Heat-induced oxidative damage is ameliorated by enhanced antioxidants activity in the tolerant wheat variety

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March 30, 2022

Abstract

Heat stress alters photosynthetic components and antioxidant scavenging system, negatively affecting plant growth and development. Plants overcome heat stress damage through an integrated network involving enzymatic and non-enzymatic antioxidants. The aim of the study was to assess physiological and biochemical responses in contrasting thermotolerant wheat varieties exposed to 25°C (control) and 35°C (heat stress), during seedling stage. Our results revealed a substantial decrease in the photosynthetic pigments, carotenoids, anthocyanin content, and increased membrane injury index, malondialdehyde, lipoxygenase, methylglyoxal and H2O2 contents compared to non-stress wheat seedlings. Comparatively the heat tolerant variety BG26 maintained a high level of stability compared to the heat susceptible variety Pavon, perpetuated by higher accumulation of proline, glycine betaine, ascorbate-glutathione cycle associated enzymes, reduced glutathione and ascorbate contents. In addition, significantly lower MG detoxification and activities of antioxidant system and ascorbate-glutathione cycle related enzymatic activities lead to increased susceptibility in Pavon. Hierarchical clustering and principal component analysis revealed BG26 possessing a combination of biochemical responses that induced higher level of tolerance. Taken together, our results provide a reference for utilizing BG26 and Pavon as highly contrasting heat-responsive varieties for comparative genomics and translational research to unravel underlying mechanisms to better adapt wheat to heat stress.

Running title- Antioxidants and heat tolerance in wheat

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Funding

We gratefully acknowledge the partial financial support of the Research Management Wing (RMW), Bangabandhu Sheikh Mujibur Rahman Agricultural University, Gazipur1706, Bangladesh.

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Summary statement-

Heat tolerance was exhibited by maintaining higher dry matter, pigment stability, osmolyte levels, and reduced oxidative stress owing to enhanced overall antioxidant capacity in the tolerant wheat variety.

ABSTRACT

Heat stress alters photosynthetic components and antioxidant scavenging system, negatively affecting plant growth and development. Plants overcome heat stress damage through an integrated network involving enzymatic and non-enzymatic antioxidants. The aim of the study was to assess physiological and biochemical responses in contrasting thermotolerant wheat varieties exposed to 25°C (control) and 35°C (heat stress), during seedling stage. Our results revealed a substantial decrease in the photosynthetic pigments, carotenoids, anthocyanin content, and increased membrane injury index, malondialdehyde, lipoxygenase, methylglyoxal and H_2O_2contents compared to non-stress wheat seedlings. Comparatively the heat tolerant variety BG26 maintained a high level of stability compared to the heat susceptible variety Pavon, perpetuated by higher accumulation of proline, glycine betaine, ascorbate-glutathione cycle associated enzymes, reduced glutathione and ascorbate contents. In addition, significantly lower MG detoxification and activities of antioxidant system and ascorbate-glutathione cycle related enzymatic activities lead to increased susceptibility in Pavon. Hierarchical clustering and principal component analysis revealed BG26 possessing a combination of biochemical responses that induced higher level of tolerance. Taken together, our results provide a reference for utilizing BG26 and Pavon as highly contrasting heat-responsive varieties for comparative genomics and translational research to unravel underlying mechanisms to better adapt wheat to heat stress.

KEYWORDS

heat stress, oxidative stress, reactive oxygen species, antioxidant, ascorbate-glutathione cycle, wheat

ABBREVIATIONS

AGE Advanced glycation end product

Anth Anthocyanins

AO Ascorbate oxidase

APX Ascorbate peroxidase

AsA Ascorbate

BARI Bangladesh Agricultural Research Institute

BSA Bovine serum albumin

Car Carotenoids

CAT Catalase

CDNB 1-chloro-2,4-dinitrobenzene

Chl Chlorophyll

DHA Dehydroascorbate

DHAR Dehydroascorbate reductase

DTNB 5,5'-dithiobis-(2-nitrobenzoic acid)

EDTA Ethylenediaminetetraacetic acid

GB Glycine betaine

Gly I Glyoxalase-I

Gly II Glyoxalase-II

GPX Glutathione peroxidase

GR Glutathione reductase

GSH Glutathione (reduced)

GSSG Glutathione (oxidized)

GST Glutathione -transferase

IPCC The Intergovernmental Panel on Climate Change

LTD Leaf temperature depression

LOX Lipoxygenase

MDA Malondialdehyde

MDHA Monodehydroascorbate

MDHAR Monodehydroascorbate reductase

MG Methylglyoxal

MII Membrane injury index

NADPH Nicotinamide adenine dinucleotide phosphate (reduced)

NBT Nitroblue tetrazolium

POD Guaiacol peroxidases

Pro Proline

PUFA Polyunsaturated fatty acid

RDM Root dry matter

RH Relative humidity

RL Root length

ROS Reactive oxygen species

SDM Shoot dry matter

SL Shoot length

SLG *S* -D-lactoylglutathione

SOD Superoxide dismutase

TBA Thiobarbituric acid

TCA Trichloroacetic acid

TDM Total dry matter

1 | INTRODUCTION

Heat stress is one of the major environmental factors that can impact crop plants negatively, leading to impairment of several physiological and biochemical processes (Paradiso et al., 2020). Global climate models predict that with increasing greenhouse gases, global mean surface temperatures are projected to increase by 0.3 to 4.8°C by the end of the 21st century (IPCC, 2014). Heat stress events leading to significant yield losses in crops and particularly in wheat has been documented under controlled environment (Aiqing et al., 2018; Bheemanahalli et al., 2019) and field conditions (Asseng et al., 2015, Tack, Barkley, & Nalley, 2015).

Heat stress is shown to have a significant negative impact during reproductive stages in crops, including wheat (Zandalinas et al., 2016; Prasad, Bheemanahalli, & Jagadish, 2017; Jagadish, 2020). Comparatively, heat stress responses at the seedling stage in wheat are poorly understood, despite heat or combined heat and drought stress are known to adversely affect early establishment of the wheat crop and seedling growth (Mufti, 2005). A large amount of wheat growing area in the north-western Bangladesh can be sown in late October, but early sown wheat crop suffers from juvenile heat stress which reduces tillers and seedling biomass, thereby negatively affecting grain yield (Rahman et al., 2019).

To ascertain the degree of sensitivity or tolerance to heat stress in wheat, different screening techniques have been proposed. Difference in leaf tissue temperature compared to ambient temperature, also known as leaf temperature depression (LTD) has been recognized as a reliable indicator of heat tolerance (Kumar et al., 2013). A proportional increase in ion leakage with increase in temperature has provided support to use ion leakage as an index for screening genotypes against heat and drought stress in wheat (Öztürk, Taşkesenligil, Haliloğlu, Aydin, & Çağlar, 2016; ElBasyoni, Saadalla, Baenziger, Bockelman, & Morsy, 2017). Almeselmani, Deshmukh and Sairam (2006) reported that increase in membrane injury index (MII) in late planted wheat genotypes, helped capture the damaging effect of high temperature during the reproductive stage. The integrity and stability of plasma membrane measured in seedlings exposed to heat and drought stress was established as a reliable physiological marker for determining stress tolerance during later stages of crop development (Rehman et al., 2016).

On exposure to heat stress, plants accumulate different signaling molecules or oxidative species and the degree to which plants can quench or balance their levels determines the degree of tolerance. Methylglyoxal is a highly reactive mutagenic, cytotoxic and genotoxic $\alpha\beta$-dicarbonyl aldehyde compound, which is highly accumulated under extreme environmental stresses, including heat stress (Hasanuzzaman, Hossain, & Fujita, 2011a, b; Hasanuzzaman and Fujita, 2011), which could act as a stress responsive signal. Methylglyoxal is synthesized in biological systems via various enzyme-catalyzed and spontaneous reactions e.g., glycolysis, lipid and protein metabolism and can react with and modify both proteins and DNA, leading to the generation of advanced glycation end products (AGEs) (Yadav, Singla-Pareek, Reddy, & Sopory, 2005; Kaur, Singla-Pareek, & Sopory, 2014; Mostofa et al., 2018). At high cellular concentrations, methylglyoxal inhibits cell proliferation and results in a number of adverse effects such as increasing the degradation of proteins and inactivating the antioxidant defense system (Hoque et al., 2010; Hossain, Teixeira da Silva, & Fujita, 2011).

The methylglyoxal detoxification system is ubiquitously spread across cellular compartments to resist methylglyoxal-mediated damage to the cellular constituents (Mostofa et al., 2018). In plants, methylglyoxal is detoxified mainly by the maintenance of GSH homeostasis via glyoxalase pathway containing enzymes, namely, glyoxalase I (Gly I) and glyoxalase II (Gly II) (Yadav, Singla-Pareek, Ray, Reddy, & Sopory, 2005; Yadav, Singla-Pareek, Reddy, & Sopory, 2005; Silva, Gomes, Ferreira, Freire, & Cordeiro, 2013; Kaur, Singla-Pareek, & Sopory, 2014). Gly I converts methylglyoxal to *S* -D-lactoylglutathione (SLG), utilizing GSH, while Gly II converts SLG to D-lactic acid and regenerates glutathione (GSH). Efficient detoxification of excess methylglyoxal produced during normal physiological processes or different abiotic stresses is one of the most important adaptive strategies of plant stress tolerance (Yadav, Singla-Pareek, Ray, Reddy, & Sopory 2005; Yadav, Singla-Pareek, Reddy, & Sopory, 2005; Hoque et al., 2010).

In addition, heat stress accelerates the generation of reactive oxygen species (ROS) including singlet oxygen ($^1O_2$), superoxide anion (O_2^{*-}), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^*), inducing oxidative stress in plants (Yin, Chen, & Yi, 2008; Hasanuzzaman et al., 2020). The main effects of ROS include autocatalytic peroxidation of membrane lipids and pigments, modification of membrane permeability and functions (Xu, Li, Zhang, Wei, & Cui, 2006). The level of lipid peroxidation has been widely used as an indicator of free radical damage to cell membranes under stress conditions. Malondialdehyde (MDA) is the principal and extensively studied product of polyunsaturated fatty acid (PUFAs) peroxidation. This aldehyde is a highly toxic molecule and needs to be considered more than just a marker of lipid peroxidation (Rio, Stewart, & Pellegrini, 2005). When PUFAs in bio-membranes are peroxidized, different aldehydes are

formed including the highly reactive aldehyde MDA. MDA is mostly generated in chloroplasts (Yamauchi, Furutera, & Sugimoto, 2008).

In general, responses of plants to heat stress may involve, among others, synthesis of various osmoprotectants, like proline and glycine betaine, heat shock proteins (Hasanuzzaman, Nahar, Alam, Roychowdhury, & Fujita, 2013), and antioxidative enzymes to reduce oxidative damage (Zhou et al., 2019). To counteract the toxicity of ROS a highly efficient anti-oxidative defense system, composed of both non-enzymatic and enzymatic constituents, is required. The enzymatic antioxidant defense mechanisms are represented by enzymes that include, superoxide dismutase (SOD); four enzymes of the ascorbate-glutathione cycle: ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and glutathione reductase (GR); catalase (CAT); glutathione peroxidase (GPX); and glutathione ?- transferase (GST) (Hasanuzzaman, Nahar, Anee, & Fujita, 2017; Noctor, Mhamdi, & Foyer, 2014). Non-enzymatic antioxidants include ascorbate (AsA), glutathione (GSH), tocopherol, flavanones, carotenoids, anthocyanins etc. (Noctor & Foyer, 1998; Gill & Tuteja, 2010). Carotenoids and anthocyanins are known to be efficient quenchers of reactive oxygen species, such as peroxide radicals and singlet oxygen molecules and thereby alleviate the oxidative damage (Eggersdorfer & Wyss, 2018; Agati, Azzarello, Pollastri, & Tattini, 2012).

The generation, effects and activities of ROS, ROS scavengers and their interplay have been reported in the seedlings of different crop species including wheat exposed to heat stress. Most studies that have taken this route, have been mostly limited to single or two wheat genotypes (Savicka & Škute, 2010). The genotypes used in the above studies are either not suitable under current growing conditions or not included in current cropping patterns Therefore, the overall aim of this study is to achieve results that are relevant to current cropping systems of Bangladesh and to address the gap in our understanding of heat stress responses during wheat seedling stage. To achieve this, we have selected four differentially thermotolerant wheat varieties and quantified the physiological and biochemical attributes to gain a mechanistic insight associated with heat-induced oxidative stress tolerance.

Specific objectives are formulated to - (i) determine changes in morpho-physiological traits and pigments content in the selected wheat varieties exposed to heat stress; (ii) capture the variation in the accumulation of oxidative stress indicators in response to heat stress; (iii) determine changes in oxidative stress alleviating antioxidants in wheat varieties under heat stress; and (iv) assess the association between traits and varieties in respect to heat-induced oxidative stress tolerance.

2 | MATERIALS AND METHODS

2.1 | Plant materials and stress treatment

Four wheat varieties differing in thermotolerance viz. a high yielding, extensively cultivated and heat tolerant BARI Gom 26 [BG26] (Hossain & Teixeira da Silva, 2013; Khatun, Ahmed, & Mohi-Ud-Din, 2015), moderately heat tolerant BARI Gom 25 [BG25] and BARI Gom 23 [BG23] (Hossain & Teixeira da Silva, 2013) and a widely used heat susceptible check variety Pavon 76 [Pavon] (Khatun, Ahmed, & Mohi-Ud-Din, 2015) were used.

