



# Juvenile heat stress tolerance in *Triticum durum*—*Aegilops tauschii* derived synthetics: a way forward for wheat improvement

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## Abstract

**Background** Exponentially increasing population and everchanging climatic conditions are two major concerns for global food security. Early sowing in the second fortnight of October is an emerging trend with farmers in Indo Gangetic Plains to avoid yield losses from terminal heat stress. This also benefits the use of residual soil moisture of rice crop, conserving about one irrigation. But most of the available wheat cultivars are not well adapted to early-season sowing.

**Methods and results** Two in-house developed SHWs, syn14128 and syn14170, were screened for juvenile heat stress. Seedling length, biochemical parameters, and expression of amylase gene immediately after heat shock (HS) of 45 °C for 12 h and 20 h, and 24 h indicated significantly lower malondialdehyde, hydrogen peroxide, and higher free radical scavenging activities. Syn14170 reported higher total soluble sugar (TSS) under both HS periods, while syn14128 had a sustainable TSS content and amylase activity under HS as well as the recovery period.

**Conclusions** Both the SHWs had lower oxidative damage along with high free radical scavenging under heat stress. The higher expression of *amy4* along with sustainable TSS after heat stress in syn14128 indicated it as a potential source of juvenile heat stress tolerance. Variable response of SHWs to different biochemical parameters under heat stress opens future perspectives to explore the enzymatic pathways underlying these responses.

**Keywords** Synthetic wheat · ROS · MDA · Seedling · Heat stress tolerance · Amylase

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## Introduction

Wheat is major crop feeding about 35% of the world population as a staple food providing major calories and proteins [1]. Starting from the green revolution tremendous efforts were made in improving wheat yields in India raising yields from 6.5 Mt in 1960 to 104 Mt in 2020–21 over a cultivation area of approximately 9.75 million hectares and 31.6 million hectares, respectively [2]. This jump in yield is the outcome of consensus efforts of improving yield parameters and counteracting biotic and abiotic stresses. Gangetic plains of South Asia are the major producer of wheat and are expected to face a serious threat to sustainable wheat production by 2050 due to climate change, decline in the groundwater table, and fading soil fertility [3, 4]. Being a cool-season crop, wheat requires an optimal temperature between 12 and 25 °C for germination and seedling establishment, while the optimum temperature range for anthesis and grain filling is between 12 and 22 °C [5]. Elevated temperature is one of the most critical factors limiting crop growth and productivity around the globe leading to a dramatic reduction in the economic yield of agricultural produce [6, 7]. The global temperature is expected to rise by 1.5–4.5 °C until the end of the current century, and with each 1 °C rise in temperature, a 3–17% reduction in the wheat yield is predicted in the wheat growing regions of South Asia especially India and Pakistan [4].

The temperatures in the second week of November are optimum for the good germination, seedling establishment, and tillering of wheat in these regions but often expose the crop to terminal heat stress reducing overall yield gains. Preponing this sowing window to the second fortnight of October will lead to early maturity in March, quite earlier than the onset of heat stress on the developing seed. Early sowing also helps the farmers to utilize the residual moisture left by the rice crop, helping to conserve water by reducing at least one irrigation, and also promoting zero tillage practices [8, 9]. Wheat-rice crops are grown sequentially in a double-cropping pattern in the Indo Gangetic plains and this rotation builds the framework of the economic backbone of this region. But this rotation is a major concern to environmental sustainability as the severely affects the underground water table.

With all these advantages associated with early sowing, there is a need to shift focus on wheat lines which perform better when sown in the second fortnight of October. Higher temperatures during this time are a matter of concern as it affects germination efficiency, seedling establishment, and vigor of the wheat plant. A high-temperature (> 27 °C) stress on wheat at the seed germination stage causes embryo death and decline in seedling

establishment rate [10] and reduces root/shoot length and dry mass, and membrane stability index [11]. Exposure to the elevated temperatures initiates signals to adjust plant metabolism, and membrane fluidity accordingly and also leads to the production of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), a secondary metabolite that involves signaling under stress; reactive oxygen species (ROS) causing oxidative damage, accumulation of osmolytes like proline in the plant which ultimately leads to death [6, 11, 12]. Heat stress elicits the production and accumulation of ROS and detoxification by antioxidants is very much necessary for protecting the plant from the detrimental effect of heat stress [13]. The major metabolic pathway affected by heat stress during seed germination and seedling establishment is the utilization of stored seed starch into simple sugars [14]. Amylases, a family of endoamylases including  $\alpha$ ,  $\beta$ , and glucoamylase,  $\alpha$ -amylase, catalyze the hydrolysis of  $\alpha$ -1, 4 glycosidic linkages of starch, converting it into soluble substrates for other enzymes to attack [15]. Among these,  $\alpha$ -amylases are known to play a significant role during the germination and seedling establishment stage and their biosynthesis is influenced by fluctuations in environmental factors such as water, temperature, and calcium levels [16, 17].  $\alpha$ -Amylase mRNAs first appear in the embryo between 0.5 day and 1 day, followed by aleurone tissue in 2 days and are increased up to 6 days and are activated by sugar starvation, and repressed by sugar provision [18, 19].

Either to avoid terminal heat stress, crave for more yield, or need to save water, the search ends with the selection of genotypes that can germinate and establish themselves at high temperatures and grow into healthier plants. To date, scientists have been focusing on terminal heat stress and so far, no significant efforts have been made to breed wheat for early heat stress tolerance under October sowing. A recent study by Uttam et al. [9] screened wheat varieties and adapted germplasm for early (October) sowing and was able to identify a few genotypes which can perform well under juvenile heat stress. But the narrow genetic base of the cultivated wheat will pose a major limiting factor for wheat improvement for juvenile heat stress tolerance. One of the easiest and most effective methods to introduce new genetic diversity and novel alleles in the wheat gene pool is the generation of synthetic hexaploid wheats (SHWs). *Triticum durum* ( $2n = 4x = 28$ ; AABB) and *Aegilops tauschii* Coss. ( $2n = 2x = 14$ ; DD) derived SHWs are the most common and extensively exploited type of wheat synthetics and have been a good resource for improvement against various quality traits as well as biotic and abiotic stresses [20, 21]. However, SHWs have primarily been exploited as a source to introduce terminal heat stress tolerance in the modern-day wheat cultivars [22, 23]. The use of SHWs to introduce juvenile stage heat stress tolerance in wheat is still an unexplored area. The present investigation was targeted to explore the

juvenile stage heat stress tolerance in SHWs based on their response to oxidative stress and free radical species and activity of  $\alpha$ -amylase in SHWs wheat.

## Material and methods

### Plant materials and growth conditions

Two *T. durum*—*Ae. tauschii* synthetic wheats (syn14170 and syn14128), one durum wheat (PBW114) used in the generation of synthetics, and two hexaploid advanced breeding lines (ABLs) were used for the present study. Syn14170 was generated from the cross of *T. durum* cv. PBW114 and *Ae. tauschii* accession pau14170, while syn1428 was generated from the cross of PBW114 and *Ae. tauschii* accession pau14128 [24]. The ABLs used for the study were improved versions of two prevalent wheat cultivars PBW343, and HD2967 having rust resistance genes. ABL derived from PBW343 had two yellow rust resistance genes (*Yr17* and *Yr70*) and one leaf rust gene (*Lr76*), while the ABL derived from HD2967 had one yellow rust resistance gene (*Yr10*). The experiment was planted in a completely randomized design (CRD) in three parallel sets with different temperature treatments—set-I, II, and III, each in three replications. Seeds of each genotype were surface sterilized with 1% (v/v) sodium hypochlorite followed by thorough washing with Milli Q water and kept in the dark at 25 °C for germination for 48 h. Eight to ten germinated seedlings with uniform radicle length were transplanted per pot containing 2:1:1 soil—FYM—cocopeat. The three sets were kept in a Conviron CMP6050 growth chamber at 25 °C/17 °C (16 h light/8 h dark) at 60% humidity. Four days after germination (4DAG), set-I was maintained at 25 °C/17 °C and was treated as a control (C). Set-II was given heat shock (HS) at 45 °C for 12 h, and set-III was given 45 °C HS for 20 h, (Fig. 1). Immediately after HS, set-II and set-III were again

shifted to 25 °C for 24 h to recover from HS. All sets were maintained at 16h light/8 h dark period. For recording biochemical parameters, the leaf samples of growing seedlings were collected from the three sets in three replicates. From set-I, at 4DAG as control, from set-II, immediately after HS of 12 h (12HS) and then after 24 h of recovery after 12 h of HS (12HS24R). In set-III, seedlings were collected 20 h immediately after HS (20HS) and then after 24 h of the recovery period after 20 h HS (20HS24R).

