resistant responses to yellow rust and near-complete resistance to stem rust is consistent with [2, 46] who attributed this broad-spectrum resistance to the intensive wheat rust resistance breeding which has been incorporated into CIMMYT wheat improvement programs. The susceptibility of Akbar-19 to yellow rust is also reported by [34], who also observed local varieties to be vulnerable to the prevalent rust races, further substantiating the breeding value of CIMMYT biofortified germplasm for rust prone environments.

Morphological traits PH and NoT did not change significantly under heat stress in either of the reproductive stages as reported by [24] and [22] which stated that the same are mechanically predetermined during vegetative growth and not affected by short duration heat stress at reproductive stages. Under both stress conditions, the significant decrease in SL, SPS, DA, TGW and GYPP was observed, which was similar to the findings of [13, 40] who reported significant reduction in these yield components under reproductive stage heat stress. The higher susceptibility of GYPP and SPS to anthesis heat stress (T1) compared with T2 is consistent with [15, 33] who reported that grain yield is more sensitive to heat stress at anthesis stage, which is due to heat sensitivity of the male gametophyte, as temperatures above 30°C have adverse effects on the function of tapetal cells, dehiscence of the anthers, and synthesis of heat shock proteins, reducing pollen viability, efficiency in fertilization and grain set. This biological explanation is further validated by the increase in trait co-regulatory network disruption in T1 (PCA variance = 64.74%) as compared to T2 (69.83%) and control (74.07%) and is consistent with the findings of [4] who observed similar reduction in cumulative PCA variance under anthesis heat stress in wheat germplasm evaluation studies. Grain filling heat stress (T2) induced more significant reduction in TGW, in line with [15, 53] reported reduction in TGW due to direct inhibition of ADP-glucose pyrophosphorylase and soluble starch synthase, both being very heat sensitive above 35 °C, and accelerated leaf senescence through accumulation of reactive oxygen species and abscisic acid, which leads to premature cessation of grain filling. The near-complete loss of genotypic TGW rank predictability under T2 ($R^2 = 0.24$ vs $R^2 = 0.93$ under T1) is consistent with [9, 10], who identified TGW as the most heat sensitive integrative yield trait, capturing cumulative effects of heat stress on source capacity, phloem transport efficiency, and sink strength. In contrast, there were contradictory patterns of changes in concentrations of Zn and Fe in response to heat stress, indicating mechanistically different accumulation pathways, as observed in previous biofortification research. The

concentration effect, as the amount of endosperm starch decreased, the amount of Zn in the aleurone layer and embryo did not change, resulting in an increase in the amount of Zn per unit grain weight, was observed and has been reported in heat-stressed wheat genotypes by [36, 54]. This concentration effect was complemented by upregulation of ZIP and HMA Zn transporter genes under heat stress, which would facilitate remobilization of Zn from vegetative tissues through the phloem as reported by [7, 50]. In contrast, under heat stress, especially under T2, there was a decrease in grain Fe concentration, which is related to reduced Fe loading via the synthesis of phytosiderophores and impaired activity of the YSL protein-mediated phloem transport, in agreement with [5] and [29], who found that Fe loading was reduced when plants were subjected to high temperatures during the grain filling stage in association with decreased synthesis of phytosiderophores and reduced activity of the YSL protein-mediated phloem transport. The interaction between genotype and treatment was significant for Zn ($P \leq 0.01$), which was expected due to the differential genotypic variation in their Zn homeostasis mechanisms under heat stress, as reported by [54] who linked the interaction with the genotypic variation in expression of Zn transporters and vacuolar sequestration capacity.

The correlation analysis showed higher correlation of traits during grain filling heat stress than during anthesis stress, findings similar to those of [18, 40] who observed higher disruption of the co-regulatory yield networks under anthesis stress. The favorable positive associations of GYPP–DH and GYPP–DA under T2 are similar to the results of [18] who found such positive associations, as a result of the advantage of later maturing genotypic configurations to avoid peak grain filling temperatures; whereas the unfavorable GYPP–TGW association as observed under T2 is consistent with [15] which found negative associations as a consequence of the compensation mechanism where genotypes with reduced grain filling ability would direct resources to grain number rather than individual grain mass. The negative correlation between GYPP and Zn under anthesis heat stress was similar to that observed by [49], who concluded that there is a dilution-concentration trade-off such that genotypes with higher grain number under stress have lower concentration of Zn per unit grain weight. The positive correlation between Fe and Zn in all treatments was maintained, which is consistent with the notion that there are common regulatory components of phloem-loading pathways, as suggested by [7, 50] and conducive to integrated selection for both micronutrients. Fe and Zn were the most stable micronutrients for stable biofortification in heat stressed treatments as their genotypic ranks remained stable across both stress timings with regression lines either parallel or higher than the 1:1 isoline ($R^2 \geq 0.97$ and 0.98, respectively), similar with the multi-environment stability of micronutrient accumulation reported by [35] and [49]. The low and variable rank stability of NoT ($R^2 = 0.17$ and 0.12) was consistent with earlier reports by [1, 39] who reported that spikelet number was the key mechanistic determinant of grain sink size and yield potential, and high tiller number plasticity under stress attributed