Uniform sized seeds of four wheat varieties were selected and surface-sterilized with 70% ethanol followed by washing several times with sterile distilled water. The seeds were then soaked with distilled water for 10 min and sown in Petri plates (15 cm diameter) filled with sterile sand moistened with distilled water for germination for 3 days. After germination, seedlings were then moved to controlled environment chamber maintained at 25±1°C during day and night, relative humidity (RH) of 75–80%, 16 hours of photoperiod with a light intensity of 200 µmol photon m^{-2} s^{-1}. After 5 days, two sets of seedlings (5 Petri plates for each variety with 20 seedings in each plates) were then grown in two growth chambers with constant temperatures of 25±1°C (control) and 35±1°C (heat stress) with RH 75–80%, 16 hours photoperiod with a light intensity of 200 µmol photon m^{-2} s^{-1} for 48 hours. Growth chamber temperature and RH were monitored by a digital humidity and temperature meter (Model: HD-306, HTC Instruments, Taiwan). Petri plates were irrigated everyday with half-strength Hoagland's nutrient solution.

After termination of heat stress, shoot and root length and dry matter was recorded. Shoot and root length was measured from the root-shoot junction to the tip of the longest leaf and root, respectively. Dry matter was measured after oven drying the shoot and root at 60°C for at least 2 days, till a constant weight was achieved. Before harvesting fully expanded leaves were collected into liquid nitrogen and used for pigment determination and biochemical assays. Expanded leaves from about 20 seedlings were collected to form a single replicate and the same repeated twice from independent set of seedlings to obtained three biological replicates. All the measurements and assays were done in triplicate. Average of three biological replicates is presented in Tables and Figures.

2.2 | Determination of chlorophyll, carotenoid and anthocyanin content

Chlorophyll (Chl) content was determined by taking fresh fully opened leaf samples (0.2 g) from randomly selected seedlings. The samples were homogenized with 5 mL of acetone (80% v/v) using pre-cooled pestle and mortar and the homogenate was centrifuged at 5,000×g for 10 min. The absorbance was measured with a UV-visible spectrophotometer (Genesys 10S UV-VIS, Thermo Scientific, USA) at 663 and 645 nm and Chl contents were calculated using the equations proposed by Arnon (1949).

Carotenoids content was estimated according the procedure described by Lachman et al. (2003) and Rahman et al. (2018) with slight modifications. Briefly, acetone extract was obtained by mixing 5 mL of HPLC grade acetone with 0.5 g homogenized leaf sample in a glass vial and allowed to stand for 24 h at 4°C in the dark. The absorbance of the acetone extract was measured spectrophotometrically (Genesys 10S UV-VIS, Thermo Scientific, USA) against acetone at 444 nm. Total carotenoids content (mg g^{-1} FW of lutein equivalent) was calculated using the molar extinction coefficient of lutein.

Anthocyanin content was determined according to Hughes & Smith (2007) and Rahman et al. (2018) with some modifications. Briefly, 0.5 g of leaf sample was placed in the 5 mL solution of methanol, 6M hydrochloric acid and distilled water (70:7:23) and placed at 4°C for 24 hours in the air tight vials. To 2 mL aliquot, 1 mL distilled water and 2 mL chloroform was added, vortexed and centrifuged at 5,000×g for 15 min at 4°C. The upper chloroform layer was separated and the absorbance was measured with a UV-visible spectrophotometer (Genesys 10S UV-VIS, Thermo Scientific, USA) at 530 nm and the anthocyanin content (μg g^{-1} FW cyanidin-3-glucoside equivalent) was calculated using the molar extinction coefficient of cyanidin-3-glucoside.

Stability index (SI) of chlorophyll, carotenoid and anthocyanin was determined according to Sairam, Deshmukh and Shukla (1997) and calculated as follows- SI = (Pigment under stress/Pigment under control) × 100.

2.3 | Leaf temperature depression (LTD)

Leaf temperature was measured by hand held infrared thermometer (Model- MT4, HTC Instruments, Taiwan; distance-spot ratio 12:1) just before harvesting the seedlings for enzymatic assays. Measurements were taken maintaining an angle of approximately 30° to horizontal line at a distance of 12 inches from the top most fully opened leaf surface. Leaf temperature depression was calculated according to the method of Fischer et al. (1998), as growth chamber temperature minus leaf temperature of the seedlings. Simultaneously, temperature inside the growth chamber was recorded from the digital humidity and temperature meter. Data for each replication was the mean of five readings.

2.4 | Membrane injury index

Membrane injury index was determined by recording the electrical conductivity of leaf leachates in deionized water described by Deshmukh, Sairam and Shukla (1991). Briefly, leaf samples (0.1 g) were cut into uniform sized squares and placed in test tubes containing 10 ml of deionized water in two sets. One set was kept at 40°C for 30 min and another set at 100°C in boiling water bath for 15 min and their electric conductivities C_1 and C_2, respectively were measured by conductivity meter (Model: EC-400L, HumanLab Instrument Co.,

Korea).

$$\text{Membrane injury index} = \frac{C_1}{C_2} \times 100$$

2.5 | Methylglyoxal

Samples were prepared according to Yadav, Singla-Pareek, Ray, Reddy and Sopory (2005a) with some modifications. One gram leaf tissue was homogenized in 5 mL of 0.5 M perchloric acid. After incubating for 15 min on ice, the mixture was centrifuged at 4 °C for 10 min at 11,000×g. The supernatant was decolorized by adding charcoal (@10 mg mL^{-1}), kept for 15 min at room temperature, and centrifuged at 11,000×g for 10 min. Before using this supernatant for methylglyoxal assay, it was neutralized with saturated solution of potassium carbonate keeping at room temperature for 15 min and centrifuged again at 11,000×g for 10 min. The neutralized supernatant was used for methylglyoxal estimation.

Methylglyoxal was estimated according to the method described by Wild, Ooi, Srikanth and Munch (2012). Briefly, 20 µL of freshly prepared 500 mM *n* -acetyl-*L* -cysteine (in 100 mM sodium phosphate buffer, p^H 7.0) was added to 980 µL of neutralized supernatant and incubated for 5 min at 22°C. A control solution was prepared without adding neutralized supernatant. After incubation, A_{288}nm was measured and control absorbance was subtracted. The final concentration of methylglyoxal was calculated from the standard curve and expressed in terms of µmol g^{-1} FW.

2.6 | Membrane lipid peroxidation and H_2O_2 content

The level of membrane lipid peroxidation was measured by estimating malondialdehyde (MDA), a decomposed product of the peroxidized polyunsaturated fatty acid component of the membrane lipid, using thiobarbituric acid (TBA) as the reactive material following the method of Heath & Packer (1968). The leaf samples (0.5 g) were homogenized in 3 mL 5% (w/v) trichloroacetic acid (TCA) and the homogenate was centrifuged at 11,500×g for 10 min. One mL supernatant was mixed with 4 mL of TBA reagent (0.5% of TBA in 20% TCA). The reaction mixture was heated at 95 °C for 30 min in a water bath and then quickly cooled in an ice bath and centrifuged at 11,500×g for 15 min. The absorbance of the colored supernatant was measured at 532 nm and was corrected for non-specific absorbance at 600 nm. The concentration of MDA was calculated by using the extinction coefficient of 155 mM^{-1} cm^{-1} and expressed as nmol of MDA g^{-1} fresh weight.

H_2O_2 was assayed according to the method described by Yu, Murphy and Lin (2003). H_2O_2 was extracted by homogenizing 0.5 g of leaf samples with 3 ml of 50 mM K-phosphate buffer p^H (6.5) at 4°C. The homogenate was centrifuged at 11,500×g for 15 min. Three mL of supernatant was mixed with 1 mL of 0.1% $TiCl_4$ in 20% H_2SO_4 (v/v), and the mixture was then centrifuged at 11,500×g for 12 min at room temperature. The optical absorption of the supernatant was measured spectrophotometrically at 410 nm to determine the H_2O_2 content (ϵ= 0.28 μM^{-1} cm^{-1}) and expressed as µmol g^{-1} fresh weight.

2.7 | Proline and glycine betaine

Free proline in leaf tissues was appraised spectrophotometrically as acid-ninhydrin method following the protocol of Bates, Waldren and Teari (1973). The proline content was determined as µmol g^{-1}FW calculated from a standard curve. Glycine betaine content was measured spectrophotometrically by 1,2-dichloroethane method following the procedure of Valadez-Bustos et al. (2016) and expressed as µmol g^{-1} FW calculated using a standard curve prepared from the series of known concentrations of betaine.

2.8 | Ascorbate and glutathione

Wheat leaves (0.5 g fresh weight) were homogenized in 3 ml ice-cold acidic extraction buffer containing 5% meta phosphoric acid and 1 mM EDTA using a mortar and pestle. Homogenates were centrifuged at 11,500×g for 15 min at 4°C, and the supernatant was used for analysis of ascorbate and glutathione following the methods described in Hasanuzzaman et al. (2014).

2.9 | Soluble protein

Fresh leaf tissue (0.5 g) were homogenized in 1 mL extraction buffer containing 1mM ascorbic acid, 1M KCl, 0.5M K-P buffer (p^H 7.0), β-marcaptoethanol and glycerol in ice-cold mortar and pestle. The homogenate was centrifuged at 11,500×g for 15 min, and the supernatant was used as a soluble protein solution for enzyme activity. The protein concentration of each sample was determined following the method of Bradford (1976) using BSA as a protein standard.

2.10 | Assays for antioxidant and glyoxalase enzyme activity

Glyoxalase I (Gly I, EC: 4.4.1.5) assay was carried out according to Hasanuzzaman et al. (2014). Briefly, the assay mixture contained 100 mM K-phosphate buffer (p^H 7.0), 15 mM magnesium sulphate, 1.7 mM GSH and 3.5 mM MG in a final volume of 700 µL. The reaction was started by the addition of methylglyoxal and the increase in absorbance was recorded at 240 nm for 1 min. The activity was calculated using the extinction coefficient of 3.37 mM^{-1} cm^{-1}.

Glyoxalase II (Gly II, EC: 3.1.2.6) activity was determined according to the method of Hasanuzzaman et al. (2014) by monitoring the formation of GSH at 412 nm for 1 min. The reaction mixture contained 100 mM Tris-HCl buffer (p^H 7.2), 0.2 mM DTNB and 1 mM S-D-lactoylglutathione (SLG) in a final volume of 1 mL. The reaction was started by the addition of SLG and the activity was calculated using extinction co-efficient of 13.6 mM^{-1} cm^{-1}.

Superoxide dismutase (SOD, EC: 1.15.1.1): SOD activity was assayed based on the competition between SOD and NBT for the production of superoxide from xanthine and xanthine oxidase interaction following Spitz and Oberley (1989).

Lipoxygenase (LOX, EC: 1.13.11.12) activity was carried out according to the method of Doderer et al. (1992). The activity was written as µmol min^{-1} mg^{-1} protein considering the extinction coefficient of 25,000 $M^{-1}cm^{-1}$.

Catalase (CAT, EC: 1.11.1.6) activity was measured according to the method of Hasanuzzaman et al. (2014) by monitoring the decrease of absorbance at 240 nm for 1 min caused by the decomposition of H_2O_2. The reaction mixture contained 50 mM K-phosphate buffer (p^H 7.0), 15 mM H_2O_2 and enzyme solution in a final volume of 700 µL. The reaction was initiated with enzyme extract and the activity was calculated using the extinction coefficient of 39.4 M^{-1} cm^{-1}.

Guaiacol peroxidase (POD, EC: 1.11.1.7) activity was measured as described by Castillo, Penel and Greppin (1984). The reaction mixture contained 10 mM phosphate buffer with p^H 6.1, 12 mM hydrogen peroxide, 96 mM guaiacol and 20 µL enzyme extract. Blank contained complete reaction mixture without H_2O_2. Absorbance was recorded at 470 nm for 1 minute and the activity was calculated using the extinction co-efficient of 26.6 mM^{-1} cm^{-1}.

Glutathione peroxidase (GPX, EC: 1.11.1.9) activity was measured as described by Elia, Galarini, Taticchi, Dörr and Mantilacci (2003) using H_2O_2 as a substrate. The reaction mixture consisted of 100 mM Na-phosphate buffer (p^H7.5), 1 mM EDTA, 1 mM NaN_3, 0.12 mM NADPH, 2 mM GSH, 1-unit GR, 0.6 mM H_2O_2 and 20 µL of sample solution. The reaction was started by the addition of H_2O_2. The oxidation of NADPH was recorded at 340 nm for 1 min and the activity was calculated using the extinction co-efficient of 6.62 mM^{-1} cm^{-1}.

Glutathione ?-transferase (GST, EC: 2.5.1.18) activity was determined spectrophotometrically by the method of Hasanuzzaman et al. (2014) with some modifications. The reaction mixture contained 100 mM Tris–HCl buffer (p^H 6.5), 1.5 mM GSH (reduced glutathione), 1 mM 1-chloro-2,4-dinitrobenzene (CDNB), and enzyme solution in a final volume of 700 µL. The enzyme reaction was initiated by the addition of CDNB and the increase in absorbance was measured at 340 nm for 1 min. The activity was calculated using the extinction coefficient of 9.6 mM^{-1} cm^{-1}.

