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Multi-trait Physio-biochemical Characterization of Contrasting Emmer Wheat (*Triticum dicoccon Schrank*) Germplasm under Controlled Post-anthesis Heat Stress

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Abstract

Emmer wheat (*Triticum dicoccon Schrank*) is a valuable genetic resource for improving nutritional and climate resilience traits in modern wheat. Fifteen dicoccon wheat genotypes, selected from multi-environment trials during Rabi 2021–22 and 2022–23 at Pune and Delhi based on grain yield HSI, including ten heat-tolerant, five heat-susceptible accessions, and five checks, were studied under control (24/14°C) and heat-stress (32/22°C) conditions at grain filling. Two-way ANOVA revealed highly significant effects of genotype, treatment, and their interaction for most of the traits. Heat stress consistently reduced RWC, MSI, CCI, and Fv/Fm, while SOD, proline, and MDA increased significantly. Under heat stress, SOD activity displayed significantly positive association with membrane stability index ($r = 0.741$) and relative water content ($r = 0.697$) and negatively with MDA ($r = -0.624$), suggesting reduced oxidative and membrane damage co-occurred with better membrane stability and water content. Principal component analysis explained 63.3% of total variation, with PC1 (48.5%) and PC2 (14.8%) primarily driven by MDA and CCI, respectively. A multi-trait matrix scoring approach categorized genotypes into four tolerance classes, identifying **G3, G4, G5, G6, G7, G9, and G10** as moderately heat-tolerant, exhibiting superior membrane integrity, water status, and antioxidant capacity under stress. Integration of HSI-based, physio-biochemical, and field-based classifications consistently identified G10 as heat-tolerant, while G3, G4, G5, G6, G7, and G9 were classified as moderately tolerant. The consistent performance across complementary approaches highlights these dicoccon wheat genotypes as promising and stable sources of heat tolerance for bread wheat improvement.

Keywords: Emmer wheat. Terminal heat stress. Antioxidant enzymes. Membrane stability. Chlorophyll fluorescence. Proline

Introduction

Wheat (*Triticum aestivum* L.) is a major cereal crop worldwide, serving as a staple food for nearly one-third of the global population and playing a crucial role in food and nutritional security, particularly in developing countries such as India (Lesk et al. 2016; Lamba et al. 2023). However, wheat productivity is increasingly threatened by various abiotic stresses, among which heat stress has emerged as one of the most critical constraints affecting crop growth and yield (Kumar et al. 2023). In India, thermal stress affects wheat more severely than other abiotic stresses (Joshi et al. 2007). Global mean temperatures are projected to rise by 1.0–1.5°C in the coming decades due to climate change, leading to more frequent and intense thermal stress events (Driedonks et al. 2016). Wheat yield is projected to decline by 4.1–6.4% per °C rise in global mean temperature by the end of the twenty-first century (Yu et al. 2014). The Indo-Gangetic Plains (IGP) of India, which contribute approximately 15% of global wheat production, are particularly vulnerable; by 2050, nearly 51% of the IGP may be reclassified as a heat-stressed, short-season mega-environment (Ortiz et al. 2008; Kuriachen et al. 2022). In India, central and peninsular zone is also affected with varied weather condition and high temperature during wheat growing season specifically grain filling duration (Chakrabarti et al. 2025). Emmer wheat (*Triticum dicoccum* Schrank), one of the oldest cultivated crops, has although served as a staple food for centuries (Nesbitt & Samuel 1996), at present, it occupies only 2% of the total wheat-growing area in India. However, its adaptability to poor soils and inherent resistance to diseases and pests make it agronomically valuable. More importantly, emmer wheat represents a rich genetic resource for improvement of nutritional traits (Angirekula et al. 2026) and biotic and abiotic stress tolerance in modern hexaploid and tetraploid wheat varieties (Raghunandan et al. 2022; Malipatil et al. 2023). Emmer wheat is mostly grown under peninsular zone and produces low yield due to high temperature and water scarcity, hence search of tolerant and high yielding germplasm is highly required to increase the production.

Being a cool-season crop, wheat requires an optimal temperature range of around 20–30°C and is particularly sensitive to elevated temperatures during the grain-filling and reproductive stages (Shirdelmoghanloo et al. 2022; Farhad et al. 2023). Exposure to high temperatures causes structural damage to cell membranes, reduces chlorophyll content, accelerates senescence, and ultimately shortens the duration of photosynthetic activity. (Wahid et al. 2007; Kumar et al. 2012; Dhyani et al. 2013; Upadhyay et al. 2020). A key biochemical consequence of heat stress is the overproduction of reactive oxygen species (ROS) including hydrogen peroxide (H_2O_2), superoxide ($O_2^{\bullet-}$), hydroxyl radicals ($OH^{\bullet}$), and singlet oxygen ($^1O_2$) which collectively cause oxidative damage to lipids, proteins, and nucleic acids (Chakraborty et al. 2011; Bitá et al. 2013). This oxidative stress leads to metabolic disturbances, enzyme denaturation, and membrane instability. Plants tolerate high temperatures through two principal mechanisms: basal thermotolerance, which is constitutive, and acquired thermotolerance (acclimation), which involves adaptive responses to otherwise lethal temperatures (Islam et al. 2017; Ding et al. 2021). At the biochemical level, thermotolerance is mediated by accumulation of compatible solutes (e.g. proline), upregulation of antioxidant enzymes including superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX), and induction of heat shock proteins (HSPs) (Mittler et al. 2012; Kumar et al. 2023). Among these, SOD, acting as the primary line of antioxidant defence, catalyses the dismutation of superoxide radicals into H_2O_2 , thereby preserving cellular integrity (Suzuki et al. 2012). Maintaining elevated concentrations of these antioxidants is essential for enhancing plant thermotolerance (Awasthi et al. 2014). In addition, compatible solutes like proline contribute to osmotic balance and cellular stability under stress. Malondialdehyde (MDA), a by-product of lipid peroxidation, serves as a reliable indicator of oxidative stress and membrane damage (Kumar et al. 2023; Sanghera et al. 2016).

Germplasm conserved in genebanks represent an invaluable reservoir of genetic diversity for trait discovery for climate resilience and heat stress adaptation (Phogat et al. 2021). Effective utilization of germplasm resources is critical for achieving the genetic gains necessary to address the challenges posed by global warming (Kumar et al. 2020; Patidar et al. 2023). Several studies have demonstrated that integrating physiological traits such as membrane stability index (MSI), relative water content (RWC), and Chlorophyll Concentration Index (CCI) with biochemical markers such as SOD, proline, and MDA is an effective strategy for identifying heat-tolerant wheat genotypes (Pandey et al. 2023; Alshaharni et al. 2025). Despite the recognized potential of emmer wheat as a valuable source of alleles for abiotic stress tolerance, the physiological mechanisms underlying heat stress adaptation remain insufficiently characterized compared with those of modern bread and durum wheat. In particular, an integrated evaluation of membrane stability index (MSI), relative water content (RWC), chlorophyll

stability index (CSI), and biochemical traits has not been systematically explored in emmer wheat under terminal heat stress. Therefore, we conducted agro-physiological evaluation of 175 dicoccum wheat germplasm accessions conserved at NBPGR genebank, during *rabi* 2021–22 and 2022–23 at New Delhi and Pune. Further, based on the heat susceptibility index (HSI) for grain yield, ten putatively tolerant and five susceptible accessions were identified for detailed evaluation under controlled condition and deciphering physio-biochemical basis of tolerance. The present investigation reports a detailed evaluation of these selected genotypes for combined multiple physiological and biochemical markers under terminal heat stress during *Rabi* 2023–24, with the following objectives: (i) to characterize variability among dicoccum wheat genotypes for physiological and biochemical traits under control and heat stress conditions; (ii) to evaluate physiological and biochemical trait responses associated with differential heat tolerance ; and (iii) to identify promising heat-tolerant candidate genotypes for potential integration into future wheat improvement programmes.