For recording length of developing seedling and expression analysis, seedlings were collected from set-II, i.e., at 12HS and 12HS24R, and from set-III at 20HS and 20HS24R, all in three replicates. For comparison with optimum growth environments, seedlings from set-I were collected at parallel time intervals as of set-II and set-III as control. Percentage increment in seedling length was calculated in sets II and III as compared to seedling length at a similar time interval from set-I.

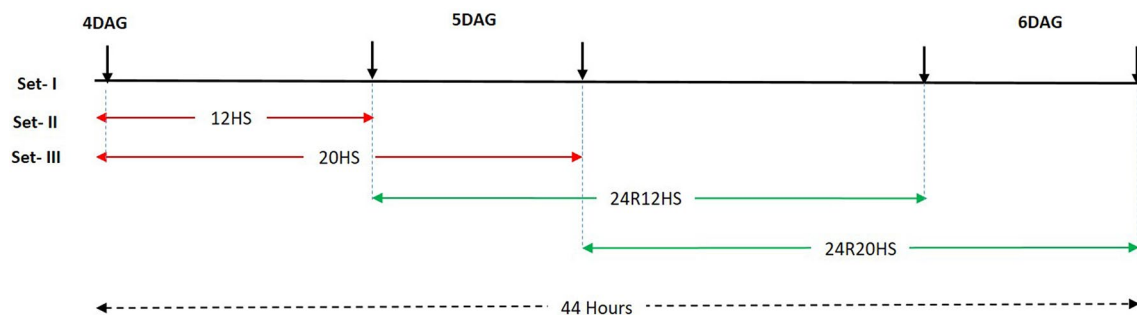
### Biochemical analysis

#### Estimation of lipid peroxidation

The content of malondialdehyde (MDA) was estimated using the method of Heath and Packer [25] with some modifications. The leaf homogenate was prepared using 0.1% (w/v) Trichloroacetic acid (TCA) and absorbance of the MDA-TBA (Thiobarbituric acid) complex of homogenate was measured at 532 nm, and the correction for non-specific absorbance was measured at 600 nm. The concentration of MDA was calculated using the Beer-Lambert law equation with an extinction coefficient of  $155 \text{ mM}^{-1} \text{ cm}^{-1}$ .

#### Estimation of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) content

H<sub>2</sub>O<sub>2</sub> content was measured spectrophotometrically after reaction with potassium iodide (KI) according to the method



**Fig. 1** Experimental setup for the study. Set I maintained at 25 °C/17 °C (16 h light/8 h dark) 4 days after germination and treated as control. Set II and set III shifted to 45 °C (16 h light/8 h dark) for 12 h and 20 h, respectively for exposure to heat stress, and

again moved to 25 °C/17 °C (16 h light/8 h dark) for a 24-h recovery period after heat stress. DAG days after germination, HS heat shock, R recovery

of Alexieva et al. [26] with slight modification. The absorbance of the reaction mixture consisting of leaf homogenate, 100 mM Potassium-phosphate buffer (pH 7.0), and reagent (1 M KI in double-distilled water) was measured at 390 nm. The amount of H<sub>2</sub>O<sub>2</sub> was determined in  $\mu$ moles/g FW using a standard curve prepared with a known concentration of H<sub>2</sub>O<sub>2</sub>.

#### Preparation of methanol extract

Leaf samples (0.05 g) collected from all three sets, were immediately frozen in liquid nitrogen. The leaf samples were extracted with 80% methanol, refluxed in a water bath at 70 °C for 1 h, followed by filtration with Whatman No. 1 and the final volume was made with 80% methanol. This extract was stored at 4 °C temperature and used to assess antioxidant activities and phenolic compounds in the biochemical tests discussed in the next sections.

#### DPPH (2,2-diphenyl-1-picrylhydrazyl) assay of antioxidant activity

DPPH assay was performed using a modified version of the Blois [27] method. 1 ml of appropriately diluted methanolic extract was mixed with 3 ml of 0.1 mM DPPH in amber-colored test tubes. The reaction mixture without sample extract served as a control. After incubation of 30 min in the dark at room temperature, the discoloration of DPPH was measured against the reagent blank at 517 nm.

#### Estimation of superoxide anion radical scavenging activity

The superoxide anion (O<sub>2</sub><sup>-</sup>) radical scavenging activity was measured by spectrophotometric monitoring of the inhibition of pyrogallol autoxidation as described by Marklund and Marklund [28]. The antioxidant activity was expressed in terms of the percentage of inhibition of pyrogallol autoxidation, which was calculated from the absorbance in the presence or absence of pyrogallol and the sample mixture. The reaction mixture containing methanol instead of the sample served as a control.

#### Estimation of nitric oxide free radical scavenging activity

Nitric oxide (NO) generated from sodium nitroprusside, and the nitrite molecule was measured by the Griess reaction [1% sulphanilamide, 0.1% naphthylethylenediamine dichloride (NED), and 2% phosphoric acid]. Aqueous sodium nitroprusside at physiological pH spontaneously generates nitric oxide, which reacts with an oxygen molecule to produce nitrite ions [29]. The nitrite ion production can be estimated by using a freshly prepared Griess reagent. 0.2 ml aqueous sodium nitroprusside and 0.2 M Na<sub>2</sub>HPO<sub>4</sub> (pH 7.8)

were added to 0.1 ml methanolic extract of leaf samples. The mixture was incubated in the dark for two and a half hour at room temperature. Finally, 0.25 ml of Griess reagent was added, and absorbance was measured at 540 nm against water blank using a UV–Visible spectrophotometer.

#### Estimation of hydroxyl radical scavenging activity

The hydroxyl radical scavenging assay is based on the Fenton-type reaction generating hydroxyl radical (OH<sup>•</sup>) and was estimated using the method described by Li et al. [30] with slight modifications. The reaction mixture consisted of 1 ml of 1,10 phenanthroline (0.75 mM), 1 ml of FeSO<sub>4</sub> (0.75 mM), 1.9 ml 0.2 M sodium phosphate buffer (pH 7.4), 0.1 ml of sample extract. 1 ml of 0.02% hydrogen peroxide was added to initiate the reaction in each sample. The samples were then incubated at 37 °C for 1 h. The absorbance of the solution was read at 536 nm against blank to check the scavenging activity. The control was prepared by adding buffer in place of the sample.

#### Estimation of total phenols

The content of total phenols (TP) was quantified with Folin's reagent using the method described by Swain and Hillis [31]. By using gallic acid in the range of 10–50  $\mu$ g, a standard curve was prepared, and total phenolic content was measured in mg/gm FW.

#### Estimation of total flavanols

The total flavanol (TF) content was estimated using the aluminum chloride colorimetric assay as described by Zhishen et al. [32]. A standard curve of rutin (40–200  $\mu$ g) was also prepared simultaneously, and the content of total flavonoids was calculated from the standard curve.

#### Estimation of total soluble sugars

Free sugars were extracted from 0.3 g of leaf samples collected from the treatment sets in three replications with 15 ml of 80% ethanol followed by 70% methanol by keeping the tubes fitted with water condensers in a boiling water bath for 20 min. Extracts were evaporated at 50 °C in a flash evaporator under a vacuum, and the final volume was made with distilled water. Total sugars were estimated by using the phenolic sulphuric method of Dubois et al. [33]. The concentration of total sugars (as glucose) was calculated from the glucose standards (10–50  $\mu$ g) which were run simultaneously.