Glutathione reductase (GR, EC: 1.6.4.2) activity was measured according to Hasanuzzaman et al. (2014). The reaction mixture contained 0.1 M K-P buffer (pH 7.0), 1 mM EDTA, 1 mM GSSG, 0.2 mM NADPH, and enzyme solution in a final volume of 1 mL. The reaction was initiated with GSSG and the decrease in absorbance at 340 nm was recorded for 1 min. The activity was calculated using an extinction coefficient of 6.2 $mM^{-1}cm^{-1}$.

Ascorbate peroxidase (APX, EC: 1.11.1.11) activity was assayed following the method of Hasanuzzaman et al. (2014). The reaction buffer solution contained 50 mM K-phosphate buffer (pH 7.0), 0.5 mM AsA, 0.1 mM H_2O_2, 0.1 mM EDTA, and enzyme extract in a final volume of 0.7 mL. The reaction was initiated by the addition of H_2O_2 and activity was measured by observing the decrease in absorbance at 290 nm for 1 min using extinction co-efficient of 2.8 mM^{-1} cm^{-1}.

Monodehydroascorbate reductase (MDHAR, EC: 1.6.5.4) activity was determined by the method of Hasanuzzaman et al. (2014). The reaction mixture contained 50 mM Tris-HCl buffer (pH 7.5), 0.2 mM NADPH, 2.5 mM AsA, 0.5 unit of AO and enzyme solution in a final volume of 700 µL. The reaction was started by the addition of AO. The activity was calculated from the change in absorbance at 340 nm for 1 min using an extinction coefficient of 6.2 $mM^{-1}cm^{-1}$.

Dehydroascorbate reductase (DHAR, EC: 1.8.5.1) activity was determined by the procedure of Hasanuzzaman et al. (2014). The reaction buffer contained 50 mM K-P buffer (pH 7.0), 2.5 mM GSH, and 0.1 mM DHA. The reaction was started by adding the sample solution to the reaction buffer solution. The activity was calculated from the change in absorbance at 265 nm for 1 min using extinction coefficient of 14 $mM^{-1}cm^{-1}$.

2.11 | Statistical analysis

All data obtained were subjected to 2-factor (treatment × varieties) analysis of variance (ANOVA) in general linear model and the mean differences were compared by Tukey's HSD test using the *R*packages *lme4* and *agricolae* . Differences at $P<0.05$ were considered significant. Radarplot was prepared using *R* packages *fmsb* and *reshape2* . The stress tolerance index (STI) for all physiological and biochemical traits (Supplementary Table S1) was calculated using the following formula: $STI = [(X_c \times X_s) / (X_c)^2]$ (Fernandez, 1992), where X_c and X_s indicates the observed values of a trait in a given variety under non-stress and heat-stress treatments, respectively, while X_c is the average value of a particular trait examined in all varieties under non-stress condition. The library *Pheatmap* of *R* version 4.0.2 was adapted to compute normalized mean values for generating heatmap and hierarchical clusters (Kolde, 2012). The principal component analysis (PCA) was carried out using the packages *ggplot2* ,*Factoextra* and *FactoMineR* in *R* version 4.0.2 (Lê, Josse, & Husson, 2008; Wickham, 2016). Correlation coefficient matrix were visualized using *R* package *corrplot* .

3 | RESULTS

3.1 | Effect of variety and heat stress on the studied traits

In the experimental setup including four wheat varieties and two growing conditions, all traits were significantly affected by both these factors but with different magnitudes (Supplementary Table S2). Variation in LTD and *Chl a /b* were significantly affected by variety but not treatment. The interaction effect was significant in half the number of measured traits, but the contribution to the variation was lesser than the main effects.

3.2 | Seedling length and dry matter

Heat stress substantially reduced shoot length (SL) and root length (RL) of all varieties (Figure 1A & B; for mean difference, see Supplementary Table S3). The reduction was the highest in Pavon and least in BG26, while in other two varieties the reduction was intermediate. The shoot dry matter (SDM) and root (RDM) and the total dry matter (TDM) was reduced in all varieties due to heat stress (Figure 1B). As a result of heat stress, the lowest TDM was recorded in Pavon (72% of the control) with the highest in BG26 (94% of the control), while on average 86% TDM was recorded in other two varieties (Figure 1B).

3.3 | Chlorophyll, carotenoid and anthocyanin content

The *Chl* concentration in the wheat leaves decreased markedly under heat stress compared to control conditions in all varieties (Table 1). Compared to the control, *Chl a* content was decreased by 14, 16, 14 and 26% in BG23, BG25, BG26 and Pavon, respectively, due to heat stress. Similar to *Chl a* , total *Chl* also decreased upon exposure to heat stress where the reduction was lower in the heat tolerant BG26 (13%) compared to heat susceptible Pavon (21%).*Chl b* and *Chl a/b* , however, remained statistically unchanged due to heat stress in all varieties. Carotenoid content in the wheat leaves decreased markedly due to heat stress in all varieties though the decrease was not statistically significant (Table 1). Anthocyanin content in the wheat leaves decreased significantly due to heat stress in all varieties (Table 1). The pigment was decreased by 22, 16, 15 and 29% in BG23, BG25, BG26 and Pavon, respectively, upon exposure to heat stress. The stability of the pigments was higher in heat tolerant BG26 and lower in Pavon (Table 1).

3.4 | Leaf temperature depression and membrane injury index

In this study, leaf temperature depression (LTD) in all wheat varieties were decreased under heat stress (35°C) conditions compared to control (25°C) (Figure 2A). Among the tested varieties, BG26 maintained relatively higher LTD (2.15 and 2.07°C in control and heat stress, respectively) while Pavon showed comparatively lower LTD (0.53 and 0.40°C) under both growing conditions. The LTD were decreased by 14, 6, 4, and 25% in BG23, BG25, BG26 and Pavon, respectively, under heat stress compared to their respective control seedlings, but none of them differed significantly between treatments.

There was a significant increase in membrane injury index (MII) in all wheat varieties except BG26 under heat stress conditions. Pavon recorded a maximum increase in MII on exposure to heat stress compared to other varieties whereas, BG26 had the lowest increase in MII under heat stress (Figure 2B). The percentage increase in MII under heat stress compared to control was 19, 16, 9 and 40% in BG23, BG25, BG26, and Pavon, respectively.

3.5 | Membrane lipid peroxidation, LOX activity and H_2O_2 level

The membrane lipid peroxidation levels in leaf tissues measured as the MDA content, increased significantly in seedlings exposed to heat stress, irrespective of the wheat variety (Figure 3A). Under heat stress condition, the relative increase in MDA content was 42, 39, 38 and 108% in BG23, BG25, BG26 and Pavon, respectively, compared to control. Comparatively higher relative increase (108%) of MDA content in Pavon indicated higher leakiness, lower thermostability and higher fluidity of membrane compared to other varieties. On average, the other three varieties recorded 39.5% increase in MDA.

LOX activity of wheat leaves recorded significant increase in all tested varieties, upon exposure to heat stress (Figure 3B). As a result of heat stress, the least 23% increase in LOX activity was recorded in BG26 with the highest (114%) in Pavon, while on average 46% increase in other two varieties.

A significant increase in the cellular H_2O_2 level was observed in wheat leaves in response to heat stress compared to control (Figure 3C). Upon heat treatment, the amount of H_2O_2 increased by 43, 39, 35, and 87% in BG23, BG25, BG26 and Pavon, respectively, compared to control. Lower relative increase in H_2O_2 level in BG25 and BG26 indicates lower cellular toxicity and oxidative damage compared to BG23 and Pavon.

3.6 | Methylglyoxal level and detoxifying enzymes

Methylglyoxal levels varied slightly and ranged from 4.60 to 5.97 μmol g^{-1} FW under control, but the level increased significantly due to heat stress and the levels ranged from 7.19 to 10.81 μmol g^{-1} FW between the wheat varieties (Figure 3D). The relative increase in methylglyoxal level were 61, 63, 56 and 81% in BG23, BG25, BG26, and Pavon, respectively relative to control. Higher relative increase in methylglyoxal level in Pavon indicated higher cellular toxicity, increased degradation of membrane proteins, lipids and nucleic acid under heat stress.

Increase in Gly I activity was observed in all wheat varieties in response to heat stress, with a significant increase in BG23, BG25 and BG26, but not in Pavon (Figure 3E). Heat stress resulted in 17, 22, 25, and 10% increase in Gly I activities in BG23, BG25, BG26, and Pavon, respectively, compared to control.

The activity of Gly II was increased slightly with heat stress in all wheat varieties but the increase was non-significant (Figure 3F). As a result of heat stress, the highest increase (19%) in Gly II activity was recorded in BG26 with the least (8%) in Pavon, while on average 14% increase was observed in other two varieties.

3.7 | Activity of osmolytes and non-enzymatic antioxidants:

Under control condition, statistically similar proline level was detected in all wheat varieties (Figure 4A). However, heat stress lead to a significant increase in proline accumulation in wheat seedlings of all four varieties. Upon exposure to heat stress, proline content increased by 17, 25, 39 and 12% in BG23, BG25, BG26 and Pavon, respectively compared to control.

Though statistically similar glycine betaine (GB) content in the wheat leaves was observed under control conditions, heat stress resulted in a substantial increase in GB content in all wheat varieties (Figure 4B). GB content increased by 90, 121, 180 and 68% in BG23, BG25, BG26 and Pavon, respectively, under heat stress conditions compared to control.

Similarly, glutathione (GSH) content was significantly increased under heat stress in all four tested wheat varieties (Figure 4C). On exposure to heat stress, BG26 recorded 99% higher GSH content with the lowest increase (25%) recorded in Pavon, while other two varieties averaged at 54% increase in GSH. Leaf AsA content decreased significantly under heat stress in BG25, BG23 and Pavon, but the reduction was not significant in BG26 (Figure 4D). Under heat stress, BG23 and BG25 on average recorded 34% lower AsA, while Pavon recorded 61% lower AsA compared to 15% decrease in BG26.

3.8 | ROS scavenging enzymes

In response to heat stress, increase in SOD activity was observed in all wheat varieties with a significant increase in BG23, BG25 and BG26, but not in Pavon (Figure 5A). Heat stress resulted in 16, 19, 36, and 8% increase in SOD activities in BG23, BG25, BG26, and Pavon, respectively, compared to control.

The CAT activity was significantly decreased under heat stress in all the wheat varieties (Figure 5B), recording a decrease by 20, 18, 15, and 38% in BG23, BG25, BG26, and Pavon, respectively, compared to control. Lower relative decrease of CAT activity in BG25, BG26 and BG23 indicated an efficient and stable ROS scavenging system in these varieties than that of Pavon.

Similarly, POD activity was significantly decreased under heat stress in BG23, BG25 and Pavon, but the decrease was not significant in BG26 (Figure 5C). Upon exposure to heat stress, the least 8% decrease in POD activity was recorded in BG26 and the highest 22% in Pavon with an average of 17% in the other two varieties.

Heat stress resulted in a significant increase in GPX activity in wheat seedlings of BG23, BG25, BG26 and but the increase was not significant in Pavon (Figure 6A). The highest GPX activity was recorded in BG26 and the lowest in Pavon under both control and heat stress conditions. Due to heat stress, the GPX activity was increased by 41, 53, 61 and 38% in BG23, BG25, BG26, and Pavon, respectively, over control.

GST activity of wheat leaves recorded a significant increase in all tested varieties, on exposure to heat stress (Figure 6B). As a result of heat stress, the lowest increase (11%) in GST activity was recorded in Pavon with the highest (24%) in BG26, while on average a 21% increase in other two varieties.

3.9 | AsA-GSH cycle enzymes

There was a significant increase in the glutathione reductase (GR) activity in all wheat varieties on exposure to heat stress (Figure 7A) and the increase ranged between 26 to 102% with BG26 recording the highest increase and Pavon the least.

Significant increase in APX activity was observed in all the tested varieties under heat stress (Figure 7B). Both under control and heat stress conditions, the highest APX activity was recorded in BG26 and the

lowest in Pavon. Under heat stress, the highest increase in APX activity was recorded in BG26 (63%) with the least (26%) in Pavon while other two were intermediate with 32 and 48% increase in APX activity.

Compared to the control, the activities of DHAR and MDHAR was decreased markedly in all wheat varieties due to heat stress (Figure 7C & D). Upon heat treatment, the activity of MDHAR decreased by 23, 22, 13 and 35% in BG23, BG25, BG26 and Pavon, respectively, compared to control, without a significant decline only in BG26. The DHAR activity was also decreased in the same manner, and the decrease ranged between 5 to 26% with BG26 having the lowest and Pavon recording the highest decline.