Materials and methods

Plant materials, experimental conditions and plant sampling

A panel of 175 dicoccum wheat (*Triticum dicoccum*) accessions was initially evaluated for agro-morphological traits for terminal heat stress tolerance under a multi-environment, replicated field trial conducted at Delhi and Pune during *Rabi* 2021–22 and *Rabi* 2022–23, using an alpha-lattice experimental design. Based on the Heat Susceptibility Index (HSI) calculated for grain yield, fifteen genotypes exhibiting contrasting thermotolerance were selected — ten putatively heat-tolerant genotypes (G1–G10) and five putatively heat-susceptible genotypes (G11–G15), consistent with HSI-based classification approaches. The selected contrasting genotypes along with five check varieties were subsequently subjected to a controlled heat stress experiment to validate their differential response and heat tolerance status under controlled temperature conditions (**Table S1** and **S2**).

Out of five checks, one check C5 (Raj 3765) specifically selected was heat tolerant bread wheat variety. The experiment was conducted in the Nanaji Deshmukh Plant Phenomics Centre (28°38'31.2"N, 77°09'39.6"E), New Delhi, India, during the *Rabi* seasons (November–April) 2023–24 . Seeds were sown in pots (0.19 m diameter, 0.4 m height, 15 L capacity) filled uniformly with 12.5 kg of soil per pot. To corroborate the findings from the controlled environment and assess their field-level relevance, the same genotype panel was concurrently evaluated under natural field conditions (ICAR-National Bureau of Plant Genetic Resources (NBPGR), Farm, New Delhi), comprising timely sown and late-sown environments each with three replications in alpha lattice design. Daily variations in rainfall (mm), maximum and minimum temperature (°C), and relative humidity (%) throughout the study period are illustrated in the **Fig.1**.

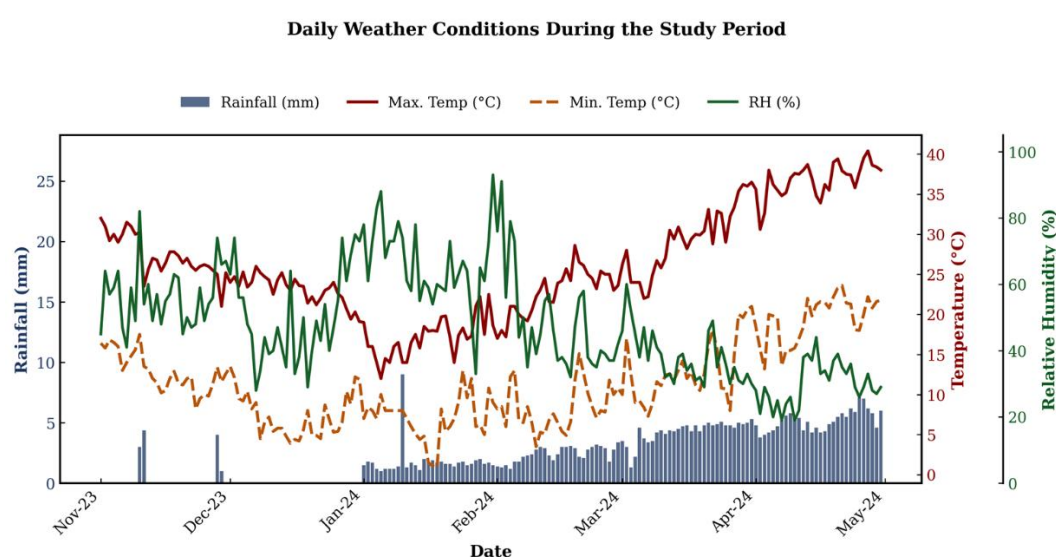


Fig.1. Daily weather conditions, including rainfall, maximum and minimum temperature, and relative humidity, during the study period in Delhi, India

The experiment was laid out in a Completely Randomised Design (CRD) with two treatments, control (C) and heat stress (S) and three replications per genotype per treatment. Five seeds were sown per pot and maintained throughout the experiment. A recommended fertilizer dose of 120:80:60 kg ha⁻¹ N:P:K was applied. All pots were kept well-watered, and standard weed, pest, and disease management practices were followed. Plants were grown under natural environmental conditions to ensure proper growth and biomass accumulation. Pots were moved to greenhouse one week after the anthesis (Feekes growth stage 10.5.4) and exposed to two temperature regimes: control (C) 24°C day/14°C and high temperature (Stress, S) 32°C day/22°C imposed within a separate growth chamber (Djanaguiraman et al. 2020). The plants were maintained under their respective C and S temperature regimes for seven consecutive days. Light intensity was maintained at 700–900 µmol, with a relative humidity of 50%. A sinusoidal temperature regime from 22 to 32 °C was applied, reaching a maximum temperature of 32°C between 11:00 AM and 2:00 PM (Bhattarai et al. 2021). The leaves were collected at afternoon time for physiological and biochemical analysis at the end of 7th day, and moved back to natural environment (**Fig. S1**).

Determination of physiological characteristics

Chlorophyll content index (CCI)

Flag leaf of the main tillers were used for the observation recording of physiologically measured traits and sampling of biochemical traits from both controlled as well as stressed experiment after seven days of stress period. Leaf chlorophyll content Index (CCI) is an indirect measure of the photosynthetic capacity of the plant. A chlorophyll content meter was employed to estimate CCI of the leaves. Three biological readings were obtained on flag leaves of main tiller from each replication in each treatment of all the genotype, and the measurements were performed at different growth stages i.e. heading, anthesis and grain filling (after stress treatment as well as control condition). Two wavelengths are used for chlorophyll determination - one wavelength within the chlorophyll absorbance peak (653 nm) while the other wavelength serves as a control (931 nm). The Chlorophyll Content Meter CCM-200 plus measures the absorbance at both wavelengths and calculates a Chlorophyll Content Index (µmol Chlorophyll·m⁻²). The CCI values of the instrument ranges from 1-100 (Kumar et al. 2020).

Chlorophyll fluorescence (CFC)

Chlorophyll Fluorometer (Model OS30P+, Opti-Sciences, Inc., USA) was used to measure the photosynthetic efficiency, as chlorophyll fluorescence of the plants under dark adapted condition. It was captured after the 20 minutes of dark adaptation by leaf clips, followed by placing the instrument at the same position with a flash of light to measure the efficiency of photosystem II in terms of Fv/Fm at heading anthesis and grain filling stage (after stress treatment) (Kumar et al. 2020).

Relative water content (RWC)

Relative water content (RWC%) was measured in three randomly selected plants following stress treatment. Fresh flag leaf samples (FW) weighing about 50 mg from both treatment and control plants were submerged in Petri-dishes with distilled water, were left for 4 hours at room temperature. The leaf samples were weighed to record the turgid weight (TW). The samples were then put in a paper bag and dried at 80 °C in a hot air oven until they reached a consistent weight, at which point their dry weight (DW) was noted. Weight measurements were performed using a precision analytical balance. RWC of a leaf was calculated as:

$$\text{RWC (\%)} = [(\text{FW} - \text{DW}) / (\text{TW} - \text{DW})] \times 100$$

where FW = fresh weight, TW = turgid weight, DW = dry weight

Membrane stability index (MSI)

Leaf Membrane stability index (MSI%) was estimated following Sairam et al. (1997). Two sets of 50mg of leaf material was immersed in 10 ml distilled water in 20 ml tubes. One set was heated at 40°C for 30 min in a water bath, and the electrical conductivity of the solution was recorded on electric conductivity meter EC1. Another sample was boiled at 100°C on a boiling water bath for 10 min, then its conductivity was measured on electric conductivity meter (EC2). MSI was calculated as: $\text{MSI} = [1 - (\text{EC1}/\text{EC2})] \times 100$.

Estimation of biochemical traits

Proline

Proline was quantified following the method of Bates et al. (1973) with minor modifications. Fresh leaf tissue (0.1 g) was homogenised in 10 ml of 3% sulfosalicylic acid, filtered through a watman N2 filter to determine the proline concentration and 2ml of this filtrate was mixed with 2ml ninhydrin reagent and 2ml acetic acid, heated at 100°C for an hour, and then rapidly cooled in an ice bath. Following this, 6 ml of toluene were added and thoroughly mixed. After mixing, the chromophore containing toluene is separated and its absorbance was read at 520 nm in spectrophotometer against toluene blank. Proline concentration was determined from a standard curve and expressed as $\mu\text{g g}^{-1}$ FW.