## Estimation of alpha-amylases

The extraction of  $\alpha$ -amylase was carried out following the method of Doehlert and Duke [34]. 100 mg tissue was extracted in 50 mM sodium acetate buffer (pH 5.0) containing 1 mM  $\text{CaCl}_2$ . The homogenate was aged for 1 h at room temperature and was centrifuged at  $10,000\times g$  for 20 min at 4 °C. The supernatant obtained was used as an enzyme extract and was incubated with 2% starch in the presence of 50 mM sodium acetate buffer (pH 5.0) containing 1 mM  $\text{CaCl}_2$  for one hour at 37 °C. The reaction was stopped by the addition of 1 ml alkaline copper tartrate and boiling for 20 min. 1 ml of arsenomolybdate was added after cooling the tubes followed by 7 ml of  $\text{H}_2\text{O}$ . The content was measured at 510 nm, and the activity was determined by subtracting the concentration of starch left after hydrolysis from the starch present in the substrate blank.

## Expression analysis of alpha-amylase gene

Gene expression levels of the  $\alpha$ -amylase gene were estimated in the seedlings under heat stress. The total RNA was isolated from the collected samples using Qiagen RNeasy® Plant Mini Kit according to the manufacturer's protocol. RNA integrity was electrophoretically verified by the ethidium bromide staining and by  $\text{OD}_{260}/\text{OD}_{280}$  nm absorption ratio of higher than 1.95. DNA-free total RNA (5 mg) from different treatments was used for the first-strand cDNA synthesis following the instruction of the PrimeScript™ 1st strand cDNA Synthesis Kit (Takara). qPCR was conducted using SYBR Green I Mastermix (Takara) using Roche LightCycler96 Real-Time PCR machine. The housekeeping (Tubulin) gene and  $\alpha$ -amylase (*amy4*) gene oligonucleotide primers along with the reaction conditions, were adapted from Kaur [18]. Melting curves were performed at 55 °C and were analyzed using the LightCycler96 software package supplied by Roche. The  $C_q$  (quantification cycle value) value and the relative expression level of amylase and the relative expression level of the housekeeping (Tubulin) were estimated in each sample. Relative fold differences were calculated based on the comparative  $C_q$  method using Tubulin as the endogenous control. For the relative differences in the target gene for each treatment, the  $C_q$  value for the target gene was normalized to the  $C_q$  value for Tubulin. The relative fold change in the target gene was calculated for each sample using the equations  $2^{-\Delta\Delta C_q}$  [35] given below. For the untreated control sample,  $\Delta\Delta C_q$  equals zero, and  $2^0$  equals one. For the treated samples,  $2^{-\Delta\Delta C_q}$  indicates the fold change in gene expression relative to the control. This method was used to calculate the relative change in gene expression. According to the  $\Delta\Delta C_q$  method:

$$\Delta C_q = \Delta C_{q\text{Target}(\alpha\text{-Amylase})} - \Delta C_{q\text{Reference}(\text{Tubulin})}$$

$$\Delta\Delta C_q = \Delta C_{q\text{Test Sample}(\text{stressed})} - \Delta C_{q\text{Calibrator Sample}(\text{Control})}$$

$2^{-\Delta\Delta C_q}$  represents the fold change in gene expression relative to untreated control.

## Statistical analysis

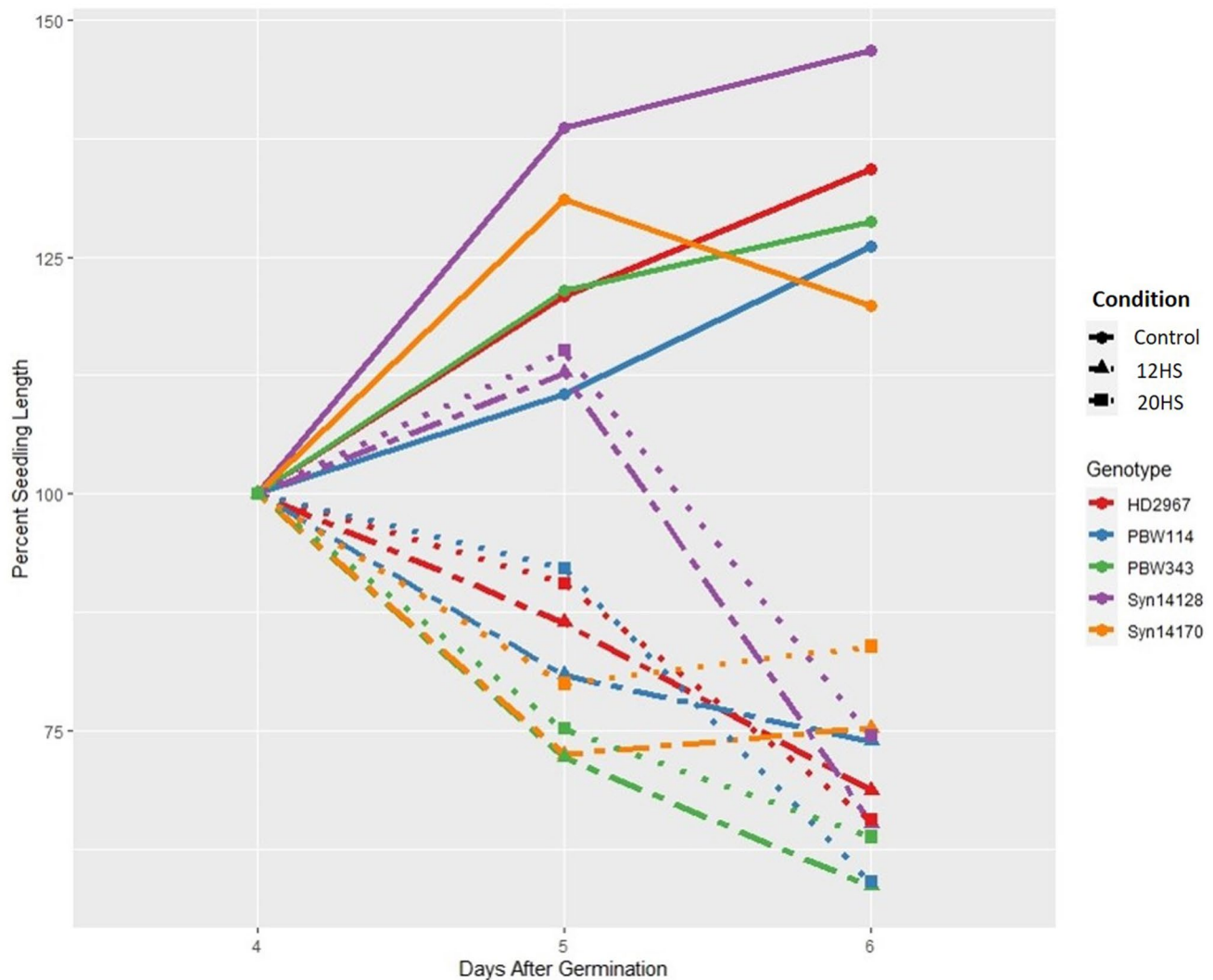
Data were analyzed by one-way ANOVA using “Agricolae” version 1.3-1 Rstudio [36]. Significant differences between treatments were identified at the 0.05 probability level (P) by the Duncan's Multiple Range Test and represented as mean  $\pm$  standard error (SE). Percent change in the level of various biochemical parameters was plotted with the help of “ggplot2” version 3.3.2 and “ggpubr” version 0.4.0 packages of RStudio [37, 38].

## Results

The selected genotypes showed seedling length variation at 4DAG with synthetics having a minimum length of 8.27 cm (syn14128) to a maximum length of 14.17 cm in PBW114 (Figs. S1, S2; Tables S1, S2). The other SHW, syn14170 reported a seedling length similar to that of HD2967 (10.53 cm) at 4DAG. The maximum percent of length increment on 5th DAG and 6th DAG was in syn14128 (38.71%, 46.80%, respectively), while the minimum percent increment in PBW114 (10.55%, 26.17%, respectively) (Fig. 2; Tables S1, S2). Syn14170, on the other hand, reported a sharp increase of 31.01% in seedling length at 5th DAG, which was further slowed down to 19.81% at 6 DAG (Fig. 2). Syn14128 reported a significantly better seedling length (56.45% and 59.68%, respectively) at 12HS and 20HS stages as compared to other synthetic, durum, and checks. Still, it was not able to maintain this increment rate during the recovery period (15.21% and 5.05% decrease compared to control respectively) (Fig. 2). However, at the 12HS24R and 20HS24R stages, syn14170 reported a better recovery (24.33% and 25.68%) than all the other genotypes. Selected ABLs and durum wheat, on the contrary, reported an 8–12% decrease in the seedling length after HS indicating severe damage to plant cellular machinery.