3.10 | Assessment of the association between treatment, varieties and traits using hierarchical clustering and principal component analysis (PCA)

The STI values of all measured physiological and biochemical traits were used to develop heatmap, hierarchical clustering and PCA. From the hierarchical clustering, two groups (Group-1 and -2) were obtained involving various physiological and biochemical traits measured among the wheat varieties (Figure 8A). Group-2 included all the measured traits, except MDA, H_2O_2, MII, LOX, MG,*Chl a* and *Chl a/b* which formed Group-1 (Figure 8A). In comparison to BG26, five of the traits in Group-1 exhibited a highly consistent increasing trend in Pavon, whereas Group-2 traits exhibited significantly decreasing pattern (Figure 8A). The other varieties like BG23 and BG25 showed varying direction in changes among traits in both groups. Specifically, variety BG26 displayed decreasing trend in Group-1 and increasing trends in the traits of Group-2, which represented opposite trends in Pavon (Figure 8A). In addition, hierarchical clustering of the varieties revealed three distinct clusters namely Cluster-1, -2 and -3. BG23 and BG25 were placed in the Cluster-1 and were strongly associated with BG26 compared to Pavon (Figure 8A). BG26 and Pavon were positioned in the Cluster-2 and -3, respectively.

Subsequently, we performed PCA analysis using STI values to assess the relationships among treatment, varieties and traits using different physiological and biochemical traits (Figure 8B). The two components PC1 and PC2 explained 78.8 and 12.7% of the total phenotypic variation, respectively (Figure 8B). Interestingly, the PCA results revealed that traits of Group-2 *viz* . DHAR, GR, GSH, SOD, Car, TDM, APX, AsA, CAT, LTD, Gly II, GB and *T.Chl* were the major contributes in PC1 and were strongly associated with BG26, while the traits from Group-1 which included H_2O_2, MDA, LOX, MG and MII were strongly associated with Pavon (Figure 8B). *Chl a* trait of Group-1 was found to be the most closely correlated with BG25, while ratio of *Chl a* to *b* was the most closely linked with BG23 (Figure 8B).

4 | DISCUSSION

4.1 | Pigment stability confers judicious light absorption under heat stress

Heat stress generates significantly higher levels of ROS including $^1O_2$, $O_2{}^{*-}$, H_2O_2 and OH^*, thereby inducing oxidative stress in plants (Mittler, 2002; Yin, Chen, & Yi, 2008). Excess energy that has not been used for photosynthesis will lead to higher amounts of ROS, which cause oxidative damage to chloroplasts and other cell structures, when exposed to heat stress (Asada, 1994; Singh & Singhal, 2001). Heat stress also reduces *Chl*biosynthesis, disintegration of chloroplast membranes and disruption of biochemical reactions in photosystems (Havaux, 1998). In this study, heat stress decrease in *Chl* content in wheat leaves could be attributed to impairment of *Chl* biosynthesis or rapid degradation (Efeoglu & Terzioglu, 2009; Aien, Khetarpal, & Pal, 2011; Reda & Mandoura, 2011; Yüzbaşioğlu, Dalyan, & Akpinar, 2017). Interestingly, the tolerant wheat variety BG26 recorded greater stability of the photosynthetic pigments under heat stress compared to the heat susceptible Pavon, with a similar finding observed with late to very late planted wheat (Almeselmani, Deshmukh, Sairam, Kushwaha, & Singh, 2006). Pigments stability particularly *Chl a* reflected by a stable *Chl a/b* ratio in the most tolerant BG26 compared to other varieties, indicated its ability to sustain a higher proportion of the vulnerable *Chl a* content under heat stress (Table 2).

Carotenoids and anthocyanins are known to protect cellular damage by stabilizing membrane fluidity (Wahid, Gelani, Ashraf, & Foolad, 2007 and Mishra, Srivastava, Prasad, & Abraham, 2008) and act as effective antioxidants (Shao et al., 2007). In this study, significantly lower reduction in photosynthesis-related pigments

and higher stability in heat tolerant BG26 compared to other varieties, imply slower degradation of chlorophylls, carotenoids and anthocyanins, allowing stability in the light absorption machinery in BG26 under heat stress (Table 1). Further, higher carotenoids and anthocyanins content would enhance heat tolerance in BG26 through stable membrane system and effective ROS scavenging system.

4.2 | Leaf temperature depression, membrane disruption and cellular toxicity correlated with oxidative damage

High leaf temperature depression (LTD) or a cooler leaf has been used as a powerful and robust selection criterion to improve tolerance of plants to heat and drought stress (Blum, 1996; Fischer et al., 1998; Reynolds, Ortiz-Monasterio, & McNab, 2001; Ayeneh, van Ginkel, Reynolds, & Ammar, 2002; Deva, Urban, Challinor, Falloon, & Svitákova, 2020). LTD was the highest in BG26, closely followed by BG25, indicating the presence of an efficient transpiration cooling mechanism in these varieties compared to the other two (Figure 2A). LTD is an important heat avoidance mechanism (Deva, Urban, Challinor, Falloon, & Svitákova, 2020) that helps to maintain higher assimilation rate in stress condition (Sharma, Sareen, Saini, & Shefali, 2017) by improving stomatal conductance (Reynolds, Ortiz-Monasterio, & McNab, 2001) and protecting chloroplasts (Luan et al., 2018). In addition to LTD, membrane injury index (MII) is a collective measure of cell membrane disruption due to stress and has been extensively used as a reliable marker for the estimation of stress injury in plants (Bajji, Kinet, & Lutts, 2002). Genotypes such as BG26 (Figure 2B) having stable and functional cell membrane under heat stress are considered as promising candidates for further enhancing heat tolerance in wheat varieties (Hemantaranjan, 2014; ElBasyoni, Saadalla, Baenziger, Bockelman, & Morsy, 2017).

H_2O_2 is a toxic compound which is harmful to cells, resulting in lipid peroxidation and membrane injury (Pastori & Trippi, 1992; Mishra, Jha, & Dubey, 2011) and thereby produce highly reactive and cytotoxic aldehyde derivative MDA (Alché, 2019) (Figure 9). Elevated levels of methylglyoxal levels is reported in different plants exposed to abiotic stressors (Yadav et al., 2005a; Singla-Pareek, Yadav, Pareek, Reddy, & Sopory, 2006). MDA levels and LOX activity are used as a measure of membrane lipid peroxidation and oxidative stress, and the impact it has on increasing membrane fluidity; increase in leakiness of the membrane; and damage to membrane proteins, enzymes, and ion channels (Garg & Manchanda, 2009; Lim et al., 2015). Significant increase in the MG levels in Pavon indicates the extent of inactivation of the vital defense system and irreparable metabolic dysfunction under heat stress. In addition, a significantly higher H_2O_2, MDA and LOX levels in Pavon adds further support to higher level of heat stress sensitivity, due to the inhibition or insufficient induction of the antioxidant defense systems compared to the tolerant BG26. MII, H_2O_2, MG, MDA and LOX collectively induce oxidative stress as these traits were significantly positively correlated under heat stress (Figure 10). Comparatively, a higher relative increase of Gly I and II in the heat tolerant BG26 (Figure 9) confers efficient MG detoxification in addition to playing a role in maintaining GSH homeostasis and subsequent ROS detoxification.

4.3 | Enhanced osmolytes accumulation alleviate heat-induced oxidative stress in wheat

Proline accumulation in plants has been associated with enhanced tolerance under different stresses, including unfavorable temperature conditions, either low or high temperatures (Hayat et al., 2012; Majláth et al., 2012). As an important osmolyte, accumulation of proline is shown to alleviate osmotic stress induced by heat stress (Li et al., 2018). In this study, differences in the proline level between the wheat genotypes were not substantial, but were markedly higher in plants exposed to heat stress at 35°C compared to control (Figure 4A). This is consistent with other studies, where even higher increases have been reported in heat stress exposed wheat plants (Khan, Din, Qayyum, Jan, & Jenks, 2015).

In vitro experiments have demonstrated glycine betaine (GB) in protecting some enzymes and protein complexes from heat-induced destabilization (Gorham, 1995; Wang, Zhang, Li, Luo, & Wang, 2010) and increased the levels of AsA and GSH in transgenic wheat by activating the synthesis of the molecules (Wang, Zhang, Li, Luo, & Wang, 2010). In the present study, higher accumulation of GSH (Figure 4C) and GB (Figure 4B) in the heat tolerant BG26 indicates lesser disruption of pathways synthesizing these antioxidants under heat stress compared to other varieties. Both glycine betaine and proline provide protection against

oxidative stress by reducing H_2O_2 and lipid peroxidation levels and by increasing the antioxidant defense and MG detoxification systems (Hossain et al., 2010).

4.4 | Stable antioxidant enzyme system is crucial for heat tolerance

In the plant cells, superoxide dismutase (SOD) provides primary protection against O_2^{*-}, which is then converted to H_2O_2 for subsequent metabolism to H_2O by catalases (CAT) and peroxidases (POD, APX and GPX), thus protecting the cell damage (Gill & Tuteja, 2010; Sarafraz-Ardakani, Khavari-Nejad, Moradi, & Najafi, 2014). In this study, the activity of both CAT and POD decreased significantly in all wheat varieties upon exposure to heat stress, which could be due to its inactivation by the accumulated H_2O_2induced by heat. But the extent of reduction varied with the relative tolerance of the varieties, *i.e.* comparatively higher reduction in susceptible Pavon than tolerant BG26 (Figure 5A B & C). Higher relative increase in SOD activity along with lower relative decrease of CAT and POD activity may be beneficial to detoxifying O_2^{*-} and H_2O_2 induced by heat stress in tolerant varieties, and *vice versa* in susceptible varieties. Our results align with other studies wherein a significant increase in SOD (Gupta et al., 2013) and reduction in CAT (Sato et al., 2001; Jiang & Huang, 2001; Anjali, Shantha, & Pathak, 2006; Lu, Sang, & Ma, 2007) and POD activity under heat stress is observed, including wheat (Almeselmani, Deshmukh, Sairam, Kushwaha, & Singh, 2006; Zhang et al., 2015) and maize (Hussain et al., 2019).

Glutathione peroxidases (GPX) are a family of isozymes that use GSH to reduce H_2O_2 and lipid hydroperoxides (ROOHs) (Figure 9), and therefore protect plant cell membrane from oxidative damage (Noctor, Gomez, Vanacker, & Foyer, 2002; Kuhn & Borchert, 2002). Our results showed a sharp increase in GPX activity in response to heat stress with a relatively higher increase in the tolerant BG26, indicating increased ability of the tolerant variety to scavenge H_2O_2 and ROOHs, similar to Hasanuzzaman, Nahar, Alam and Fujita (2012). The plant glutathione-*S* -transferases (GST) are a large and diverse group of enzymes that catalyze the conjugation of electrophilic xenobiotic substrates with the GSH and are associated with inducing tolerance to different abiotic stresses (Hossain, Hossain, & Fujita, 2006; Dixon, Skipsey, & Edwards, 2010). More than 2-fold increase in GST activity was recorded in the tolerant BG26 compared to the susceptible Pavon (Figure 6B), thereby decreasing the levels of MDA and H_2O_2 in wheat seedlings exposed to heat stress (Figure 3A & C). Similar increases in GST activity under heat stress were observed in wheat (Hasanuzzaman, Hossain, & Fujita, 2011a; Hasanuzzaman, Nahar, Alam, & Fujita, 2012) and maize (Tiwari & Yadav, 2019).

Taken together, apart from the AsA-GSH cycle, SOD, CAT, POD, GPX and GST are important antioxidant enzymes in plants. Our results demonstrated that the heat tolerant BG26 seedlings countered ROS production by maintaining relatively higher amount of CAT and POD, and increasing the activities of SOD, GPX and GST under heat stress (Figure 5 & 6).

4.5 | Efficient operation of Ascorbate-glutathione cycle is pivotal for heat tolerance

Ascorbate–glutathione cycle is the core metabolic pathway to detoxify ROS and recycling of non-enzymatic antioxidants. This vital cycle contains four enzymes: APX, MDHAR, DHAR and GR which are systematically and proportionately involved in the H_2O_2detoxification (Figure 9). Other than H_2O_2 detoxification, these enzymes are also actively involved in the regeneration of non-enzymatic antioxidants like AsA and GSH. The presence of AsA and GSH has been reported to improve osmoregulation, plant water status and nutrient status, water use efficiency, photosynthetic performance, reduce oxidative stress and improve overall productivity in plants (Hasanuzzaman et al., 2019; Tiwari & Yadav, 2019). Our results demonstrate that AsA content markedly decreased under heat stress in all wheat varieties and it was less pronounced in heat tolerant BG26 (Figure 4D). A decline in AsA is probably due to its direct role in scavenging ROS, since AsA is considered to be the first line of defense against oxidative stress. The greater decline of AsA content in heat susceptible Pavon indicated the increased extent of AsA utilization to counter the higher amount of ROS produced under heat stress.

The central role of GSH in the antioxidant defense system is due to its ability to regenerate AsA through reduction of DHA in the AsA-GSH cycle (Noctor & Foyer, 1998; Hasanuzzaman et al., 2019) (Figure 9). Our results indicated a significant increase in the level of GSH in seedlings exposed to heat stress (Figure

4C) with a significantly higher accumulation in heat tolerant BG26 than others. This finding is in agreement with Tiwari & Yadav (2019) and Kocsy, Szalai and Galiba (2002), a multi-fold increase in GSH content was recorded in tolerant maize and wheat varieties under heat stress exposure. They also suggested that an increase in GSH content under heat stress was due to higher rate of GSH synthesis which was further accelerated by the enhanced GR activity. Similarly, in the present study, higher increase in the GSH content in BG26 was well coordinated leading to an increased GR activity under heat stress (Figure 7A).