Malondialdehyde content (MDA)

Further, the method by Heath R.L. and Packer L. (1968) were used to estimate the level of lipid peroxidation in the leaf tissue in terms of MDA. Fresh leaf tissue (0.1 g) was homogenised in 10 milliliters of 0.1% trichloroacetic acid (TCA) and centrifuged at 15,000 rpm for 15 min. Supernatant was used for the estimation of TBARS contents. To 1.0 ml aliquot of the supernatant, 4.0 ml of 0.5% thiobarbituric acid (TBA) in 20% TCA is added. The mixture was heated at 95°C for 30 min in the laboratory electric oven and then cooled in an ice bath. After centrifugation at 10000 rpm for 10 min, the absorbance of the supernatant was recorded at 532 nm. Values of non-specific absorption recorded at 600 nm are subtracted from the values recorded at 532 nm. The TBARS content is calculated according to its extinction coefficient $\epsilon = 155 \text{ mM}^{-1} \text{ cm}^{-1}$.

Superoxide dismutase (SOD)

SOD activity was measured following the methods of Dhindsa et al. (1981) with minor modifications. A sample of 0.1g of leaves was macerated with 2ml of 50mM phosphate buffer and the extract was centrifuged at 13000 rpm for 30 minutes. Reaction mixture was prepared by adding 50 μl of extract, 2.150 ml 50 mM phosphate buffer, 0.2ml of methionine, 0.2 ml of NBT, 0.2ml of EDTA and 0.2ml of riboflavin solution in glass tubes. After adding riboflavin (0.2 ml), the tubes were exposed to two 15 W fluorescent lamps for 15 minutes to initiate the reaction. The control was a full reaction mixture devoid of enzyme that produced the most color. The reaction was stopped by turning off the light and placing the tubes in the dark. A non-irradiated complete reaction mixture served as a blank. Separate controls (lacking enzymes) were used for total SOD and inhibitor studies. The absorbency was recorded at 560 nm, and one unit of enzyme activity was taken as that amount of enzyme.

$$\text{SOD activity} = \frac{\text{Absorbance of blank} - \text{Absorbance of sample}}{\text{Absorbance of blank}/2}$$

Statistical analysis

Heat susceptibility index calculated based on grain yield in Microsoft excel 2019 using the formula given below (Fischer and Maurer, 1978). Based on, HSI_{pv} genotypes were grouped into three classes, tolerant ($HSI_{pv} < 0.5$), moderately tolerant ($HSI_{pv} 0.5–0.99$), and susceptible ($HSI_{pv} \geq 1.0$) (Suppl. Table 1)

$$HSI = \frac{1 - (Y_{si}/Y_{pi})}{1 - (\frac{Y_s}{Y_p})}$$

Where, yield of genotypes under normal condition (Y_{pi}), yield of genotypes under heat stress (Y_{si}), mean yield under irrigated environment (Y_p), mean yield under both stress environment (Y_s).

The statistical analyses were performed using R software version 4.1.1. Two-way analysis of variance for CRD performed using agricolae package of R software. Treatment means were compared using Fisher's Least Significant Difference (LSD) test at $p \leq 0.05$ performed using the LSD.test() function of the agricolae package in R. Correlation and principal component analysis (PCA) using the Performace Analytics, FactoMineR, factoextra, devtools, ggplot2, ggpubr, gplots, function of R version 4.1.1. A multi-trait matrix scoring system was applied to classify genotypes for heat tolerance across nine traits (Pandey et al. 2022). First, the percentage change (stress relative to control) for each trait was normalized to a scale of (0–100%) and divided into quartiles to assign initial categories: Tolerant (0–25%), Moderately Tolerant (25–50%), Moderately Sensitive (50–75%), and Sensitive (75–100%). These categories were assigned numerical weights (T = 3, MT = 2, MS = 1, S = 0) and summed across traits to generate a final tolerance score out of a maximum of 27. Genotypes were then classified based on their score range: 0–25% = Susceptible, 25–50% = Moderately Susceptible, 50–75% = Moderately Tolerant, >75% = Tolerant

Results

Phenomics-based evaluation of fifteen dicoccum wheat germplasm lines along with five check genotypes was conducted under control and heat stress conditions to assess physio-biochemical traits. Heat stress imposed at the reproductive stage exerted a significant influence on the physio-biochemical characteristics of the genotypes, indicating differential responses to terminal heat stress.

Analysis of variance and phenotypic variation under terminal heat stress

Two-way ANOVA revealed significant effects of genotype, treatment, and their interaction for most physiological and biochemical traits studied (**Table 1**). Significant differences among genotypes were observed for SOD (84***), MSI (163.6***), MDA (50.8*), Proline (22*), RWC (66.8**), CCI_GF (41.6**), and CF_GF (0.0514***), indicating substantial genetic diversity for heat stress responses among the evaluated accessions for most of the traits. The treatment effect (control vs. heat stress) was highly significant ($p < 0.001$) for all traits, including proline (1423.4***), SOD (5416***), MDA (2842.2***), RWC (738.5***), and MSI (2769.9***). For proline (62.1**), SOD (41***), MDA (34.8*), and MSI (109.1*), the genotype $\times$ treatment interaction was significant, demonstrating that genotypes varied in their biochemical response to heat stress.

Table 1 Analysis of variance for physiological and biochemical traits in dicoccum wheat under control and heat stress conditions

Source of Variation	DF	Proline	SOD	MDA	RWC	MSI	CCI_H	CCI_A	CCI_GF	CF_H	CF_A	CF_GF
Genotype	19	22*	84***	50.8*	66.8**	163.6***	17.67	20.12	41.6**	0.00146	0.0018	0.0514***
Treatment	1	1423.4***	5416***	2842.2***	738.5***	2769.9***	32.76	142.79**	1633.2***	0.0385***	0.0276***	0.568***
Replication	2	10.2	0.3	28.6	0.1	134.7*	16.52	41.27	109.6	0.01408	0.018752	0.0677
Genotype: Treatment	19	30*	41***	34.8*	28.4	109.1*	18.13	9.33	20.8	0.00148	0.001823	0.0102
Residuals	78	16.7	14	27.4	26.8	56.9	13.52	19.5	18.2	0.00124	0.00205	0.0093
SE		1.66	1.544	2.136	2.115	3.080	1.501	1.803	1.741	1.741	0.018	0.039
CV (%)		30.26	18.745	39.146	6.128	9.344	12.526	16.892	24.673	24.673	6.198	16.404

*, **, *** indicate significance at $p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.001$, respectively. Proline ($\mu\text{g/g FW}$); MDA ($\mu\text{mol/g FW}$), Malondialdehyde; SOD ($\mu\text{mol/g FW}$), superoxide dismutase; MSI (%), Membrane stability index; RWC (%), Relative water content; CCI_H, CCI_A and CCI_GF, Chlorophyll content index at heading, anthesis and grain filling stage respectively; CF_H, CF_A, and CF_GF, Chlorophyll fluorescence at heading, anthesis and grain filling respectively.