## Heat stress-induced oxidative stress in wheat seedlings

The increase in MDA content was observed in seedlings after 12HS and 20HS as compared to control, followed by a decrease in the MDA content at 12HS24R and 20HS24R (Fig. 3a; Table 1). Under the control conditions, no

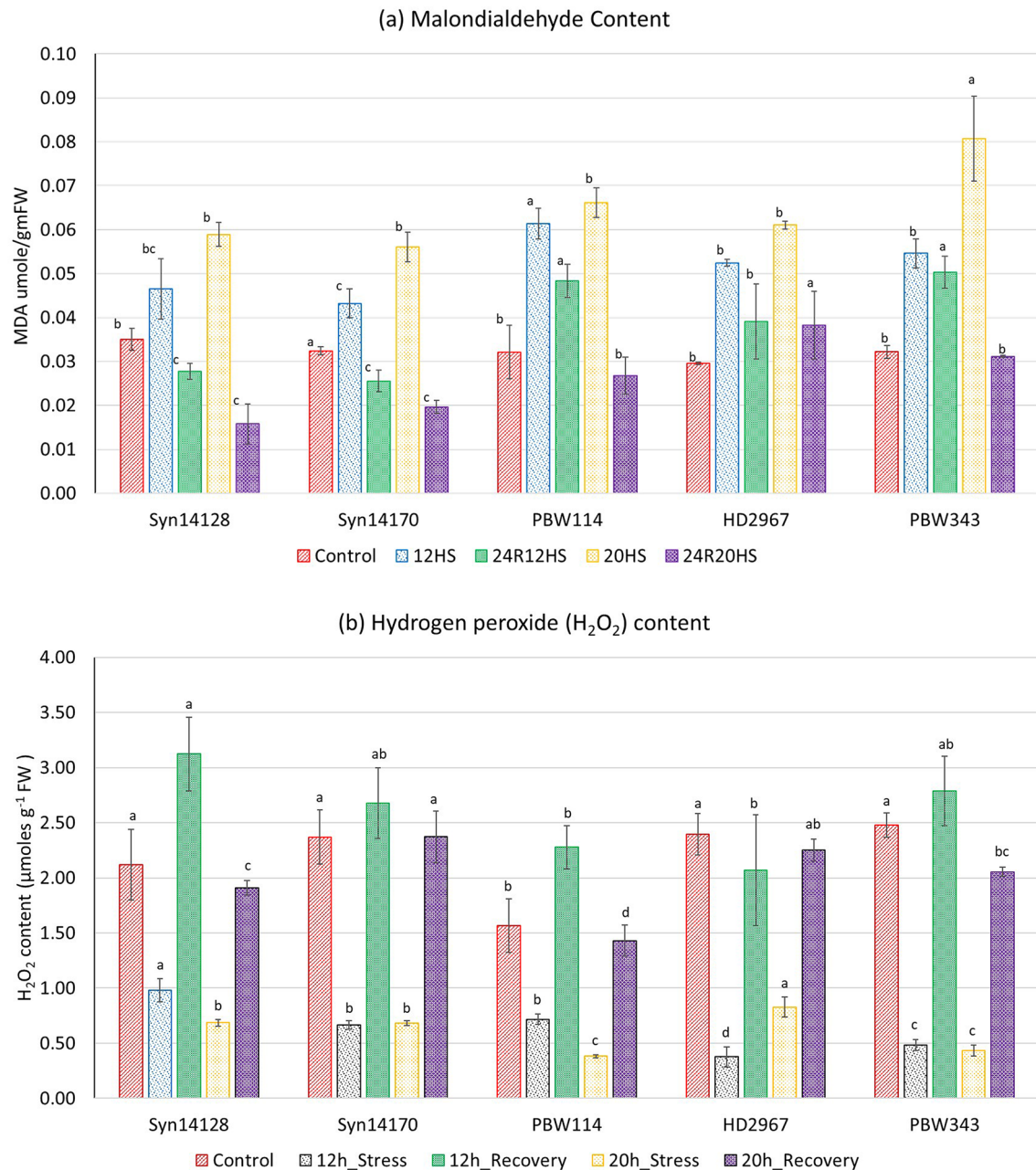


**Fig. 2** Percent change in the seedling length in selected wheat genotypes under control, heat shock (12 h- and 20 h-), and recovery period. *HS* heat shock

significant difference in MDA content was observed among selected genotypes. When exposed to 12HS, ABLs and durum wheat reported an abrupt increase in MDA content with the maximum increase in PBW114 (0.061  $\mu\text{moles/g}$  FW, 91.06%), followed by HD2967 (0.052  $\mu\text{moles/g}$  FW, 77.36%) (Fig. 3a; Table 1). Syn14128 reported the minimum increase in MDA content after 12HS as well as 20HS (33.08% and 68.25%, respectively). At 12HS24R, MDA content decreased by 20–40% in SHWs while ABLs and durum wheat still reported higher MDA content (30–56%). However, at 20HS24R only HD2967 had higher MDA content while the rest of the genotypes indicated a decreased MDA level.

$\text{H}_2\text{O}_2$  level of the control set was lower in PBW114 as compared to other genotypes (1.57  $\mu\text{moles/g}$  FW, Fig. 3b; Table 1). 12HS and 20HS resulted in a significant

decrease in  $\text{H}_2\text{O}_2$  content in the seedlings. After 12HS, syn14128 reported a minimum reduction in the  $\text{H}_2\text{O}_2$  content (0.98  $\mu\text{moles/g}$  FW, 53.76%) while syn14170 had a 71.92% decrease in  $\text{H}_2\text{O}_2$  content compared to the control set. However, durum wheat, PBW114, reported a reduction similar to that of syn14128 (54.20%, Fig. 3b). At 12HS24R,  $\text{H}_2\text{O}_2$  content increased significantly with no significant difference in syn14128 (47.48%) and PBW114 (45.43%). However, at 20HS, HD2967 reported maximum  $\text{H}_2\text{O}_2$  content (0.83  $\mu\text{moles/g}$  FW; 82.55% decrease compared to control) and at 20HS24R syn14170 was able to recover its  $\text{H}_2\text{O}_2$  content similar to that of control conditions (0.09%) (Fig. 3b; Table 1). Both the synthetics had a minimum percent increase in the MDA content along with lower  $\text{H}_2\text{O}_2$  content under 12HS, 20HS as well as 12HS24R, and 20HS24R.



**Fig. 3** **a** Malondialdehyde content and **b** H<sub>2</sub>O<sub>2</sub> content in wheat seedlings under control, heat stress (HS), and recovery (R) period. Error bars represent the standard deviation of three replicates. The different

alphabetical letters in superscript indicate the significant differences with the Duncan test at a p-value 0.05

### Free radical scavenging activities in wheat seedlings under heat stress

Free radical scavenging activities were estimated for four different radicals viz., DPPH, O<sup>2-</sup>, NO and OH<sup>·</sup>. Under the control conditions, all selected lines had statistically similar DPPH scavenging activities ranging from 76 to 79% (Fig. 4a; Table 1). While after 12HS, DPPH scavenging activity was reduced with the lowest in PBW343 (13.37%),

followed by HD2967 (21.34%) with a significant reduction (82.81% and 72.56%; Table 1) as compared to the control. In synthetics, ~43% percent decrease in DPPH scavenging activities was recorded at 12HS with ~45% reduction as compared to control. A similar trend was also observed at 20HS, with maximum DPPH reduction in PBW343 (74.21%) followed by HD2967 (70.58%), while syn14170 had the minimum reduction (56.59%) in the DPPH scavenging activity followed by syn14128 (61.90% reduction).