APX is a key enzyme in the AsA-GSH cycle and plays a vital role in plant defense against oxidative stress by catalyzing the conversion of H_2O_2 to water (Figure 9). Its essential role in the scavenging of H_2O_2 in chloroplasts, where CAT is absent, has been well established (Pandey et al., 2017). Our results show higher relative increase in APX activity during heat stress in the tolerant BG26 and lower in heat susceptible Pavon, which is in agreement with Dash & Mohanty (2002); Almeselmani, Deshmukh, Sairam, Kushwaha and Singh (2006) and Tiwari & Yadav (2019). In addition, a significantly higher GR activity observed (Figure 7A) would complement APX in H_2O_2 scavenging (Apel & Hirt, 2004).

Results demonstrated that the activities of MDHAR and DHAR decreased markedly due to heat stress, which are partially supported by Rivero, Ruiz and Romero (2004). The slight increase in APX and GR activities were not sufficient to protect the seedlings from ROS induced damages in susceptible variety Pavon. As the MDHAR and DHAR are equally important in regulating the level of AsA and its redox state under oxidative stress (Eltayeb et al., 2006; 2007; Wang, Zhang, Li, Luo, & Wang, 2010), decreases in the activities of these enzymes were followed by a decrease in AsA content; and these decreases was more pronounced in heat susceptible variety Pavon (Figure 4D). Findings from this study revealed AsA–GSH cycle to be more efficiently operating in tolerant variety BG26 than that of Pavon (Figure 9). The differential response of these varieties to heat stress as a consequence of variation in the levels of enzymatic and non-enzymatic antioxidants suggests that manipulation of AsA–GSH pathway may be a promising route to enhance heat tolerance in wheat.

5 | CONCLUSION

In conclusion, a comprehensive analysis of the oxidative species and antioxidant interactions allowed us to capture the pathways that induced greater heat tolerance in BG26 seedlings exposed to heat stress, compared to other varieties. BG26 was able to maintain highest dry matter and pigment stability, due to increased proline and glycine betaine levels, lower accumulation of H_2O_2, MDA, LOX and MG content and reduced oxidative stress with enhanced antioxidant capacity. Our findings demonstrated that heat-tolerant attributes are closely associated with the overall antioxidant activities to aid plants to maintain cellular homeostasis under heat stress. Our results establish a reference for further molecular analysis in wheat at seedling stage, using the contrasting responses between BG26 (heat-tolerant), BG23 and BG25 (moderate) and Pavon (heat-sensitive), to better adapt wheat to early-stage heat stress. Further investigations are needed to establish a pipeline for translating findings from seedling stage to different developmental stages including yield and yield related parameters.

ACKNOWLEDGMENTS

We gratefully acknowledge the partial financial support of the Research Management Wing (RMW), Bangabandhu Sheikh Mujibur Rahman Agricultural University, Gazipur, Bangladesh. We also extend our thanks to Molecular Breeding Laboratory, Bangladesh Agricultural Research Institute, Gazipur, Bangladesh for providing lab facilities during the research work. This is contribution no. 21-156-J from the Kansas Agricultural Experiment Station.

Conflict of Interest

The authors declare no conflict of interest

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TABLE 1 . Leaf pigment contents of wheat varieties grown under control and heat stress conditions.

Variety	Growing Condition	Chl a (mg g^{-1} FW)	Chl b (mg g^{-1} FW)	Total Chl (mg g^{-1} FW)	Chl a/b ratio	Carotenoid (mg g^{-1} FW)	Anthocyanin (µg g^{-1} FW)¶
BG23	Control	0.49±0.01^{ab}	0.26±0.01^{c-e}	0.75±0.01^{a-c}	1.88±0.13^{a}	0.09±0.008^{b-d}	71.51±0.55^{b}
	Heat stress	0.42±0.01^{c}	0.23±0.01^{e}	0.65±0.01^{de}	1.83±0.12^{ab}	0.07±0.012^{c-e}	55.73±0.47^{d}
		(85.6±1.45)	(88.8±1.58)	(86.7±1.24)		(77.8±5.88)	(78.0±1.25)
BG25	Control	0.50±0.02^{a}	0.32±0.02^{ab}	0.83±0.02^{a}	1.57±0.12^{a-c}	0.13±0.015^{ab}	80.40±0.67^{a}
	Heat stress	0.42±0.01^{c}	0.28±0.01^{b-e}	0.70±0.02^{cd}	1.50±0.01^{bc}	0.11±0.012^{bc}	67.40±0.50^{c}
		(83.5±0.83)	(87.2±5.61)	(84.7±1.67)		(84.6±1.04)	(83.9±1.26)
BG26	Control	0.44±0.01^{bc}	0.35±0.01^{a}	0.79±0.01^{ab}	1.26±0.03^{c}	0.17±0.009^{a}	79.25±0.83^{a}
	Heat stress	0.38±0.01^{cd}	0.31±0.01^{a-c}	0.70±0.01^{cd}	1.23±0.08^{c}	0.15±0.014^{ab}	67.56±0.59^{c}
		(87.2±2.58)	(89.5±2.61)	(88.2±0.35)		(88.2±8.48)	(85.3±0.77)
Pavon	Control	0.43±0.01^{bc}	0.30±0.01^{a-d}	0.73±0.02^{bc}	1.42±0.05^{c}	0.05±0.012^{de}	71.21±0.74^{b}
	Heat stress	0.32±0.02^{d}	0.25±0.01^{de}	0.58±0.02^{e}	1.28±0.10^{c}	0.03±0.009^{e}	50.74±0.65^{e}
		(75.3±4.41)	(83.6±4.88)	(78.6±1.62)		(60.0±8.45)	(71.3±0.91)

Values represented as mean ±SE. Figures in the parentheses indicate stability of that pigment. Values in a column with different letter(s) are significantly different at P[?]0.05.lutein equivalent, and¶cyanidin-3-glucoside equivalent.

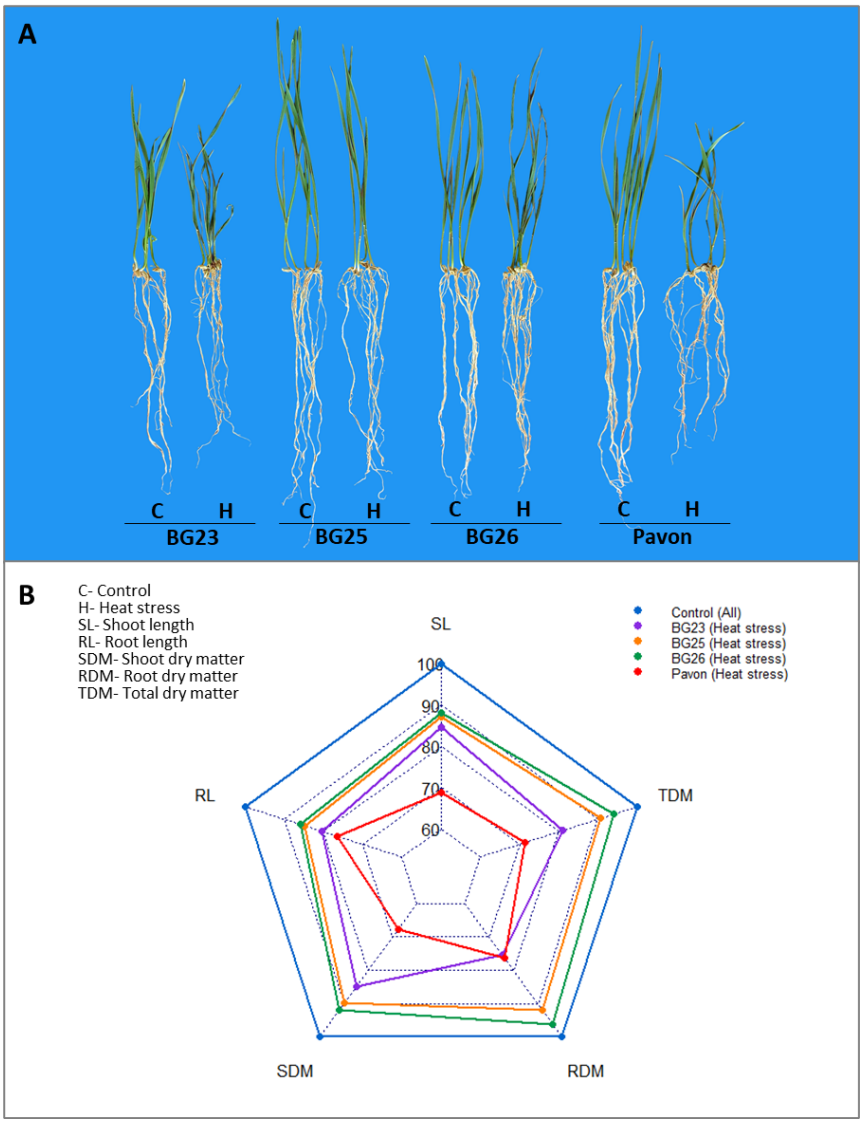


FIGURE 1 (A) Comparative changes in shoot and root length of wheat varieties under control and heat stressed conditions. (B) Radar plot showing changes in various morphological traits in different wheat varieties caused by heat stress. The values are expressed as % of the control.

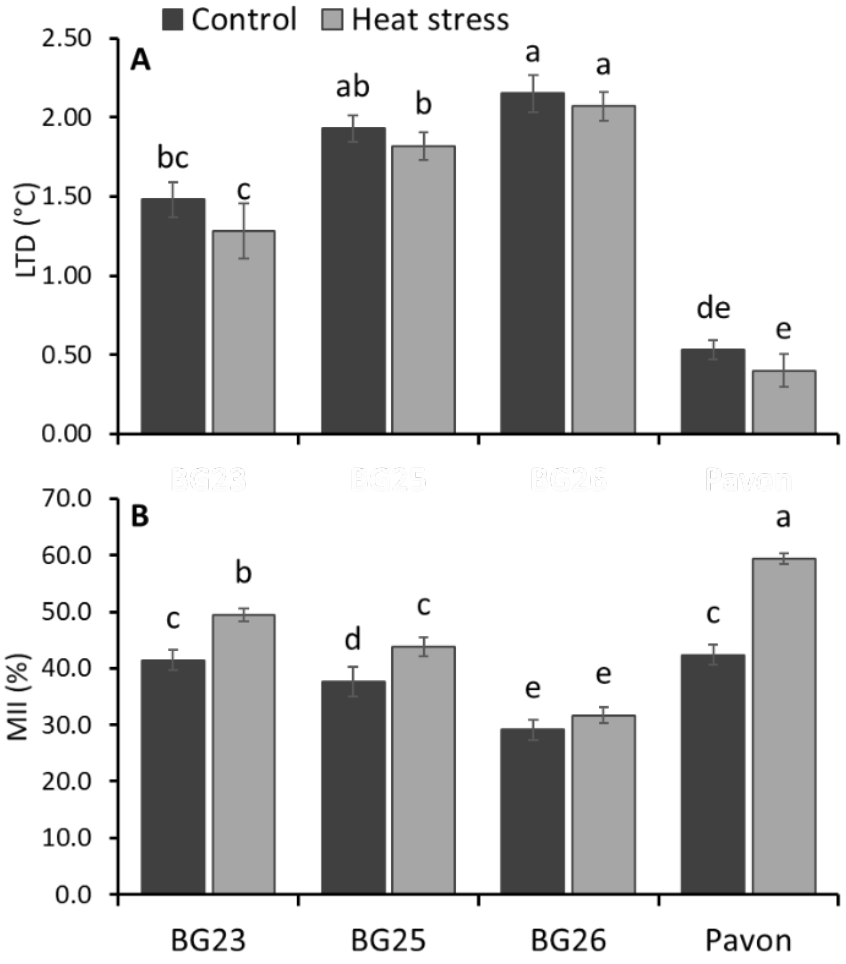


FIGURE 2 (A) Leaf temperature depression (LTD) and (B) membrane injury index (MII) of wheat varieties grown in the growth chamber under control and heat stressed conditions. Vertical bars represent +/- SE values. Different letter(s) indicate significant difference at $P<0.05$.

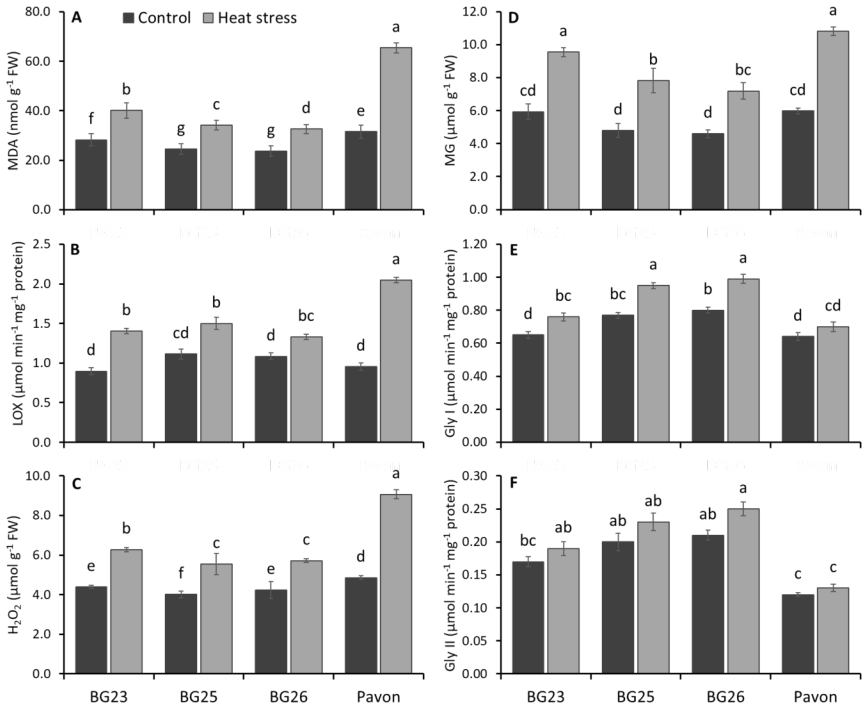


FIGURE 3 Changes in- (A) malondialdehyde (MDA), (B) lipoxygenase (LOX), (C) hydrogen peroxide (H_2O_2), (D) methylglyoxal (MG) content and the specific activity of (E) glyoxalase I (Gly I) and (F) glyoxalase I (Gly II) enzymes of wheat varieties grown under control and heat stressed conditions. Vertical bars represent +/- SE values. Different letter(s) indicate significant difference at $P<0.05$.