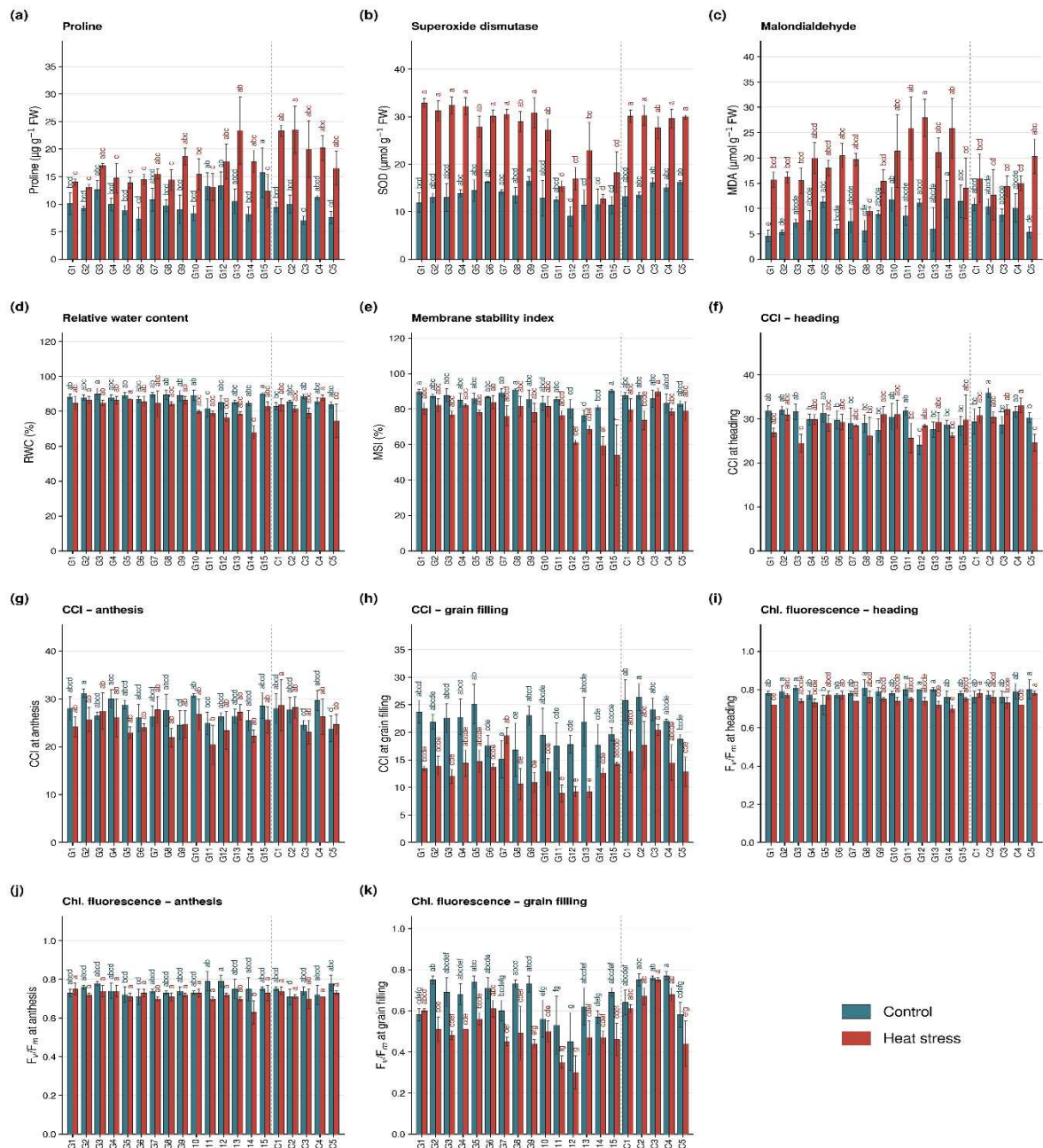


Fig.2. Physio-biochemical responses of emmer wheat genotypes under control and post-anthesis heat stress conditions. **(a)** Proline content, **(b)** superoxide dismutase (SOD) activity, **(c)** malondialdehyde (MDA) content, **(d)** relative water content (RWC), **(e)** membrane stability index (MSI), **(f–h)** chlorophyll concentration index (CCI) recorded at heading, anthesis and grain filling, respectively, and **(i–k)** chlorophyll fluorescence recorded at heading, anthesis and grain filling. Different letters above the bars indicate significant differences among genotypes according to Fisher's protected least significant difference (LSD) test at $p \leq 0.05$

Replication means with standard errors for control and stress conditions are presented in **Fig.2** and **Table S3**. The Fisher's protected LSD test revealed significant genotypic variation in physiological and biochemical traits in response to temperature stress. Percent change for each genotype for across traits are depicted in the **Table 2**

Table 2 Percent change (increase/decrease) under heat stress for all the genotypes across the traits

Genotype	Proline	SOD	MDA	MSI	RWC	CCI_H	CCI_A	CCI_GF	CF_H	CF_A	CF_GF
G1	-38.73	-175.62	-237.07	10.52	4.36	15.51	13.71	43.32	7.66	-1.64	0.31
G2	-42.16	-141.27	-205.69	5.86	1.56	3.24	17.54	36.28	2.53	5.65	31.87
G3	-33.99	-149.87	-118.08	12.28	5.62	22.41	0.78	47.05	8.33	4.50	29.97
G4	-48.11	-132.22	-161.58	3.75	1.83	0.00	13.22	36.90	5.43	-0.14	24.33
G5	-56.00	-93.43	-59.60	9.61	2.47	7.25	20.28	41.57	-6.15	0.33	24.17
G6	-97.67	-85.36	-245.41	3.21	1.25	1.46	7.68	21.63	-0.74	-2.76	13.37
G7	-27.09	-115.74	-161.21	14.74	5.54	2.18	0.19	-28.48	4.57	5.20	24.42
G8	-47.59	-115.60	-70.53	10.14	5.64	9.91	20.65	36.90	5.83	3.42	32.29
G9	-107.04	-86.62	-74.09	8.97	3.00	-13.43	0.82	52.60	4.41	3.15	40.05
G10	-85.78	-112.67	-80.83	2.09	10.80	0.86	12.92	34.13	4.88	-0.73	9.47
G11	-0.91	-21.86	-200.87	10.66	3.71	19.31	18.07	49.14	7.04	11.13	34.11
G12	-32.87	-87.83	-150.04	23.75	10.18	-18.50	10.93	48.03	7.67	8.65	33.31
G13	-123.37	-101.36	-253.40	10.16	7.61	-6.17	0.29	57.84	10.15	6.35	24.12
G14	-115.35	-10.72	-117.10	26.42	19.71	8.49	12.83	28.36	9.07	16.27	18.10
G15	-0.48	-61.00	-22.97	40.19	7.78	-4.69	10.40	27.50	4.60	2.44	33.09
C1	-148.33	-127.96	-47.62	9.33	-1.03	-5.12	-2.38	35.79	-1.97	1.82	4.23
C2	-135.23	-122.69	-21.80	15.98	4.92	15.51	-2.05	33.04	1.81	-0.66	9.86
C3	-184.02	-72.25	-65.19	0.45	10.83	-12.22	6.11	15.59	5.02	5.80	2.01
C4	-80.36	-96.60	-48.35	5.75	-2.50	-4.76	11.35	34.44	7.77	1.80	11.42
C5	-119.39	-84.58	-283.60	5.14	10.87	18.96	-3.93	31.67	2.95	6.31	25.64

Proline ($\mu\text{g/g}$ FW); MDA ($\mu\text{mol/g}$ FW), Malondialdehyde; SOD ($\mu\text{mol/g}$ FW), superoxide dismutase; MSI (%), Membrane stability index; RWC (%), Relative water content; CCI_H, CCI_A and CCI_GF, Chlorophyll content index at heading, anthesis and grain filling stage respectively; CF_H, CF_A, and CF_GF, Chlorophyll fluorescence at heading, anthesis and grain filling respectively.

Effect of Terminal Heat Stress on Proline content, superoxide dismutase and Malondialdehyde content

Heat stress is linked to an increase in ROS production in different cell compartments and raised activities of antioxidant enzymes is an adaptation by plants to ameliorate the heat stress-induced oxidative stress. Activities of SOD, MDA and proline showed a significant increase in all the tested varieties under stress. Proline accumulation varied significantly across genotypes under heat stress (**Table 1**). Stressed plants consistently exhibited higher proline levels than their control counterparts, confirming the role of proline as a key osmolyte under heat stress (**Fig.2a** and **Table S3**). Under stress, proline content ranged from 12.33 to 23.51 $\mu\text{g/mol}$ FW, while under control condition it varied from 7.04 to 15.7 $\mu\text{g/mol}$ FW. Among the genotypes, C2 (23.51 $\mu\text{g/mol}$ FW) and C1 (23.32 $\mu\text{g/mol}$ FW) recorded the highest proline under stress, followed by G13 (23.39 $\mu\text{g/mol}$ FW), while G11 (13.11 $\mu\text{g/mol}$ FW) and G2 (13.1 $\mu\text{g/mol}$ FW) showed the lowest accumulation. Under control conditions, G15 (15.7

µg/mol FW) had the highest proline, while C3 (7.04 µg/mol FW) and G14 (8.2 µg/mol FW) were among the lowest. The greatest percentage increases in proline from control to stress were observed in G13 (123.4%), G14 (115.3%), and G9 (107.0%), suggesting pronounced stress-responsive proline biosynthesis in these genotypes.