**Table 1** Percent change in different parameters as compared to 4 DAG control after different treatments

Genotype	DAG	Treatment	MDA	H <sub>2</sub> O <sub>2</sub>	DPPH	O <sub>2</sub> <sup>-</sup>	NO	OH <sup>·</sup>	TP	TF	TSS	Starch	Amy
Syn14128	5	12HS	33.08	-53.76	-45.02	84.84	97.68	245.60	90.82	-29.88	-21.72	-14.72	-38.41
Syn14170	5	12HS	33.27	-71.91	-44.28	53.08	139.27	158.10	97.54	-37.67	-53.31	-12.60	-49.22
PBW114	5	12HS	91.06	-54.20	-63.03	137.66	97.38	367.63	221.12	-13.84	-10.77	-9.40	-26.76
HD2967	5	12HS	77.36	-84.27	-72.56	82.56	59.74	482.11	164.88	-28.01	-40.17	-21.70	-56.13
PBW343	5	12HS	69.54	-80.52	-82.81	18.05	72.33	283.95	272.31	-32.98	-10.05	-23.62	-50.09
Syn14128	6	24R12HS	-20.69	47.48	1.13	17.23	6.29	-12.36	-38.12	2.69	-28.17	-52.03	-10.35
Syn14170	6	24R12HS	-21.24	12.97	1.49	6.61	4.95	-45.53	-60.99	-8.25	-64.48	-65.33	-31.28
PBW114	6	24R12HS	50.34	45.43	3.36	-14.43	-15.18	-15.98	-21.12	21.10	-39.38	-45.98	-3.22
HD2967	6	24R12HS	32.33	-13.68	2.81	-18.12	-27.96	-1.79	-9.65	6.46	-63.68	-53.78	-22.67
PBW343	6	24R12HS	56.19	12.41	2.98	-38.33	-15.85	-25.04	-8.08	2.02	-40.42	-43.38	-32.26
Syn14128	5	20HS	68.25	-67.58	-61.90	258.59	86.42	107.30	20.56	-23.09	-53.39	14.09	-49.01
Syn14170	5	20HS	73.07	-71.20	-56.55	122.16	71.42	256.12	49.08	-27.59	-48.72	4.87	-59.17
PBW114	5	20HS	105.64	-75.62	-66.89	170.37	41.71	227.18	165.02	4.68	-3.00	2.88	-45.56
HD2967	5	20HS	106.41	-65.49	-70.58	188.85	21.83	293.49	127.88	-29.46	-7.28	-20.00	-69.90
PBW343	5	20HS	150.58	-82.55	-74.21	249.84	23.63	89.44	207.31	-32.05	-33.03	-22.33	-66.13
Syn14128	6	24R20HS	-54.98	-9.74	-18.02	-7.51	59.93	-31.94	-47.90	-11.51	-55.14	-8.13	-39.04
Syn14170	6	24R20HS	-39.28	0.09	-10.28	-49.78	72.28	50.68	-51.75	-16.53	-57.91	-7.82	-49.76
PBW114	6	24R20HS	-16.80	-8.80	-9.75	-50.76	24.16	44.19	19.47	17.95	-25.62	-39.03	-35.75
HD2967	6	24R20HS	29.32	-6.01	-12.07	24.77	0.89	55.79	3.75	-12.19	-12.37	-39.63	-57.83
PBW343	6	24R20HS	-3.21	-17.14	-14.87	6.30	20.09	-18.52	32.69	-28.23	-40.07	-30.13	-63.88

Values represented as percent increase or decrease as compared to control

The different alphabetical letters in superscript indicate the significant differences with Duncan test at p value 0.05

DAG days after germination, MDA malondialdehyde content, H<sub>2</sub>O<sub>2</sub> hydrogen peroxide content, DPPH percent DPPH content, O<sub>2</sub><sup>-</sup> superoxide anion free radical scavenging activity, NO nitric oxide free radical scavenging activity, OH<sup>·</sup> hydroxyl anion free radical scavenging activity, TP total phenol content, TF total flavanol contents, TSS total soluble sugar content, Starch starch content, Amy amylase activity

However, no significant change in DPPH scavenging was observed at 12HS24R and 20HS24R.

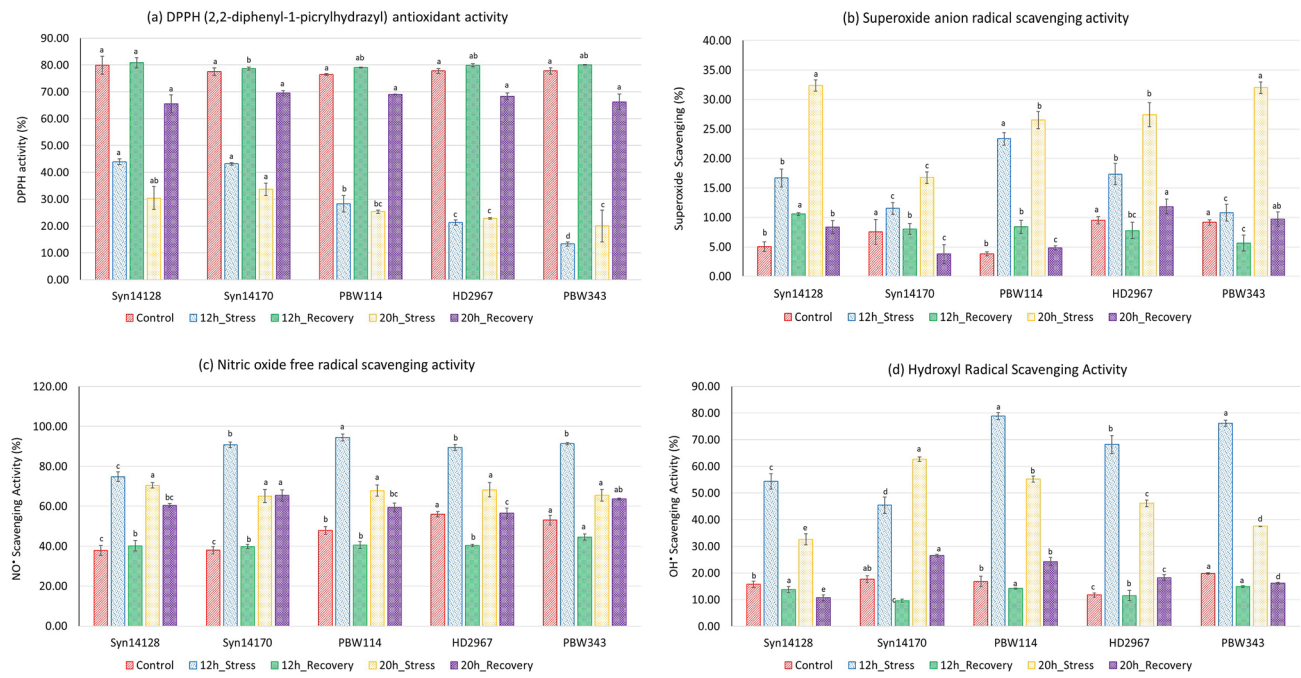
Further, O<sub>2</sub><sup>-</sup> radical scavenging activity was low in the control set and increased significantly after HS (Fig. 4b; Table 1). At 12HS, PBW114 reported a maximum spike in the level of O<sub>2</sub><sup>-</sup> radical scavenging activity (137.66% increase) followed by syn14128 (84.84%). In the case of 20HS, an abrupt percent increase in O<sub>2</sub><sup>-</sup> radical scavenging activity was observed in all the genotypes with the maximum growth in the case of PBW343 (249.84%). However, increased O<sub>2</sub><sup>-</sup> radical scavenging activity was observed in the case of two selected synthetics, syn14128 (17.23%) and syn14170 (6.61%), in 12HS24R, while the other three genotypes indicated a decreased percent scavenging (Fig. 4b). 20HS24R indicated decreased O<sub>2</sub><sup>-</sup> radical scavenging activity as compared to control in the case of synthetics and PBW114, while increased scavenging in the case of ABLs.