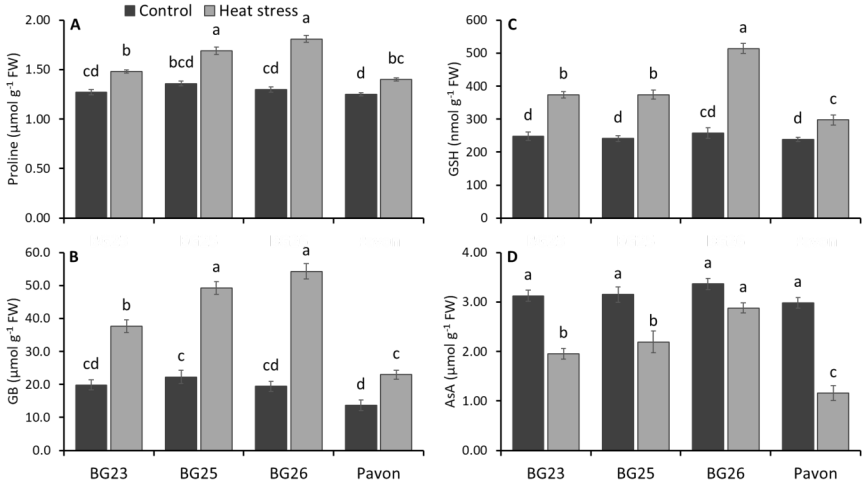


FIGURE 4 Changes in osmolytes and non-enzymatic antioxidants- (A) proline, (B) glycine betaine (GB), (C) glutathione (GSH) and (D) ascorbate (AsA) content of wheat varieties under control and heat stressed conditions. Vertical bars represent +/- SE values. Different letter(s) indicate significant difference at $P<0.05$.

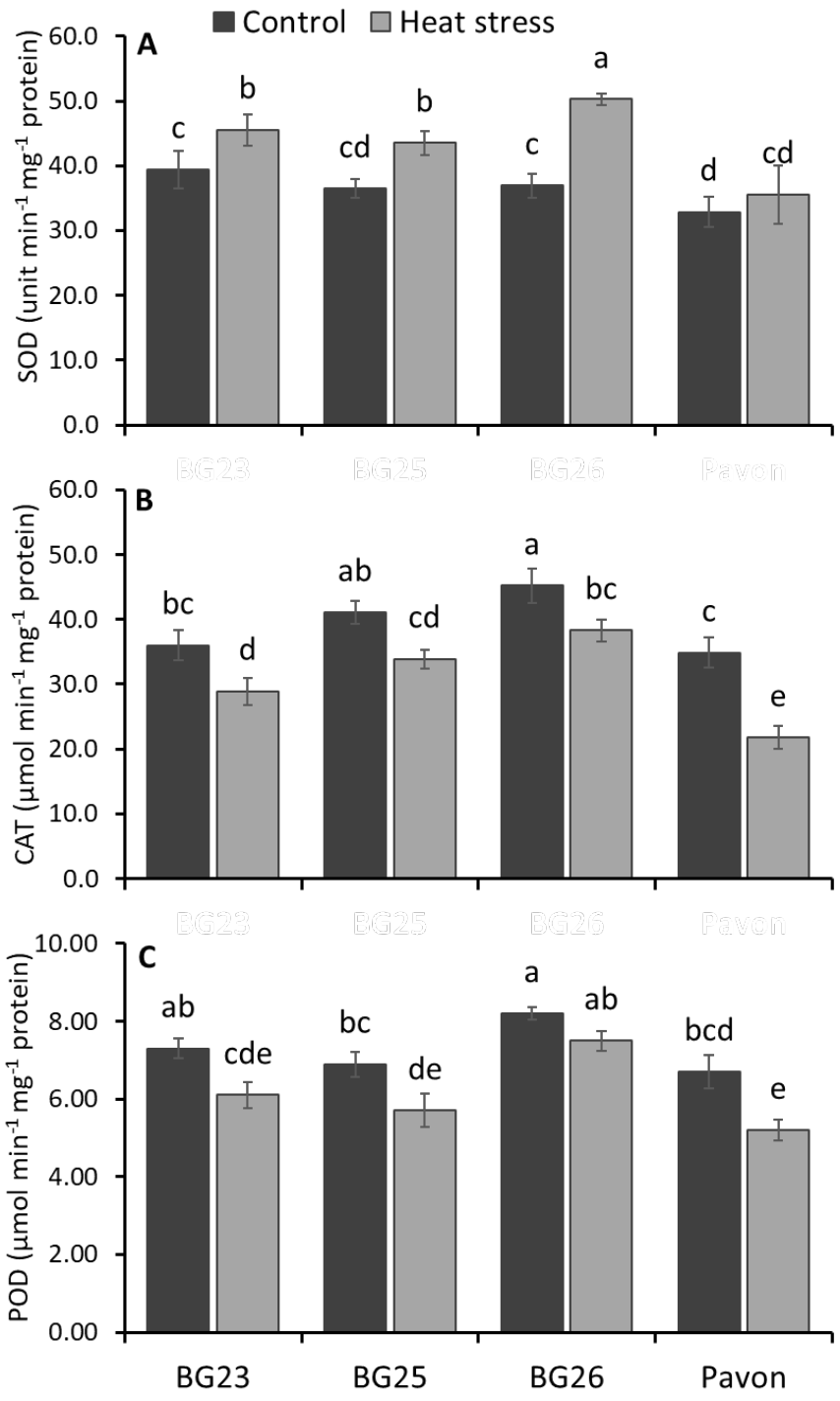


FIGURE 5 Specific activity of (A) superoxide dismutase (SOD), (B) catalase (CAT), and (C) guaiacol peroxidases (POD) enzymes of wheat varieties under control and heat stressed conditions. Vertical bars represent +/- SE values. Different letter(s) indicate significant difference at $P<0.05$.

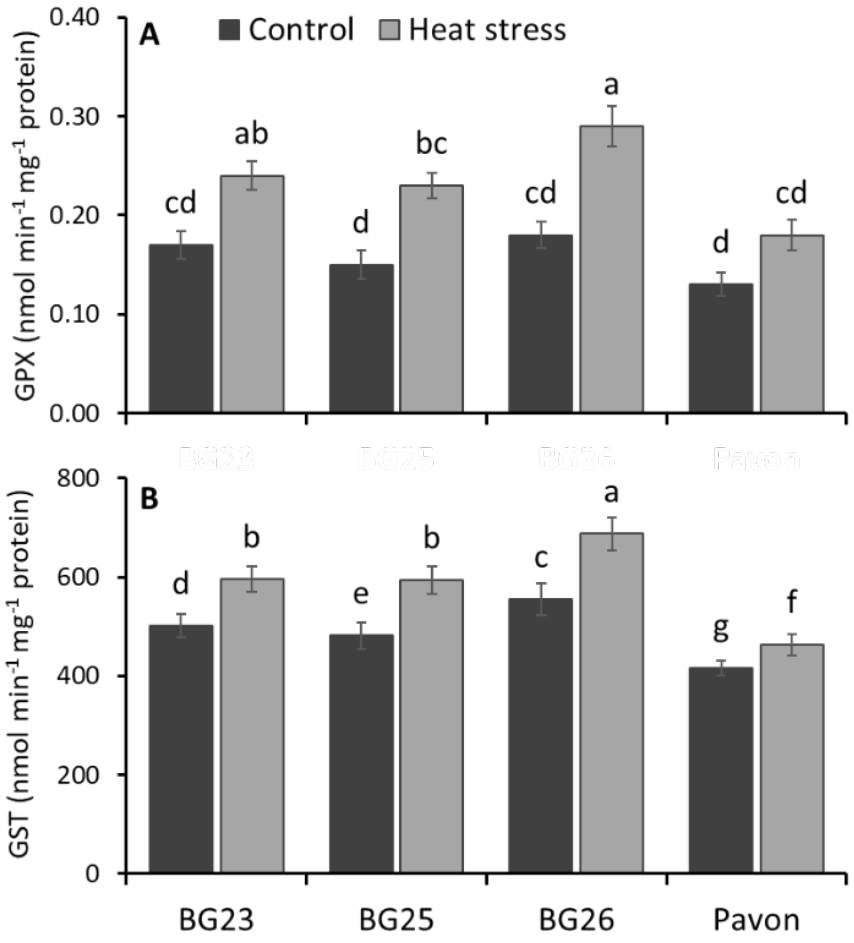


FIGURE 6 Specific activity of (A) glutathione peroxidase (GPX) and (B) glutathione-S-transferase (GST) enzymes of wheat varieties under control and heat stressed conditions. Vertical bars represent +/- SE values. Different letter(s) indicate significant difference at $P<0.05$.

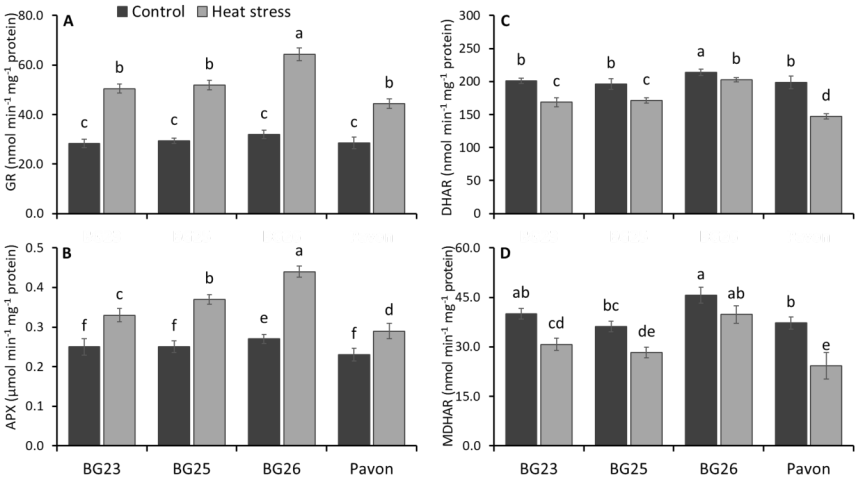


FIGURE 7 Specific activity of (A) glutathione reductase (GR), (B) ascorbate peroxidase (APX), (C) dehydroascorbate reductase (DHAR) and (D) monodehydroascorbate reductase (MDHAR) of wheat varieties under control and heat stressed conditions. Vertical bars represent +/- SE values. Different letter(s) indicate significant difference at $P<0.05$.