SOD activity exhibited significant difference for genotypes under heat stress and its activity increased markedly under stress in most genotypes, indicating upregulation of antioxidant defence (**Table 2 and Table S3**) Supplementary Table 2). Under stress, SOD activity ranged from 15.35 to 32.95 µmol g⁻¹ FW, compared with 9.03–16.50 µmol g⁻¹ FW under control conditions. Genotypes G1, G2, G3, G4, C1, and C2 recorded the highest SOD activity under stress (≥ 30 µmol g⁻¹ FW) (**Fig.2b**). In contrast, G14 (12.69 µmol g⁻¹ FW) and G11 (15.35 µmol g⁻¹ FW) showed the lowest SOD activity under stress. Highest percentage increase SOD activity observed G1, G2, G3, and G4 among tested genotypes.

A significant variation in MDA content was observed among all genotypes under heat stress conditions, with a substantial increase in value compared to normal conditions (**Table 2 and Table S3**). The MDA content varied from 9.5 to 27.96 µmol/g FW in heat stress condition and G11 (25.86 µmol/g FW) exhibited the highest MDA under stress, indicating greater oxidative damage (**Fig.2c**). G8 (9.5 µmol/g FW) showed the lowest MDA under stress, suggesting superior membrane integrity. The lowest percentage increases in MDA under heat stress indicative of superior membrane integrity were recorded in G15 (22.97%), G5 (59.60%), G8 (70.53%), and G9 (74.09%).

Effect of Terminal Heat Stress on Relative water content, Membrane stability index, Chlorophyll content, and Chlorophyll fluorescence

Relative water content (%) declined under heat stress in most genotypes, reflecting impaired water relations (**Fig.2d and Table S3**). Under stress, RWC ranged from 67.95% (G14) to 87.04% (G5), compared with 81.74% (G11) to 90.13% (G15) under control conditions (**Table 2**). Whereas G14 (67.95) and G12 (76.46) recorded the lowest, indicating poor water retention capacity. Genotype G (3.74%), G10 (2.08%) and G6 (3.21%) showed the less percent reduction in the RWC under heat stress indicating superior water status maintenance.

MSI varied significantly among genotypes and showed a consistent decline across all genotypes under stress compared to control conditions (**Fig.2e and Table S3**). In stress condition, MSI ranged from 53.89%(G15) to 83.85%(G6) compared with 76.18% (G13) to 90.86% (G8) under control conditions (**Table 2**). Genotypes G2 (1.5%), G4 (1.83%), and G6 (1.25%) showed minimal percent reduction in the MSI under thermal stress condition, confirming strong membrane structural integrity even under heat stress.

Chlorophyll content index (CCI) exhibited significant variation among all genotypes under both control and heat stress conditions, with a consistent reduction of chlorophyll content at anthesis and grain filling stage under heat stress (**Fig.2f-h and Table S3**). At anthesis CCI values ranged from 20.40 (G11) to 27.23 (G13) under stress, compared with 24.50 (G9) to 31.17 (G2) under control conditions (**Table 2**). At grain filling CCI values ranged from 8.9 (G11) to 19.4 (G7) under stress, compared with 15.1–25.1 under control. Genotypes G3 (0.78%), G7 (0.19%), and G9 (0.82%) showed the smallest percentage reduction in CCI_A, whereas G6 (21.63%) and G15 (27.50%) showed comparatively lower reduction in CCI_GF. Chlorophyll degradation was more pronounced at the grain-filling stage than at anthesis under heat stress, consistent with accelerated senescence under thermal stress. Chlorophyll degradation was more pronounced at the grain-filling stage than at anthesis under heat stress, consistent with accelerated senescence under thermal stress.

CF (Fv/Fm) declined under heat stress, particularly at the grain-filling stage (**Fig.2i-k and Table S3**). At anthesis (CF_A), Fv/Fm ranged from 0.63 (G14) to 0.75 (G1) under stress, compared with 0.71 (G6) to 0.79 (G11) under control conditions. At grain filling (CF_GF), values ranged from 0.30 (G12) to 0.61 (G6) under stress, compared with 0.45 (G12) to 0.75 (G2) under control conditions. The more pronounced reduction in CF at grain filling than at anthesis confirms that terminal heat stress specifically impairs PSII photochemical efficiency during the grain-filling period.

Correlation between biochemical and physiological traits

Pearson's correlation analysis revealed distinct trait relationships under control and heat stress conditions (**Fig. 3**). Under heat stress condition, proline showed a positive correlation with SOD ($r = 0.109$), whereas it exhibited negative correlations with MSI ($r = -0.089$). MDA displayed significant negative associations with RWC ($r = -0.596^{**}$), and negative correlation with with MSI ($r = -0.356$) and consistent with a pattern of elevated oxidative markers under stress conditions. MSI and RWC showed significant positive correlations ($r = 0.631^{***}$), suggesting a link between membrane stability and tissue water content across genotypes. SOD showed significantly negative correlation (-0.624^{**}) with MDA and strong positive association with MSI (0.741^{***}), RWC (0.697^{***}) and CF_GF (0.518^{*}) under heat stress. MSI exhibited positive correlations with CCI and CF at the heading, anthesis, and grain-filling stages. Furthermore, MSI showed significant positive associations with RWC (0.457^{*}) and CF_GF (0.511^{*}) under heat stress. RWC displayed positive relation with CCI and CF at all three stages under heat stress condition. Positive correlations were also observed among chlorophyll content indices (CCI_H, CCI_A, and CCI_GF) and fluorescence parameters, particularly between CCI_GF and CF_GF ($r = 0.758^{**}$).

Principal component Analysis (PCA)

Principal Component Analysis (PCA) revealed that the first two components (PC1: 48.5%, PC2: 14.8%) cumulatively explained 63.3% of the total variability in physio-biochemical traits. While both temperature regimes showed distinct trait clustering, heat stress induced greater dispersion (**Fig.4a** and **4b**). MSI, RWC, chlorophyll fluorescence were strong positive contributors, while MDA, Proline, SOD were negative contributors to PC1. MDA (15.1%) and CLC_H (27.73%) emerged as the primary contributors to PC1 and PC2, respectively. Traits such as MDA (15.1%), CCI_GF (13%), RWC (11%), and CF_GF (10.85%) were accounted for major contribution towards diversity among PC1, whereas CCI_H (27.73%), CF_A (15.78%), CF_H (13.84%), and CF_GF emerged as the predominant contributing traits to PC2.