Syn14170 and syn14128 had significantly lower (P < 0.05) (~37%) NO scavenging activity than elite (~55%) and durum (47%) wheats under control conditions which was increased further as the genotypes were exposed to HS (Fig. 4c; Table 1). After 12HS ABLs showed a minimum percent increase (59.74% and 72.33%, respectively) followed

by syn14128 and PBW114 (97%). Although syn14170 reported a maximum percent increase (139.27%) after 12HS, syn14128 reported the overall maximum percent after 20HS (86.42%). ABLs again had the minimum increase (~22%) at 20HS. In the case of 12HS24R, NO free radical scavenging activity was decreased (15–27%) in ABLs and durum wheat while SHWs had 4–6% higher scavenging as compared to the control set. At 20HS24R, an overall percentage increase of NO scavenging was observed in all the genotypes.

OH<sup>·</sup> radical scavenging had an activity pattern similar to that of O<sub>2</sub><sup>-</sup> scavenging activity, with lower levels of scavenging in control and recovery stages but higher scavenging after HS (Fig. 4d; Table 1). In control, PBW343 showed maximum OH<sup>·</sup> radical scavenging activity (19.84%), while HD2967 had the lowest (11.72%) (Fig. 4d). At 12HS treatment, PBW114 reported maximum levels of OH<sup>·</sup> scavenging (78.87%) followed by PBW343 (76.17%). However, the maximum percent increase compared to control was reported in HD2967 (482.11%) followed by PBW114 (367.63%). Both the SHWs had minimum OH<sup>·</sup> scavenging (45.43% syn14128, 54.36% syn14170) and minimum percent change (245.60%, 158.10%, respectively) compared to the control. At 12HS24R syn14170 indicated a better recovery (45.55%)





**Fig. 4** **a** DPPH, **b** superoxide anion free radical, **c** nitric oxide free radical, and **d** hydroxyl anion free radical scavenging activities in control, heat stress (12 h- and 20 h-) and recovery period. Error bars

represent the standard deviation of three replicates. The different alphabetical letters in superscript indicate the significant differences with the Duncan test at a p-value 0.05

than syn14128 (12.36%). However, a significant difference in the  $\text{OH}^\bullet$  free radical scavenging activity was observed for all the lines after 20HS, and 20HS24R with a maximum percent change was observed in HD2967 (293.49%, 55.79%) followed by syn14170 (256.12%, 50.68%). Overall DPPH scavenging was decreased, while  $\text{O}_2^-$ ,  $\text{OH}^\bullet$ , and NO scavenging increased under both 12HS and 20HS. Syn14170 had a minimum decrease in the DPPH scavenging (44.28%) along with maximum NO scavenging (139.27%) for 12HS set, while a better  $\text{OH}^\bullet$  radical scavenging (256.42%) in this synthetic was observed in the 20HS set. On the other hand, syn14128 also reported a minimum decrease in the DPPH scavenging (45.02%) along with the highest  $\text{OH}^\bullet$  radical scavenging (245.60%) for the 12HS set. In the case of 20HS syn14128 had maximum  $\text{O}_2^-$  radical scavenging (258.59%), maximum NO scavenging (86.42%), and average  $\text{OH}^\bullet$  radical scavenging (107.30%).

### Total phenol and total flavanol content in wheat seedlings under heat stress

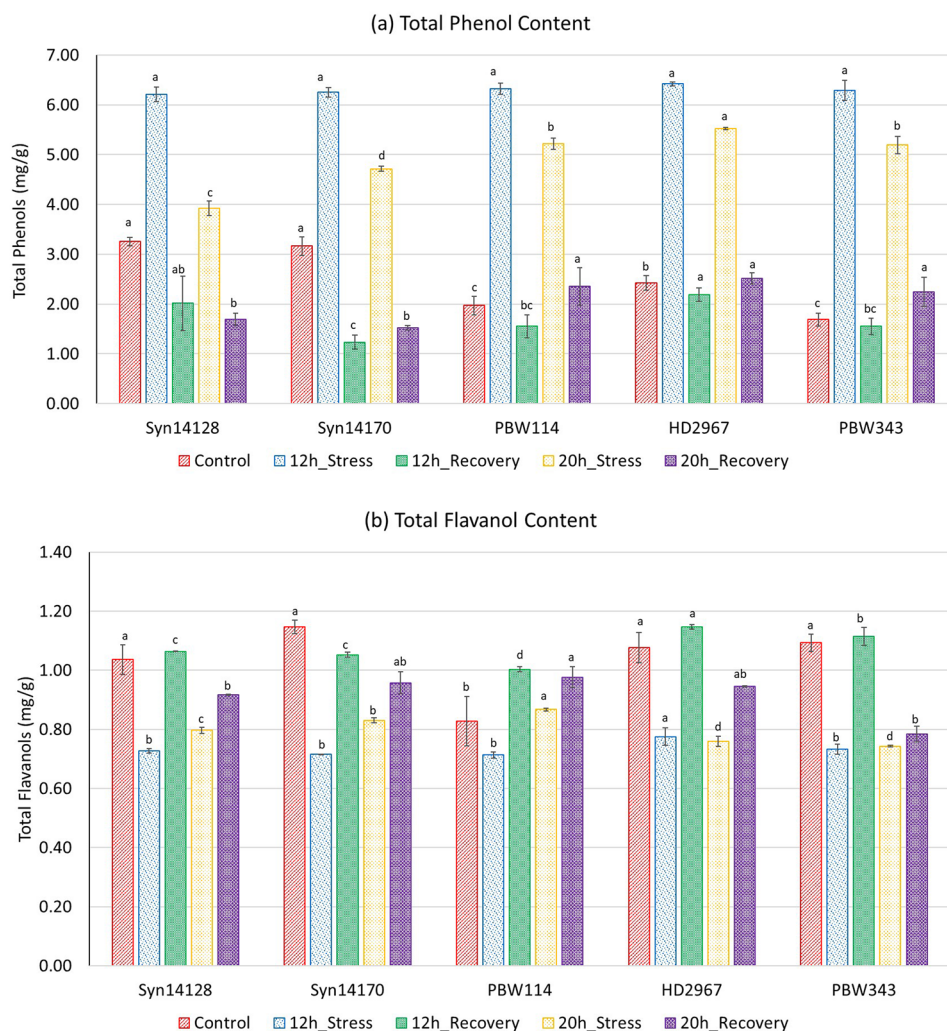
In the present study, TP increased under the HS condition while the TF decreased when seedlings were exposed to HS. TP content was significantly higher in SHWs in the control set (~3 mg/g FW) as compared to ABLs and durum wheat (1-2 mg/g FW) (Fig. 5a; Table 1). An overall increase in the phenolic content was observed at 12HS, but there was

no significant variation among selected genotypes (~6 mg/g FW). PBW343 (272.31%) followed by PBW114 (221.12%) showed a significantly higher increase in the phenolic content, while SHWs reported the minimum percent change in total phenol content under 12HS (Table 1). However, syn14170 recovered back quickly from heat damage after 12HS24R with minimum phenolic content (1.23 mg/g FW) and a maximum percent decrease in total phenol content (60%). SHWs reported significantly less phenolic content (3-4 mg/g FW) as compared to the elite and durum wheat (~5 mg/g FW) in 20HS treatment. Syn14170 was still observed to recover back quickly after 20HS24R with minimum phenolic content (1.53 mg/g FW, 51.75%) followed by syn14128 (1.70 mg/g FW, 47.90%) (Fig. 5a). An increase in total phenol levels was observed in ABLs and durum wheat with a maximum percent increase in PBW343 (32.69%) followed by PBW114 (19.47%) in recovery post 20 h HS.