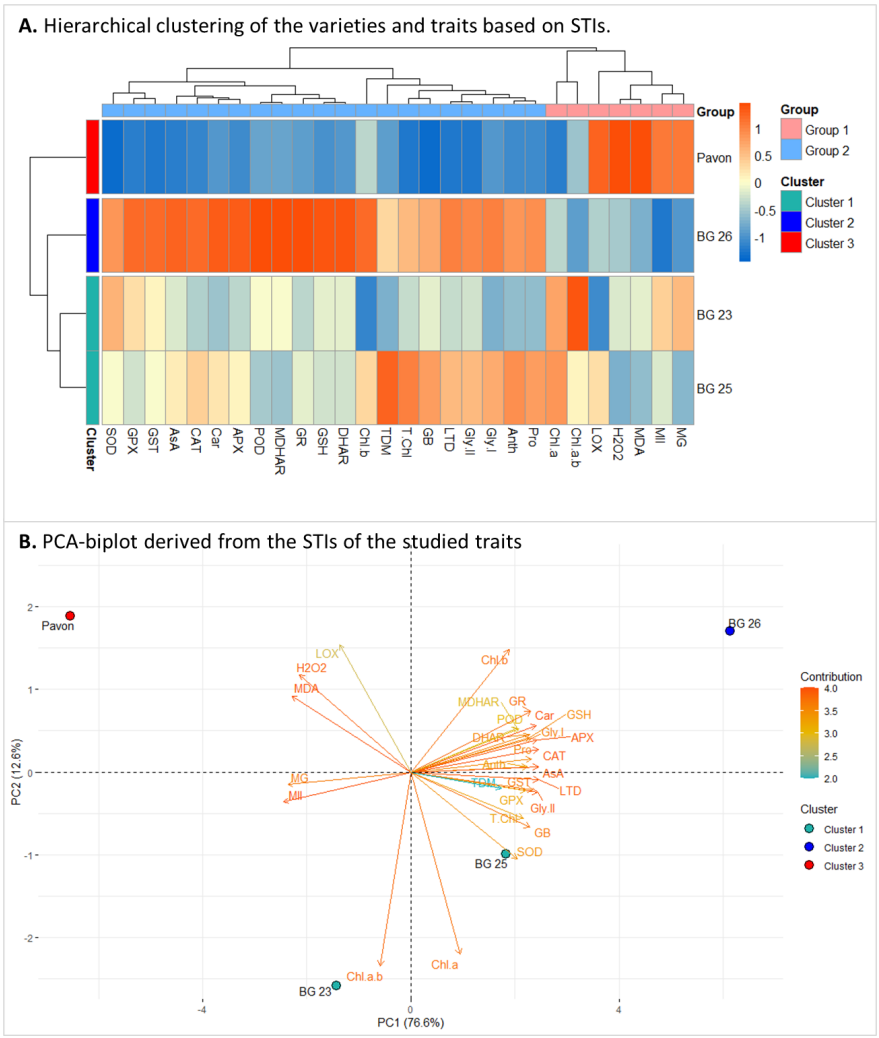


FIGURE 8 Hierarchical clustering and PCA-Biplot indicates association between treatments, variables and varieties.**(A) Hierarchical clustering:** The STI (Stress Tolerance Index) mean values obtained from the studied traits of all varieties, which were then normalized and clustered. Two distinct groups were obtained at the variable levels (Group-1 & 2) for all varieties. Different color scale expresses the intensity of the normalized mean values of various traits. **(B) PCA-Biplot:** Varieties dispersed in different ordinates based on the dissimilarity among them. The length and color intensity of a vector in the biplot indicate the quality of representation and the contribution of the variable on the principal component, respectively. The angles between the vectors derived from the middle point of biplots exhibit positive or negative interactions among the traits. The traits included LTD (leaf temperature depression), MII (membrane injury index), MG (methylglyoxal), MDA (malondialdehyde), LOX (lipoxygenase) H_2O_2 (hydrogen peroxide), Pro (proline), GB (glycine betaine), TDM (total dry matter), STI (stress tolerance index), GSH (glutathione), AsA (ascorbate), Chl *a*(chlorophyll *a*), Chl *b* (chlorophyll *b*), T.Chl (total chlorophyll), Chl.a.b (chlorophyll a/b), Car (carotenoids), Anth (anthocyanin), Gly.I (glyoxalase I), Gly.II (glyoxalase II), SOD (superoxide dismutase), CAT (catalase), POD (peroxidase), GPX (Glutathione peroxidase), GST (Glutathione *S* -transferases), GR (glutathione reductase), APX (ascorbate peroxidase), MDHAR (monodehydroascorbate reductase) and DHAR (dehydroascorbate reductase).

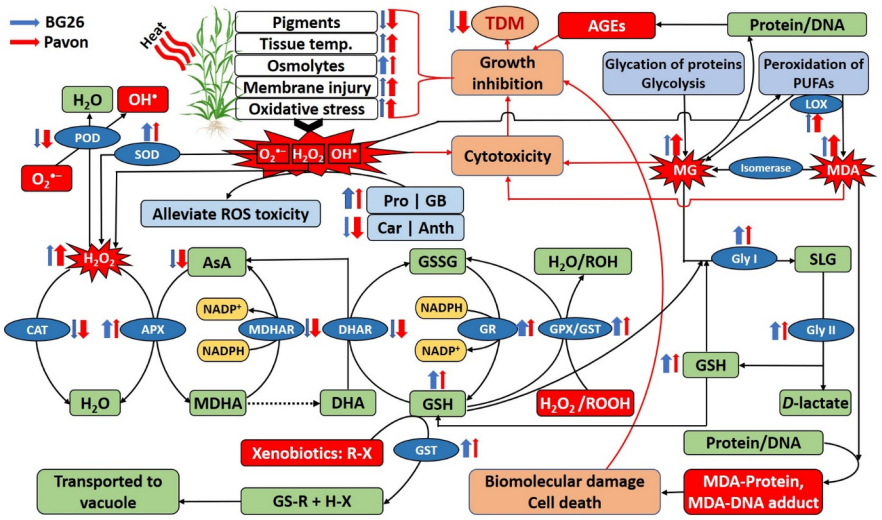


FIGURE 9 Schematic representation of the generation of ROS, MG and MDA due to heat stress and parametabolic reactions of AsA-GSH cycle and glyoxalase system in plants involved in ROS and MG detoxification. Red boxes and explosions indicate cellular oxidative stressors. Blue ellipses and green boxes represent the enzymes and substrate/product of the reactions, respectively. The blue and red arrows represent BG26 and Pavon, respectively. Direction of the arrows indicate an overall increase (upwards) or decrease (downwards) on exposure to heat stress within a variety. Size of the arrow in terms of thickness indicates the amount/activity level with thicker arrows indicating higher change (increase or decrease) under heat stress compared to control between the varieties.

AGE- advanced glycation end product, Anth- anthocyanin, APX- ascorbate peroxidase, AsA- ascorbate, Car- carotenoids, CAT- catalase, DHA- dehydroascorbate, DHAR- dehydroascorbate reductase, DNA- deoxyribonucleic acid, GB- glycine betaine, Gly I- glyoxalase I, Gly II- glyoxalase II, GPX- Glutathione peroxidase, GR- glutathione reductase, GSH- glutathione (reduced), GSSG- glutathione (oxidized), GST- Glutathione *S* -transferases, H_2O_2- hydrogen peroxide, LOX- lipoxygenase, MDA- malondialdehyde, MDHA- monodehydroascorbate, MDHAR- monodehydroascorbate reductase, MG- methylglyoxal, NADPH- nicotinamide adenine dinucleotide phosphate (reduced), POD- peroxidase, Pro- proline, PUFA- polyunsaturated fatty acid, ROS- reactive oxygen species, SLG- *S* -D-lactoylglutathione, SOD- superoxide dismutase, and TDM- total dry matter.

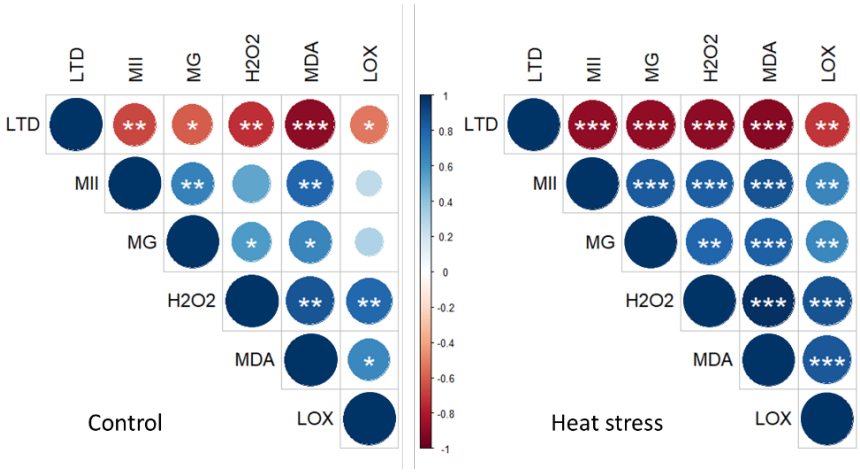
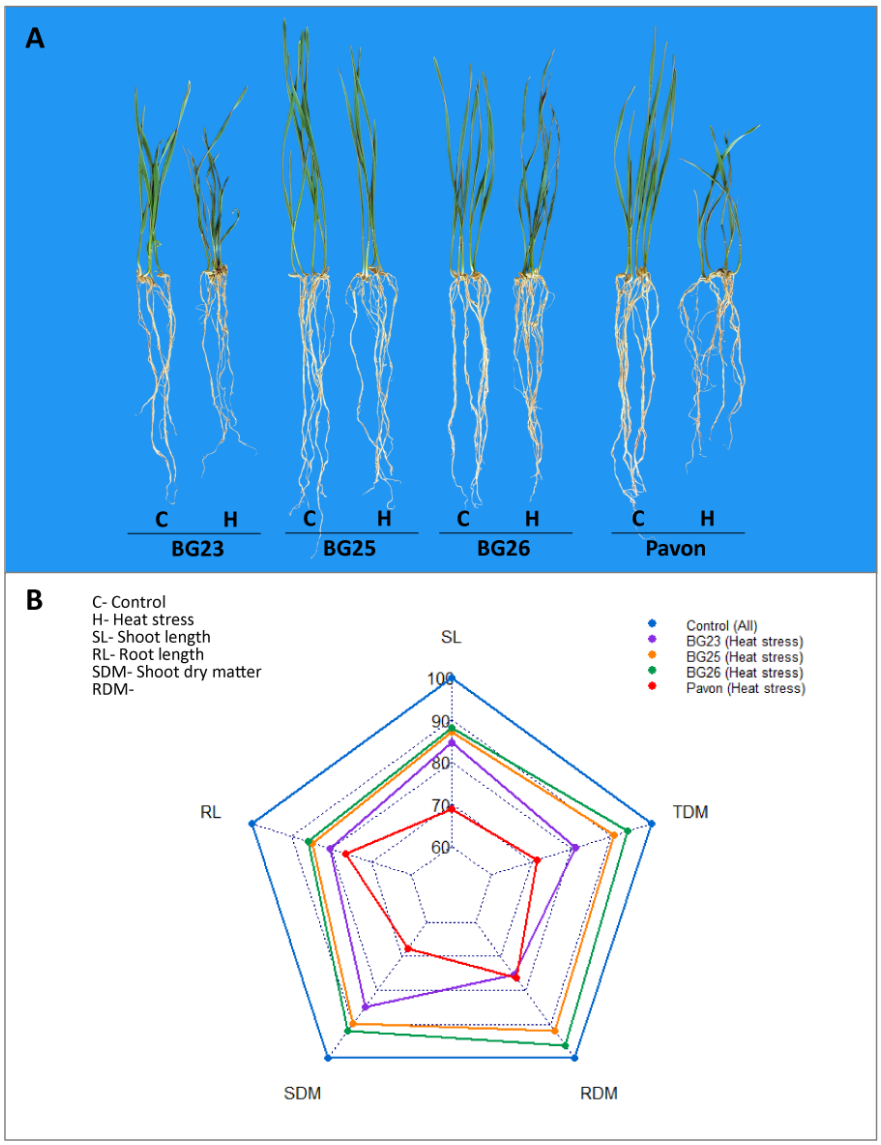
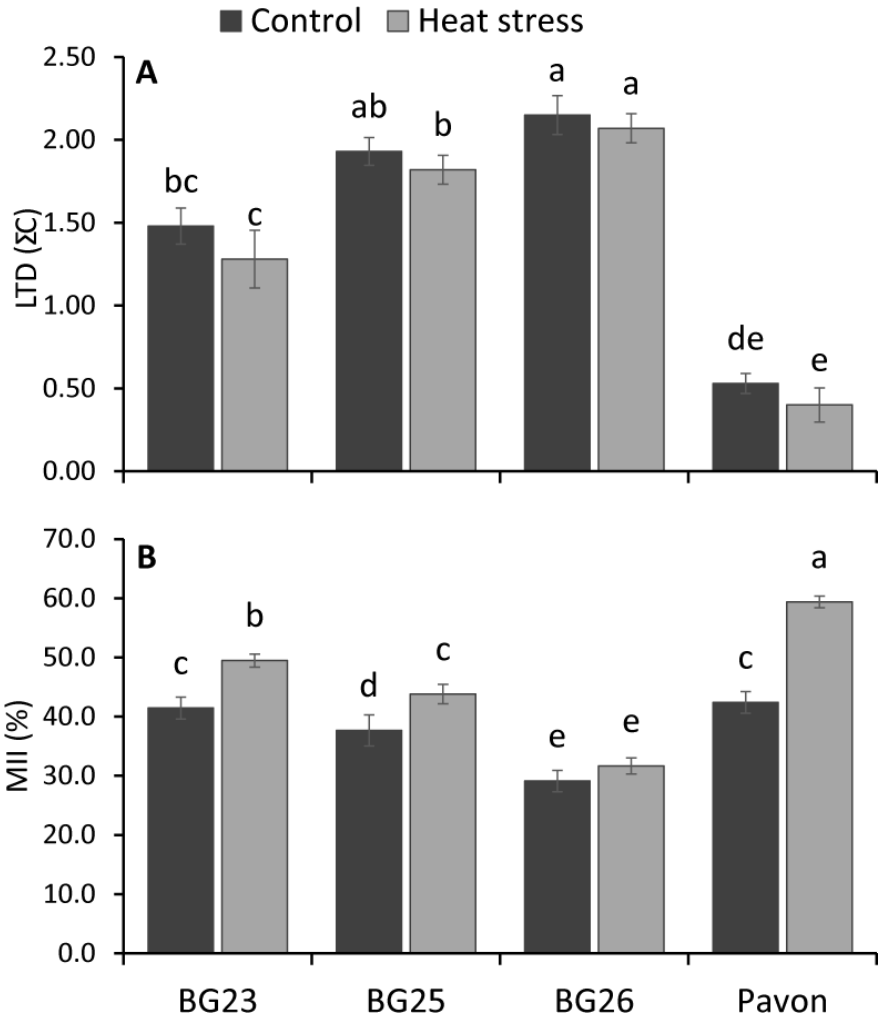
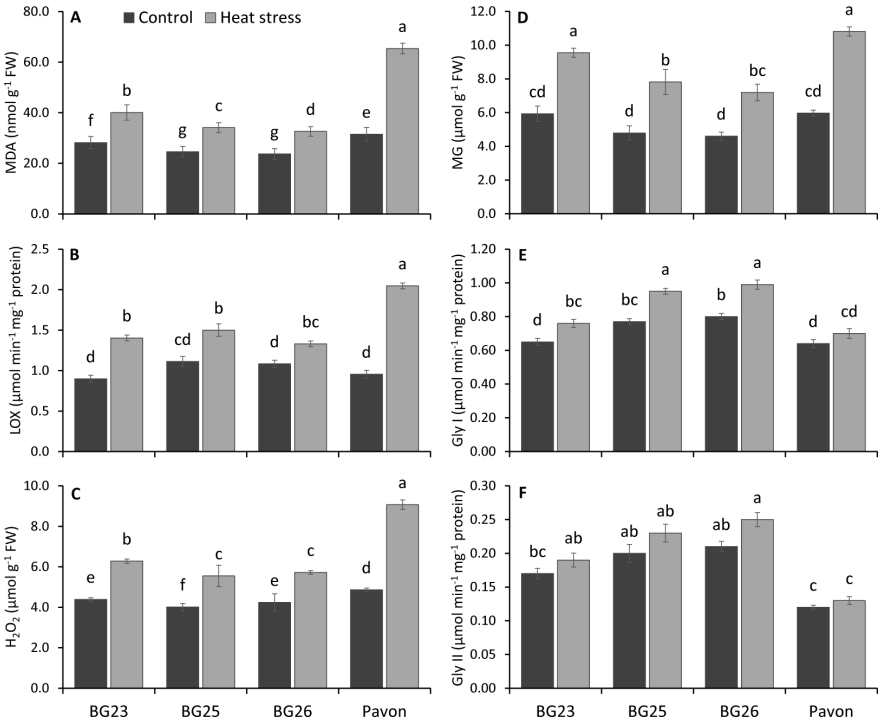
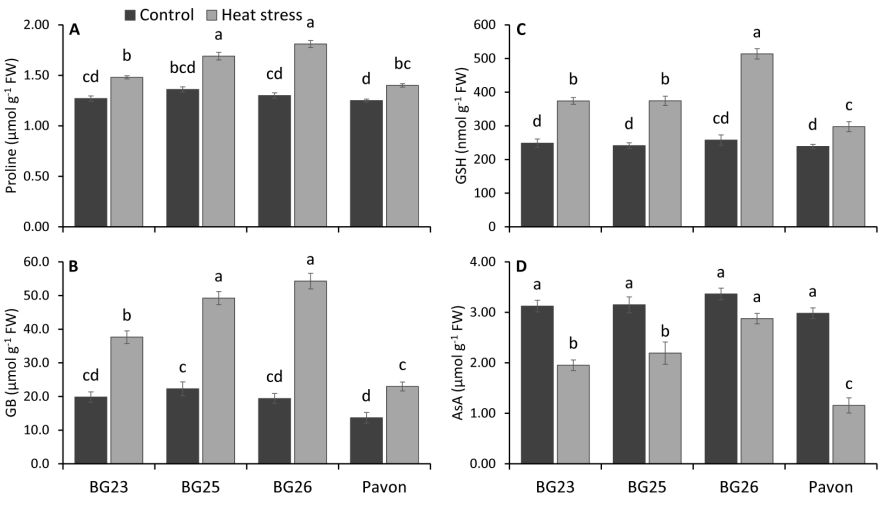