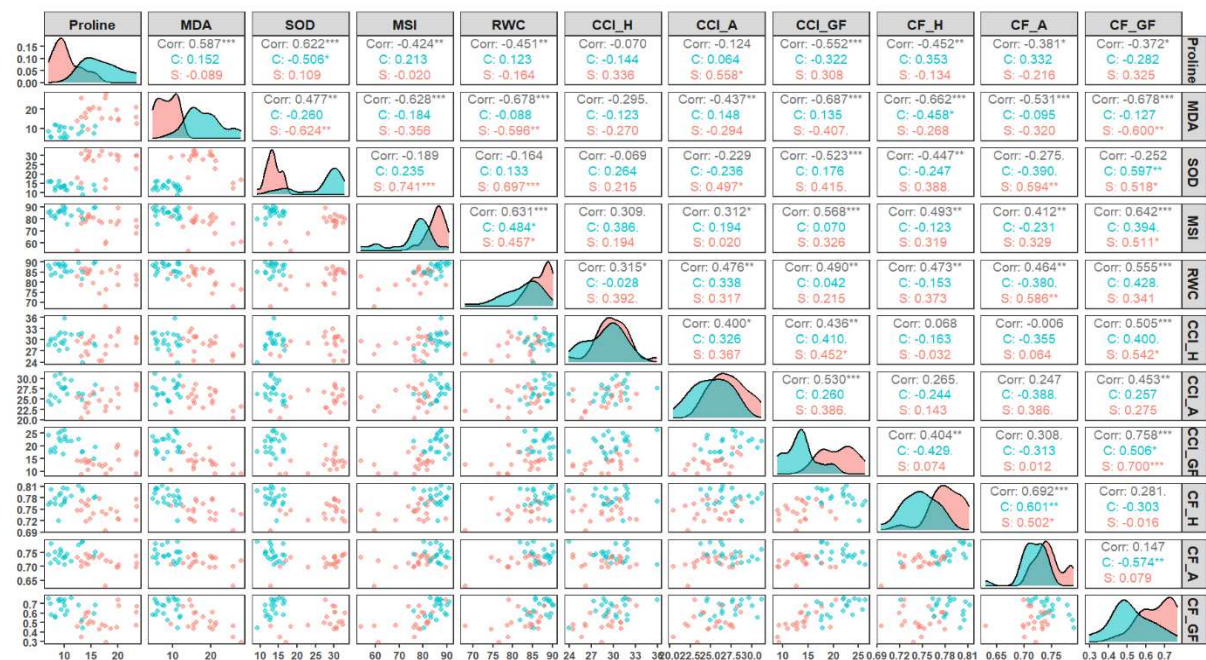


Fig. 3 Significance of correlation among all the physiological and biochemical studies under control and heat stress condition. *** = Significant at $p \leq 0.001$; ** = Significant at $p \leq 0.01$; * = Significant at $p \leq 0.05$. Proline; MDA, Malondialdehyde; SOD, superoxide dismutase; MSI, Membrane stability index; RWC, Relative water content; CCI_H, CCI_A and CCI_GF, Chlorophyll content at heading, anthesis and grain filling stage respectively; CF_H, CF_A, and CF_GF, Chlorophyll fluorescence at heading, anthesis and grain filling respectively.

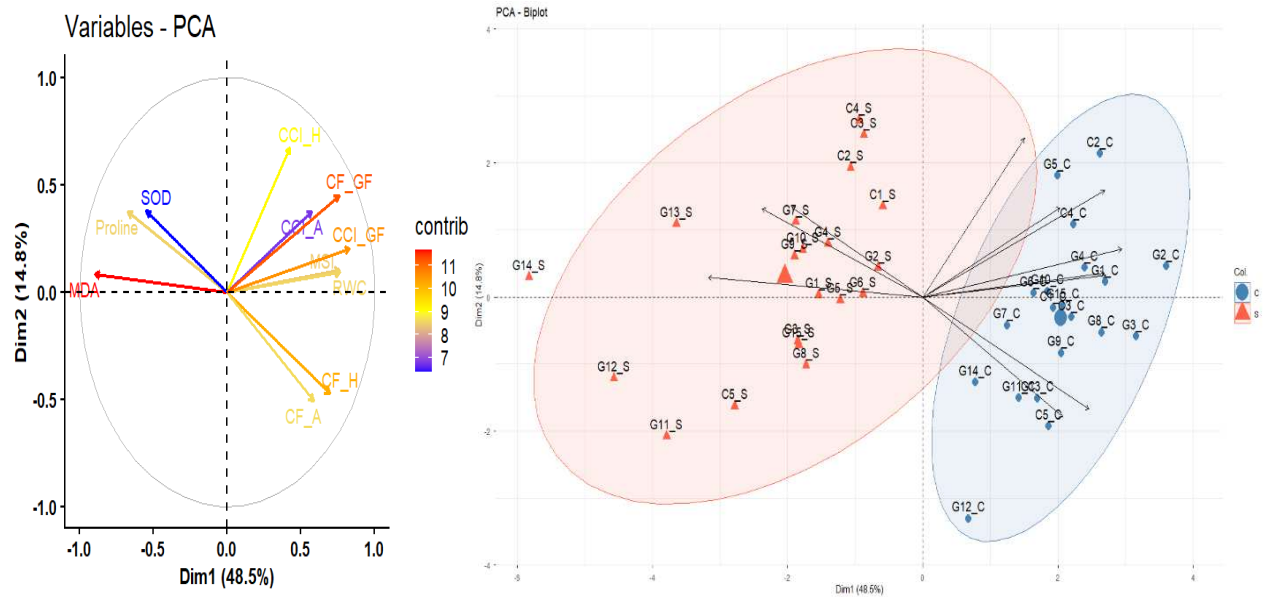


Fig. 4 PCA scatter plot showing: **a)** the position of physiological and biochemical traits. **b)** genotypes under control and heat stress condition

Matrix scoring

The overall findings showed a considerable increase in biochemical traits, including proline, SOD and MDA in heat stress condition. Whereas traits such as RWC, MSI, CCI and CF at anthesis and grain filling were reduced under heat stress. Based on the composite matrix scoring system across nine traits, genotypes were classified into four tolerance categories (**Table 3**). Genotypes G1(Score 13), G2 (Score 12), G8(Score 13), G11(Score 12), G12 (Score 11), G13(Score 13) and G14(Score 11) were moderately susceptible. Genotypes G3(Score 14), G4(Score 15), G5(Score 13), G6(Score 16), G7(Score 18), G15(Score 14), G16(Score 20), G17(Score 18), G18(Score 17), G19(Score 19) and G20(Score 14) were classified as moderately tolerant.

Table 3 Heat tolerance matrix of emmer wheat genotypes for physiological and biochemical traits

Genotype	Proline	SOD	MDA	MSI	RWC	CCI A	CCI GF	CF A	CF GF	Score
G1	T	S	S	MT	MT	S	S	T	T	13
G2	T	S	MS	T	T	S	S	MT	S	12
G3	T	S	MT	MT	MT	T	S	MT	S	14
G4	MT	MS	MS	T	T	MS	S	T	MS	15
G5	MT	MS	T	T	T	S	S	T	MS	16
G6	MS	MT	S	T	T	MS	MS	T	MT	16
G7	T	MS	MS	MT	MT	T	T	MT	MS	18
G8	MT	MS	T	T	MT	S	S	MT	S	13
G9	MS	MT	T	T	T	T	S	MT	S	17
G10	MT	MS	T	T	MS	MS	MS	T	MT	17
G11	T	T	MS	MT	MT	S	S	MS	S	12
G12	T	MT	MT	MS	MS	MS	S	MS	S	11
G13	MS	MS	S	T	MT	T	S	MT	MS	13
G14	MS	T	MT	MS	S	MS	MS	S	MT	11
G15	T	MT	T	S	MT	MS	MS	MT	S	14
C1	S	MS	T	T	T	T	MS	T	T	20
C2	MS	MS	T	MT	MT	T	MS	T	MT	18
C3	S	MT	T	T	MS	MT	MS	MT	T	17
C4	MT	MS	T	T	T	MS	MS	T	MT	19

C5	MS	MT	S	T	MS	T	MS	MT	MS	14
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T, Tolerant; MT, Moderately tolerant; MS, Moderately susceptible; S, Susceptible

Validation of Moderately tolerant genotype through field experiment

The genotypes evaluated under controlled conditions are also planted in the field at ICAR-National Bureau of Plant Genetic Resources (NBPGR), Farm, New Delhi in timely sown and late sown condition and Heat susceptibility index calculated based on grain yield. Among the 20 wheat genotypes based on HSI (grain yield), genotype G10 emerged as the sole heat-tolerant line, recording the lowest HSI value of 0.47, well below the tolerance threshold of 0.5 (**Fig. S2**). Genotypes G3(0.55), G6(0.69), G2(0.77), G4(0.88), G1(0.90), G9(0.91), G8(0.96), G5(0.97) were confirmed as moderately tolerant.