On the other hand, TF content decreased after HS. In the control set, there was no significant difference SHWs and ABLs (~1 mg/g FW), while durum wheat had minimum TF content (0.82 mg/g FW) (Fig. 5b; Table 1). All the genotypes showed a decrease in flavanol content at 12HS, with maximum percent decrease in syn14170 (0.72 mg/g FW, 37.67%) followed by PBW343 (0.73 mg/g FW, 32.98%) and syn14128 (0.73 mg/g FW, 29.88%). However, syn14170 reported an 8.25% decrease in the TF content at 12HS24R, and in all the other genotypes 2–21% increase in TF content



**Fig. 5** Content and percent change in **a** total phenols and **b** total flavanols contents in control, heat stress (12 h- and 20 h-) and recovery period. Error bars represent the standard deviation of three replicates. The different alphabetical letters in superscript indicate the significant differences with the Duncan test at a p-value 0.05



was recorded as compared to the control set. Durum wheat, on the other hand, reported an increase in the TF content (4.68%) for 20HS treatment, while ABLs and SHWs reported a 23–32% decline in the levels of flavanol content. A similar trend was observed when these seedlings were given a recovery period of 24 h.

### Effect of heat stress on total soluble sugars and total starch

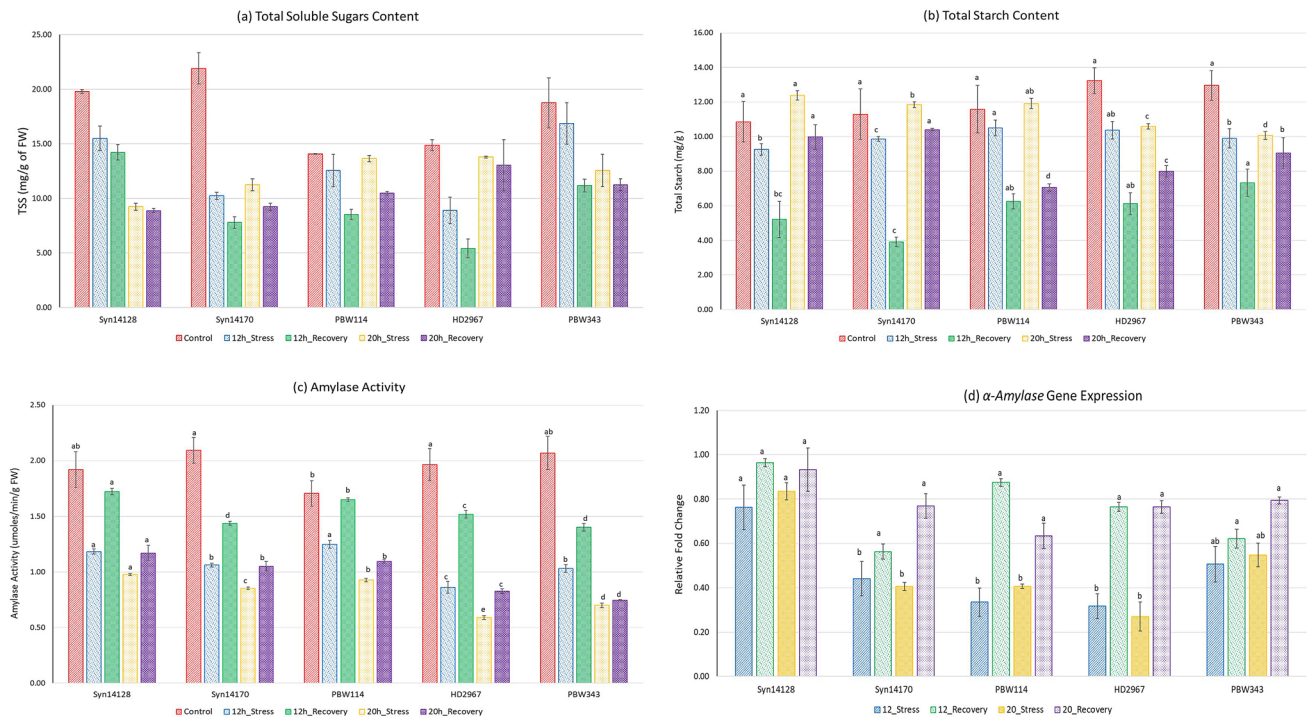
In the control set, total soluble sugar (TSS) was highest for syn14170 (21.90 mg/g FW) followed by syn14128 (19.78 mg/g FW) (Fig. 6a; Table 1). However, at 12HS, a decrease in TSS was observed for all the genotypes with a maximum percent decrease in syn14170 (53.31%) followed by HD2967 (40.17%). At 12HS24R treatment, syn14128 reported the highest recovery potential with only 28% decline in the amount of available sugars, while syn14170 reported the minimum recovery with a 64.48% decline in

TSS. Further, TSS content was decreased by ~ 50% in the case of SHWs for 20HS and 20HS24R.

Selected wheat genotypes did not show a significant difference in the levels of starch content in the control set (Fig. 6b; Table 1). However, at 12HS, minimum starch content was observed in the case of syn14128 (9.26 mg/g FW) followed by syn14170 (9.87 mg/g FW) which was further decreased (52.03% and 65.33%, respectively) when at 12HS24R, indicating a better utilization of starch under HS. At 20HS and 20HS24R, ABLs reported a decline in starch content (~ 21%) while SHWs and durum wheat reported an increased starch availability, indicating a decreased starch metabolism in these genotypes.

### Amylase activity and gene expression of $\alpha$ -Amylase under heat stress

Syn14170 (2.09  $\mu$ moles/min/g FW) and PBW343 (2.07  $\mu$ moles/min/g FW) reported the highest activity of amylase in the control set. At 12HS, reduced activity of amylase was observed



**Fig. 6** Content and percent change in **a** total soluble sugar content, **b** starch content, **c** amylase activity along with the **d** gene expression of  $\alpha$ -amylase, *amy4*, in control, heat stress (12 h- and 20 h-) and recovery

in all the genotypes, but the reduction was more significant in HD2967 (56.13%) followed by PBW343 (50.09%) and syn14170 (49.22%) (Fig. 6c; Table 1). Amylase activity was least affected in PBW114 (1.25  $\mu$ moles/min/g FW, 26.76% reduction) followed by syn14128 (1.18  $\mu$ moles/min/g FW, 38.41% reduction) at 12HS and a similar trend was observed at 12HS24R. In the 20HS set, PBW114 (0.93  $\mu$ moles/min/g FW) again showed minimum reduction (45.56%) in amylase activity, followed by syn14128 (0.98  $\mu$ moles/min/g FW, 49.01% reduction) and a similar trend was observed for 20HS24R.

In real-time PCR expression profiling of  $\alpha$ -amylase targeting the *amy4* gene (Fig. 6d), a general trend of downregulation due to HS was observed in all the selected wheat lines. However, syn14128 reported significant minimum downregulation in *amy4* activity than the other selected lines at both 12HS as well as 20HS. Further, *amy4* expression in syn14128 also recovered back quickly to the control level for 12HS24R as well as 20HS24R (Fig. 6d). Syn14170, however, has low expression than durum as well as ABL checks.

## Discussion

Practicing early planting of wheat after the rice crop is a common trend adopted by the Indian farmers, as it avoids the terminal heat stress, can help apply zero tillage and utilizes

ery period. Error bars represent the standard deviation of three replicates. The different alphabetical letters in superscript indicate the significant differences with the Duncan test at a p-value 0.05

the residual moisture left by the rice crop saving both time and energy. Early planting coincides with a higher temperature which affects the seed germination, establishment, growth, and yield. There is a need to expand the heat stress resistance both ways, exploring wheat varieties that could endure HS during the germination and seedling establishment stage along with work on terminal heat stress [39]. Being a newer thought of work, significantly fewer studies have targeted the juvenile stage heat stress. The potential of SHWs for juvenile stage heat stress tolerance is a significantly less explored area and the present study targets two SHW lines, already evaluated for terminal heat stress tolerance, for their potential use in the early/seedling stage heat stress tolerance. The seedlings from the selected wheat genotypes were managed in three different sets as control (set I, at 25 °C), 12HS (set II), and 20HS (set III) at 45 °C followed by a 24 h- recovery period at 25 °C (Fig. 1). Oxidative stress, free radical scavenging activities, total phenol, and flavanols along with the starch metabolizing enzyme (amylase) were estimated for the selected lines to understand the level of seedling stage heat stress tolerance.