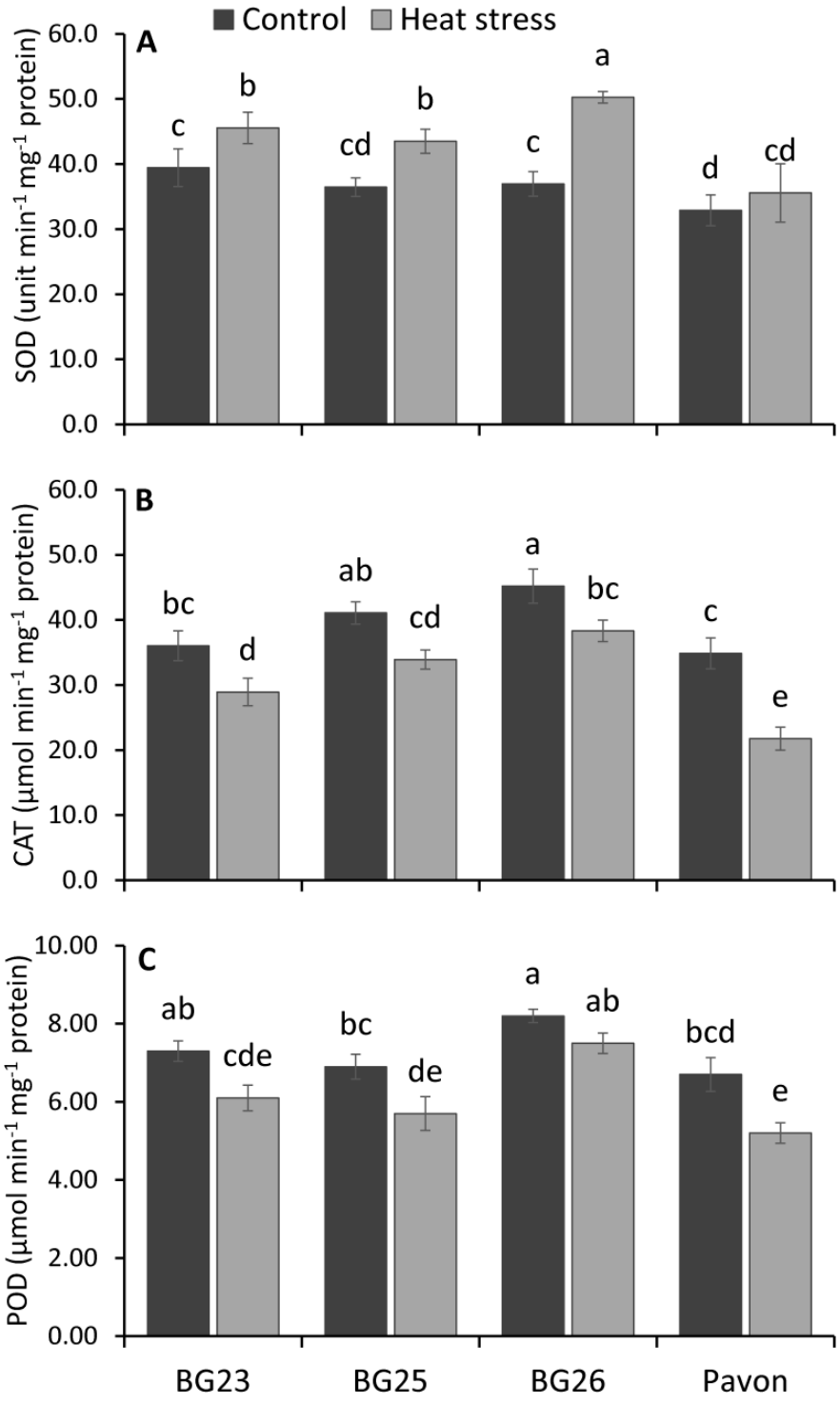
FIGURE 10 Correlation coefficients among the parameters that collectively induced oxidative stress. The parameters included LTD (leaf temperature depression), MII (membrane injury index), MG (methylglyoxal), MDA (malondialdehyde), H_2O_2 (hydrogen peroxide) and LOX (lipoxygenase). ***, ** and * indicate significant at P[?]0.001, P[?]0.01 and P[?]0.05, respectively.

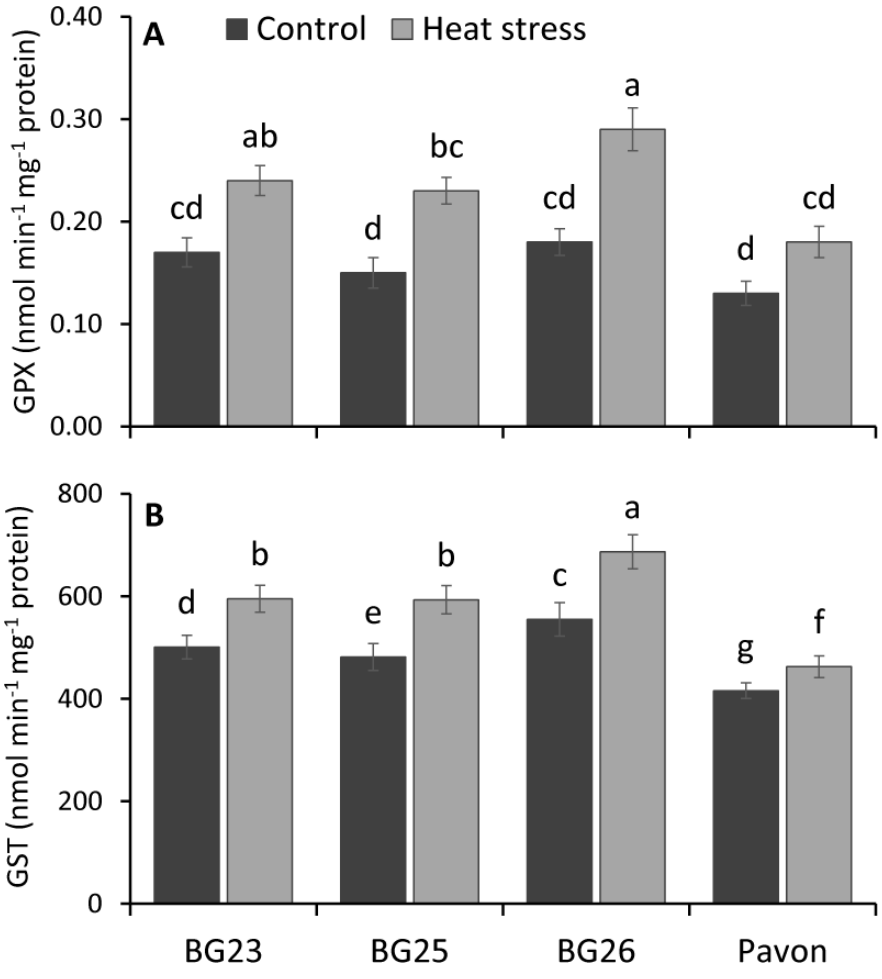


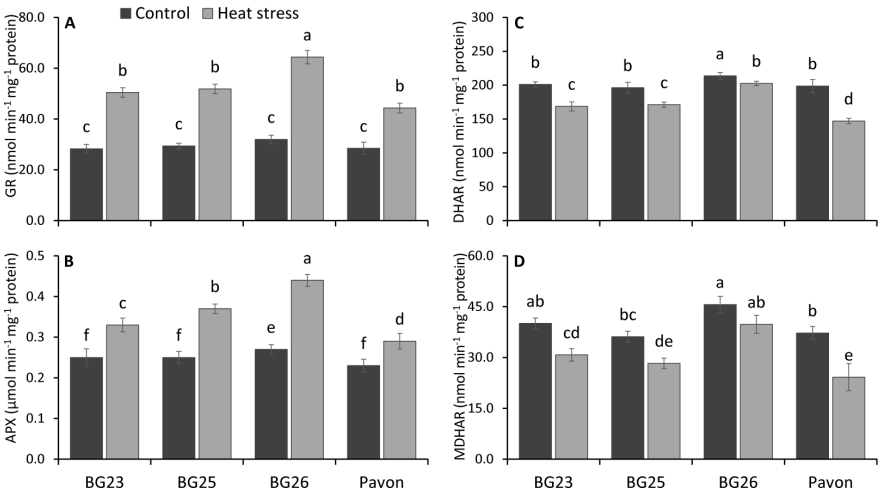




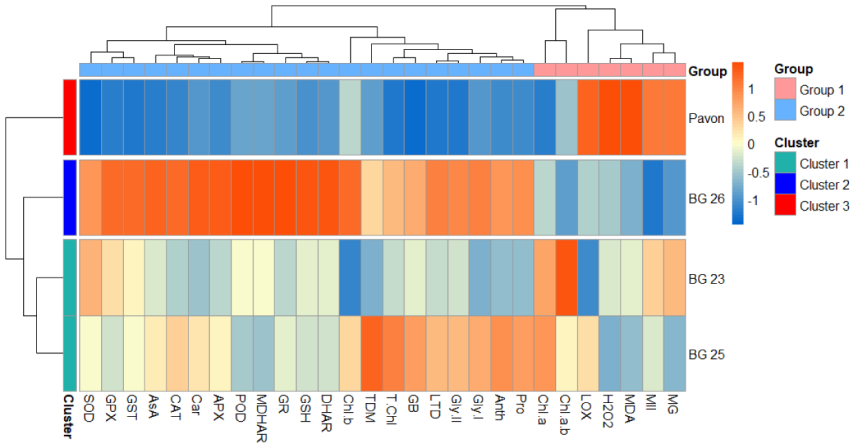








A. Hierarchical clustering of the varieties and traits based on STIs.



B. PCA-biplot derived from the STIs of the studied traits