Discussion

Terminal heat stress during grain filling is a major constraint to wheat yield globally, causing significant disruptions to grain filling, photosynthetic activity, and membrane integrity (Yadav et al. 2019). Therefore, identifying and developing heat-tolerant wheat genotypes is essential to ensure stable and sustainable crop productivity under rising temperature conditions. This study was conducted to assess the effect of heat stress on physiological and biochemical traits in ten putative heat-tolerant and five heat-susceptible emmer wheat genotypes and to our knowledge, the first such investigation in this species. The panel of 15 dicoccum lines plus 5 checks was intentionally sized based on multi environment HSI for precise, replicated multi-trait evaluation rather than large-scale unreplicated screening. Unlike hexaploid bread wheat, tetraploid emmer wheat retains stress-adaptive alleles for antioxidant defence and membrane stability that may have been lost during wheat domestication and breeding, making it a valuable genetic resource for heat-tolerance breeding (Ullah S et al. 2021). While individual traits such as SOD, MDA, proline, and RWC are indeed established heat-stress indicators in bread wheat (*Triticum aestivum*), the novelty of our study lies in applying this integrated physio-biochemical panel to dicoccum wheat (*T. dicoccum*), a tetraploid, climate-resilient wheat species that remains comparatively underexplored for terminal heat stress response. Analysis of variance revealed significant variation among genotypes of emmer wheat for physiological and biochemical traits under stress conditions, highlighting differences in their stress tolerance levels. Such genotypic variation for oxidative stress markers, membrane stability and physiological traits has been well documented in wheat under high-temperature conditions (Almeselmani et al. 2009; Rehman et al. 2021; Kumar et al. 2023). The existence of significant genotypic differences provides the basis for identifying superior heat-tolerant accessions.

The highly significant treatment effects observed for all measured traits confirmed that the imposed heat stress was effective in inducing physiological and biochemical stress responses. The dramatic increase in SOD activity under heat stress aligns with findings of Almeselmani et al. (2009), who reported enhanced antioxidant enzyme activity as a protective mechanism in heat-stressed wheat. Elevated MDA content under heat stress, may reflect enhanced lipid peroxidation and membrane damage, is consistent with earlier reports in bread wheat (Nayyar et al. 2006). The significant decline in RWC and MSI under heat stress corroborates findings of Sairam et al. (2000), who identified these as reliable indicators of heat injury in wheat. Reduction in chlorophyll content and chlorophyll fluorescence (Fv/Fm) across anthesis and grain filling stages reflects heat-induced photoinhibition and accelerated chlorophyll degradation, as documented by Ashraf & Harris (2013) and Feng et al. (2014) in wheat. The significant genotype $\times$ treatment interaction for Proline, SOD, MDA and MSI demonstrated that genotypes differed not only in their mean performance but also in their plasticity of biochemical response to heat stress, which is consistent with earlier reports in wheat by Gupta et al. (2013) and Kumar et al. (2023) underlining the importance of evaluating genotypes under both stressed and non-stressed conditions for meaningful selection. This is likely because proline and SOD are stress-induced defence responses, while MDA and MSI reflect the resulting damage — both of which naturally vary more between genotypes than fixed traits.

High temperature reduces leaf water potential by limiting water uptake and disrupting stomatal regulation of transpiration. Most of the tolerant lines identified based on HSI displayed relatively low reduction in RWC as compare to susceptible lines indicating their relevance in high temperature tolerance by maintaining high RWC. Previous studies have reported that the ability of plants to sustain higher RWC is one of the mechanisms of heat

tolerance (Pandey et al. 2019). The membrane stability index, declined substantially under stress conditions across all genotypes and the majority of putative tolerant genotypes based on HSI, exhibited lower reduction.

Lipid peroxidation begins and electrolyte leakage rises with increasing heat stress. Lower membrane leakage of the genotypes might be due to their heat tolerant nature as they maintain stronger membrane integrity as compared to sensitive genotypes. According to Poudel & Poudel (2020), adaptation to high temperatures appears to be controlled by a stable cell membrane system that continues to operate during heat stress. Combinedly, genotypes G2, G4, G5, G6, G8 and G9 showed less reduction in MSI and RWC demonstrating superior resilience through higher Membrane stability index (MSI) and relative water content (RWC) retention. Notably, the magnitude of MSI and RWC reduction in our putative tolerant emmer genotypes (3.2–10.1% and 1.3–5.6%, respectively) was lower than the percent reduction reported in heat-tolerant hexaploid wheat genotypes under similar stress conditions (MSI PR: 4.7–18.9%; LRWC PR: 8.5–24.6%), suggesting that it may confer membrane better stability and water status maintenance mechanisms than those found in elite hexaploid tolerant lines (Jainth et al. 2026). According to earlier research (Iqbal et al. 2017) plants capacity to sustain increased RWC is one of the mechanisms of heat tolerance. Thus, the identification of these genotypes as superior in maintaining membrane integrity and water status provides the first evidence of this adaptive mechanism operating in dicoccum under heat stress. Although these findings are based on physiological evaluation, they provide a strong foundation for future molecular marker-assisted validation, QTL mapping, and gene discovery to elucidate the genetic mechanisms underlying heat tolerance.

Heat stress causes a decrease in the synthesis of chlorophyll and an increase in its loss. The most heat-labile cell structure in chloroplasts that contains chlorophyll is the thylakoid. Thus, chlorophyll loss will occur from any heat-induced damage to thylakoids (Mamrutha et al. 2020). All the genotypes displayed pronounced reduction of chlorophyll content at grain filling stage than the anthesis stage under terminal heat stress. Similar findings were reported by Miroslavljević et al. (2024) indicating the effect of terminal heat stress in chlorophyll degradation. Urban et al. (2018) highlighted, that heat stress during grain filling significantly reduced the primary photochemical reactions measured as a maximum quantum yield of PSII photochemistry. In the present study also all the genotypes showed high reduction in chlorophyll fluorescence at grain filling stage. However, the putative tolerant genotypes G1 and G10 were found to have high PS-II efficacy and may maintain better photosynthetic performance under thermal stress. Importantly, field evaluation of all twenty genotypes for terminal heat tolerance supported the controlled-condition results. G10 was the only genotype classified as highly tolerant based on grain yield-derived HSI (0.47), linking its superior PSII efficiency with better field-level performance and highlighting its potential as a promising source for terminal heat tolerance breeding (Supplementary Fig 2). Our findings are in accordance with the previous finding of Sharma et al. (2015) where he reported that heat-tolerant wheat lines retain greater values of important chlorophyll fluorescence metrics under heat stress than susceptible lines, such as F_v/F_m (maximum quantum efficiency of PSII).

Under environmental stress, production of ROS exceeds the antioxidative capacity resulting in cellular damage. In the present study SOD activity was higher in genotypes under stress than in control plants. This trend agrees with Ravindra et al. (2025), who reported increased SOD activity under stress, reflecting enhanced production of harmful ROS and activation of antioxidant defence mechanisms. Most of the putative tolerant genotypes based on HSI displayed higher SOD activity compared to susceptible genotypes. This reveals that these tolerant genotypes are more effective at scavenging superoxide, where it converts superoxide radicals into H_2O_2 and O_2 under heat stress and offer a plausible explanation for their tolerant nature under stress conditions. The significant positive correlation of SOD with MSI, RWC and CF_{GF} under stress suggests these traits are not independent responses but components of a coordinated physiological-biochemical defence network: enhanced antioxidant (SOD) activity limits ROS-driven membrane damage (reflected in higher MSI), which in turn preserves cellular turgor and water retention (RWC), sustaining photosynthetic apparatus integrity and PSII efficiency (CF_{GF}) under heat stress (Kaur et al. 2021). Thus, results obtained was extending the findings of Pantha et al. (2024) in hexaploid wheat under stress condition to a novel tetraploid emmer background under heat stress.