Malondialdehyde (MDA) levels after exposure to stress can be used as an indicator of cellular membrane damage as MDA causes lipid peroxidation of heat stress damaged cell membranes due to higher lipoxygenase [40]. Heat stress generally increases the MDA content in all the organs of

the wheat plant, tolerant genotypes of wheat showed lower lipid peroxidation levels than the susceptible ones [41, 42]. The two synthetics used in the present study reported the minimum increase in the MDA levels under two levels of HS and at recovery after HS as compared to ABLs and PBW114. This indicated that the lower membrane damage occurred in these genotypes under HS and their ability to recover minimum damage of membrane integrity after the stress is over.  $H_2O_2$  is recognized as an integral component of cell signaling cascades [43].

Along with MDA,  $H_2O_2$  is a strong oxidant and a second messenger in biotic and abiotic stresses [44]. Syn14128 and syn14170 had lower  $H_2O_2$  content after 12HS and 20HS and better recovery than ABLs and durum wheat, indicating lower oxidative damage in synthetics. Thus on exposure to HS, the decreased  $H_2O_2$  content might have initiated its action as a signaling molecule, triggering the tolerance responses in the seedlings of two synthetics used in the present study [45]. PBW343 and HD2967 with higher  $H_2O_2$  content under HS were not able to recover quickly from the heat damage, suggesting a toxic role of  $H_2O_2$  [43].

In cellular redox homeostasis, there is the overproduction of ROS that overwhelms the detoxification system of the plant and makes the plant vulnerable. Regular scavenging of these toxic species under stress helps the plants to thrive under adverse conditions. DPPH free radical scavenging activity decreased significantly under heat stress indicating a higher expression of ascorbate peroxidase in the selected wheat lines. Higher levels of DPPH activities have already been correlated with higher levels of ascorbate peroxidase in the D-genome donor of wheat, *Ae. tauschii* by Hairat and Khurana [46]. These higher levels of DPPH and decreased  $H_2O_2$  content have also been associated with salinity tolerance and drought tolerance [12, 47]. The production of ROS, mediated by alternate  $O_2^-$  reduction and subsequent oxidative damage in heat-stressed plants, has been reported by several researchers [13, 48]. Savicka [49] also reported an increased  $O_2^-$  scavenging post 24 h heat stress in 4 day old and 7-day old seedlings.  $OH^\cdot$  is the most reactive free radical and can damage different cellular components by lipid peroxidation, protein damage, and membrane destruction. Also, there is no known enzymatic system to scavenge it, making it the most toxic ROS [6, 11, 12]. In addition to the generation of ROS, several reactive nitrogen species (RNS) are also formed in the plants under heat stress. Increased NO scavenging under heat stress in the present study indicated a role of this molecule in stress tolerance, as per the earlier studies [6, 29].

Phenolic compounds are an essential class of secondary metabolites, produced under optimal and suboptimal conditions in plants playing critical roles in developmental processes like cell division, and photosynthetic activity. Under temperature stress, plants synthesize more phenols

and flavanols to protect the plant cells [50]. The lower increment in the levels of phenols in the selected SHW genotypes under HS suggested that these may be supporting the other biochemical parameters to maintain cell integrity during the heat stress instead of directly causing any impact. Lower, DPPH content has also been reported associated with the high phenolic and flavonoid contents under heat stress in wheat [51].

Heat stress during seed germination and seedling growth affects the activity of carbohydrate metabolizing enzymes like amylase. As a seed gets suitable conditions for germination, all the enzymes and other proteins leave their dormant phase and start hydrolyzing the starch to soluble sugars. Up to 7 days during seed germination and seedling growth, the plant uses the energy from its stored reserves, endosperm. Due to higher temperature, the activity of these proteins was impaired leading to lower availability of soluble sugars. In the present study, a continuous increase in the seedling length was observed from the 4th to 6th DAG (Table S1). In set-I (Control), syn14128 indicated minimum seedling length on 4th DAG (8.27 cm) that might have been caused by the initial slow metabolism of starch in this genotype which showed an abrupt increase of length on 5th DAG (138%) and 6th DAG (146%). A similar trend was observed at 12HS and 20HS treatment, suggesting the involvement of the starch metabolizing enzyme, which can work even under HS conditions. To further understand the metabolism and utilization of stored starch, total soluble sugar content, total starch, and total amylase activity and gene expression of *amy4* were estimated in selected lines (Fig. 6). Higher TSS under heat stress indicated the potential of the amylase enzyme to metabolize endosperm starch. Syn14170 and syn14128 suggested a lower starch content along with higher TSS and higher amylase activity under both 12HS and 20HS sets indicating a stable and functioning amylase enzyme causing degradation of starch and providing sufficient energy to seedlings for survival even under heat stress conditions [16, 17].

Further, the minimum percent decrease in amylase activity after HS in the case of syn14128 suggested the capacity of amylase to recover quickly from the heat shock. In contrast, amylase activity in syn14170 reported a decrease similar to that of ABL, PBW343 in 12HS, 12HS24R, 20HS, and 20HS24R indicating higher damage and lower recovery of starch metabolism system in this SHW. Syn14170 had high TSS under both 12HS and 20HS and while syn14128 had less reduction in TSS in 12HS, nominating both of these synthetics as promising candidates for juvenile stage heat stress tolerance.

The germinating wheat seed requires a constant supply of sugars, and amylases are the primary set of enzymes involved in this process. Amylases can act in two different forms and are categorized as endoamylases and exoamylases.



Endoamylases, such as  $\alpha$ -amylase can cleave  $\alpha$ ,1-4 glycosidic bonds present in the inner part of the amylose or amylopectin chain.  $\alpha$ -amylase constitutes a significant portion and is more sensitive to heat as compared to  $\beta$ -amylase. To check the effect of heat stress on the utilization of starch in wheat seedlings,  $\alpha$ -amylase (*Amy4*) gene expression was quantified using quantitative real-time PCR. *Amy4* gene has already been characterized for seedling stage heat stress in wheat genotypes by Kaur [18]. Syn14128 followed by PBW114 were showing less reduction in total amylase activity in both 12HS and 20HS sets as well as under 12HS24R and 20HS24R sets as confirmed by expression analysis using quantitative real-time PCR for the  $\alpha$ -amylase gene. It suggested that better amylase activity in syn14128 might be a combined effect of PBW114 and *Ae. tauschii* parent and lower amylase expression and activity in syn14170 might be a trait derived solely from either one of the parents.

The two synthetics targeted as the potential candidates for juvenile stage heat stress tolerance in the present study had lower MDA content, lower  $H_2O_2$  content along with better free radical scavenging activities under 12HS, 20HS as well as 12HS24R, and 20HS24R indicating less membrane damage and lesser injury to the cellular machinery and a better ability to recover after facing short periods of high temperature. This suggested that these synthetics were better able to mitigate the oxidative stress caused by heat shock as compared to the durum wheat as well as the selected ABLs. This improved capacity of synthetics to endure heat stress is expected to be a probable trait inherited by synthetics from the D-genome donor of the modern wheat, *Ae. tauschii*. Further, better total amylase activity along with higher TSS and lower starch content in synthetics indicated a heat-efficient amylase and starch metabolizing pathway. In literature, various epigenetic and genetic changes occurring at the genome level during the formation of SHWs have been reported to cause the superiority of different SHWs with similar parents for different traits, which might be a reason for the differential expression of the amylase activity in selected SHWs. However, the differential biochemical and gene expression results of amylases in the SHWs and durum parents open up a future perspective for identifying the novel allelic forms of genes involved in starch metabolism along with various enzymes involved in heat stress tolerance.

**Supplementary Information** The online version contains supplementary material available at <https://doi.org/10.1007/s11033-022-07595-8>.

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**Author contributions** SK and KS conceived and designed the research. AK conducted the experiments and collected and analyzed the data. SKG helped in all biochemical analysis work, SK and SHW helped in

the analysis of results SK, MG, PC, SHW and AK produced the final draft of the manuscript. SK, KS and PC reviewed the experiments and manuscript.

**Data availability** NA.

**Code availability** NA.

## Declarations

**Conflict of interest** The authors declare that there is no conflict of interest.

**Ethical approval** NA.

**Consent to participate** NA.

**Consent for publication** All the authors have agreed to the publication of this manuscript.

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