to a complex strigolactone, cytokinin and auxin signaling network that was sensitive to unpredictable thermal perturbations by [21].

In PCA biplots, G-445, G-439, G-434, G-419, G-443, and G-405 consistently displayed favorable positions near the yield and micronutrient trait vectors, representing stability in their performance under heat stress irrespective of the timing of heat stress, as recommended by [15, 37] for the identification of heat stress tolerant wheat germplasm. The G-445 and G-439 vectors clustered around Fe, Zn and TGW in all treatments, suggesting that heat stress did not appear to compromise the heat tolerance of their starch biosynthetic machinery or micronutrient loading systems, in agreement with [35, 54], who highlighted the importance of maintaining the capacity to accumulate micronutrients under projected climate warming scenarios. However, G-416, G-426, G-406 and Akbar-19 maintained negative position in F1, which is similar to the findings of [34] that these are heat susceptible, cannot be advanced. The G-445, G-439, G-434, G-419, G-443 and G-405 are priority candidates for the breeding program for biofortification for heat tolerance and micronutrient density in heat-prone and micronutrient-deficient agro-ecological zones because these cultivars have a direct opportunity to be integrated into national wheat improvement programs [6, 35]

5 Conclusion

In this study, genotypes screened from the CIMMYT 13th High Zinc Wheat Trial were subjected to a two-stage screening process under both normal and heat-stress conditions to identify biofortified wheat lines with both high yield and high grain Zn and Fe concentration in combination with heat tolerance. Significant genotypic variation ($P \leq 0.001$) was observed for all traits studied, confirming substantial genetic variability within the germplasm. There was a significant decrease in spike length, spikelets per spike, thousand-grain weight, and grain yield per plant caused by heat stress at anthesis stage compared to stress at the grain-filling stage, suggesting that the yield related traits are more sensitive to heat stress at anthesis stage. The heat sensitivity of Zn and Fe loading to the grain was different, with increases in grain Zn concentration with heat stress, and decreases in grain Fe concentration, particularly during grain filling stage stress. The six wheat lines (G-445, G-439, G-434, G-419, G-443, and G-405) from CIMMYT's biofortified wheat germplasm were consistently superior in grain yield, yield components and micronutrient concentrations under heat stress as well as under control conditions and are recommended as heat-tolerant high micronutrient parental lines for development of climate resilient, biofortified wheat. To our knowledge, this is the first study to comprehensively evaluate grain yield, heat tolerance, and Zn and Fe biofortification potential among wheat genotypes developed by CIMMYT under heat stress at reproductive stages (anthesis versus grain filling), offering novel insights into stage-specific and micronutrient-specific heat stress response and a practical selection framework for advancing CIMMYT's high-zinc wheat germplasm to high

yielding, heat-tolerant, biofortified wheat cultivars for regions that are both subject to heat stress and micronutrient malnutrition.

Contribution of authors

RR conceived the idea and contributed to the development of the research concept and experimental design. RR and ZA jointly planned the experiments and supervised the research activities. RF and RK conducted experiments. AA and SR assisted in data collection. RK and RF prepared the first draft of the manuscript. SS, MA and MHK contributed to data analysis and manuscript preparation. All the authors contributed to the manuscript write-up. VG, HA and HM commented on the first draft and suggestions were incorporated and manuscript was finalized by ZA and RR.

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Data availability

Additional data underlying this article will be made available upon reasonable request to the corresponding author.

Declarations

Ethics approval and consent to participate

The manuscript does not include any studies involving human or animal subjects conducted by the authors. Seeds of *Triticum aestivum* were kindly provided by the CIMMYT, Mexico.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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