Both susceptible and tolerant genotypes accumulated more proline than control under heat stress. Kaur et al. (2018) indicated that proline accumulation is a prevalent plant response to environmental stresses such as

terminal heat stress. HSI based tolerant genotypes **G5, G6, G9** and **G10** accumulated markedly higher proline levels under heat stress, reinforcing the association between proline accumulation and thermotolerance. Liang et al. (2013), Dikilitas et al. (2020) and Jainth et al. (2026) also reported higher amount of proline in heat tolerant genotypes. Protective roles of proline in protein stabilization, ROS scavenging, redox balance and signalling, suggest that higher levels of proline in tolerant wheat genotypes enhance heat stress survival and serve as an early screening marker (Sihag et al. 2023).

Lipid peroxidation of cell membranes represents one of the most severe consequences of heat stress, and MDA content acts as a primary determinant for gauging stress intensity (Kumari et al. 2020). According to Pantha et al. (2024), *Triticum dicoccoides* exhibits higher levels of MDA during stress condition than *Triticum aestivum*. HSI based tolerant genotypes **G5, G8, G9** and **G10** displayed minimal increase in MDA values and were classified as tolerant. Our results were in accordance with the report of Turkan et al. (2005) and Pandey et al. (2023) who observed lower MDA content in the leaves of heat tolerant genotypes as compared to heat sensitive genotypes.

Proline exhibited a positive correlation with SOD and CCI_A, CCI_GF and CF_GF, whereas it showed negative correlation with MDA, MSI, RWC, CF_A under heat stress condition. This pattern was earlier observed in the case of proline under drought stress in wheat (Alshaharni et al. 2025). It suggests that higher proline accumulation supports ROS scavenging and protects chlorophyll function. Conversely, the negative association with MDA suggests that higher proline may be associated less oxidative damage consistent with reports by Patwa et al. (2024) in wheat. Under controlled condition proline was negatively associated with CCI and CF at grain filling stage. This transition from negative correlation under normal conditions to positive correlation under stress may reflect physiological changes at that stage, though experimental validation would be needed to confirm causality. SOD exhibited a strong positive association with MSI, RWC and CF_GF under stress, consistent with a link between antioxidant enzyme levels and membrane water status. Under stress conditions, MDA exhibited negative correlations with all evaluated traits, suggesting that higher oxidative stress was associated with lower membrane stability, reduced water status, and decreased chlorophyll-related traits. The negative SOD and MDA association may reflect co-occurring antioxidant and oxidative stress responses (Abid et al. 2018). The first two principal components collectively explained 63.3% of the total variability, with PC1 accounting for 48.5% and PC2 for 14.8%, indicating that these components effectively captured the major sources of variation among the evaluated traits. Heat-stressed plants demonstrated notably greater dispersion in the PCA biplot compared to control conditions, suggesting variable genotypic responses to elevated temperature stress. These findings confirm that multidimensional trait analysis effectively differentiates wheat genotypes and facilitates the selection of heat-tolerant lines (Mansour et al. 2018; Habibullah et al. 2021). Collectively, these trait associations underscore the potential of proline, SOD, and membrane-stability indicators reliable physiological markers for heat-tolerance screening, providing a valuable basis for future mechanistic investigations and breeding programs in this underexplored species.

The multi-trait matrix scoring system effectively integrated responses across nine traits, enabling an objective and multidimensional classification of genotypes. Across all the traits tolerant genotypes based on HSI, G3(EC06912), G4(IC535082), G5(IC534587), G6(EC577404), G7(IC535090), G9(IC535108) and G10(IC138418) were classified as moderately tolerant as they are falling in this category (25-50%). Notably, the heat-tolerant check Raj 3765 (C5) achieved a tolerance matrix score of 14, consistent with its well-established thermotolerant status (Azameti et al. 2022; Pandey et al. 2023). The convergence of HSI-based classification, physiological scoring, and molecular evidence for the Raj 3765 check strengthens confidence in the tolerance matrix for identifying heat-tolerant emmer wheat germplasm. These genotypes emerge as promising candidates for use as donors in dicoccum or bread wheat improvement programmes targeting heat stress adaptation. Among them, G4(IC535082), G7(IC535090) and G9 (IC535108), originated from the Karnataka region of India, Similarly, G5(IC534587; PI-248991) was collected from Telangana and G10(IC138418; Kenphed), from the Delhi region. G5(PI 248991) germplasm accession is recognized for its valuable genetic traits, including broad resistance to highly virulent stem rust races (such as TTKSK/Ug99) (Olivera et al., 2012). Karnataka-origin genotypes G4(IC535082), G7(IC535090), and G9(IC535108) scored consistently high in the tolerance matrix, aligning with the strong performance of released Karnataka checks C1(DDK1025, score 20) and C2(DDK1029, score 18). Genotype G3 (EC06912) originated from the USA, but the origin of G6(EC577404), with an alternate identity of E-2623, is exotic material. Additionally, field validation under timely and late-sown conditions

corroborated these findings: G10 remained the sole heat-tolerant genotype (HSI = 0.47), while G3, G6, G2, G4, G1, G9, G8, and G5 were confirmed moderately tolerant, providing independent evidence of tolerance stability across environments. The field-based assessment therefore corroborated the differential heat responses observed under controlled conditions and provided independent evidence for the stability of heat tolerance among the selected genotype. The comprehensive physiological and biochemical profiling of dicoccum wheat germplasm enabled the clear classification of genotypes into heat-tolerant and susceptible categories. Furthermore, these results validated the use of trait-based selection for future research and advocated for incorporating tolerant lines into wheat breeding programs. Our results revealed that heat stress profoundly impacted all biochemical and physiological traits, leading to marked declines in photosynthetic performance and water retention capacity. However, the activation of defence mechanisms such as elevated proline levels and increased antioxidant activity may play role in the tolerance of genotypes for heat stress.

Conclusions

This study demonstrates significant genotypic variation among emmer wheat accessions for physiological and biochemical traits under terminal heat stress. Heat stress profoundly reduced RWC, MSI, chlorophyll content, and PSII efficiency (Fv/Fm), while elevating SOD activity, proline accumulation, and MDA content. The significant genotype $\times$ treatment interactions for key biochemical traits confirm that genotypes differ in their biochemical plasticity under heat stress. Traits including MSI, RWC, SOD activity, and Fv/Fm at grain filling proved to be the most informative indicators for differentiating heat-tolerant from susceptible genotypes. The multi-trait matrix scoring system identified genotypes G3(EC06912), G4(IC535082), G5(IC534587), G6(EC577404), G7(IC535090), G9(IC535108) and G10(IC138418) as moderately tolerant genotypes. The agreement between the tolerance matrix and the performance of the heat-tolerant check Raj 3765 supports the robustness of this approach for identifying promising heat-tolerant germplasm. These genotypes represent valuable donor resources for heat tolerance breeding in dicoccum and bread wheat. Integration of HSI-based, physio-biochemical matrix, and field-validated classifications consistently identified G10 as heat-tolerant and G3, G4, G5, G6, G7, and G9 as moderately tolerant, confirming these dicoccum wheat genotypes as reliable, stable sources of heat tolerance. Collectively this study provides a strong foundation for future breeding efforts combining physiological phenotyping with molecular approaches to accelerate the development of climate-resilient wheat cultivars.

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Author contribution

Jyoti Kumari, Pavan K Naik, Shailendra Kumar Jha and Harikrishana made substantial contributions to the conception or design of the work. Pavan K Naik, Jyoti Kumari, Rohit Kumar Singh conducted phenotyping experiment and data compilation. Pavan K Naik, Jayaluxmi Devi Khumukcham and Jyoti Kumari done the statistical analysis and wrote the manuscript. Jyoti Kumari, PV Vara Prasad, GP Singh edited the manuscript. All authors edited and approved the manuscript for publication.

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Data availability

The data that supports the findings of this study are available in the supplementary material of this article

Declarations

Conflict of interest The authors declare no conflict of interest

AI-assisted figure preparation statement: Fig. S1 presents a schematic illustration of the experimental setup, including growth conditions, temperature treatments, and biochemical analyses. The figure was initially generated using ChatGPT and was subsequently reviewed and refined by the authors to ensure scientific accuracy and appropriate presentation.

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