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Enhanced antioxidant enzyme activities in developing anther contributes to heat stress alleviation and sustains grain yield in wheat

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Abstract. Climatic variations along with a rise in temperature during the winter season impose severe heat stress during the anthesis stage of spring wheat, resulting in severe yield losses. The present study was conducted to evaluate the influence of heat stress on redox homeostasis in developing anthers and flag leaves of wheat. Five Indian bread wheat genotypes were studied under field conditions during the dry season, with two extreme sowing dates (timely and very late sown) to explore the effect of heat stress on anthesis stage. Results showed that elevated temperature during anthesis caused significant increase in reactive oxygen species (ROS) content and malondialdehyde (MDA) accumulation in developing anthers, triggering pollen mortality. Moreover, defective source (leaf) to the sink (anthers) mobilisation of starch also contributes in reducing pollen viability. However, ROS-induced oxidative damage of developing anthers under heat stress varied among the wheat genotypes depending upon differential antioxidant enzyme activities. Wheat genotype with enhanced antioxidant activities and reduced ROS built up in developing anthers sustained their grain yield, suggesting thermo-tolerance in wheat to be associated with antioxidant enzyme-mediated improved ROS-scavenging mechanism not only in leaves even in developing anther also. In the present study, heat stressed wheat genotype WH 730 exhibited effective source to sink mobilisation and sustainable grain yield with improved ROS scavenging, conferring greater potential for heat tolerance. We conclude that redox homeostasis and balanced source sink activity played a significant role for sustainable yield and heat tolerance in wheat.

Additional keywords: anther, antioxidant enzymes, ROS, terminal heat stress.

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Introduction

Global climatic changes along with constant elevation in atmospheric temperature severely influence plant growth and agricultural productivity. Due to global climate change, the annual mean maximum and minimum of global temperatures have increased by 0.35 and 1.13°C, respectively, from 1979 to 2003 (Peng *et al*. 2004). Continuous climate change has been predicted to result in the rise of average temperature of earth with extreme heat events, such as heat waves throughout the world (IPCC 2014). Heat stress frequently affects greatest part of the world's wheat cultivating regions during the growing season (Mondal *et al*. 2016). Food and Agriculture Organisation (FAO 2012) has revealed the heat stress to cause a 5.7% overall decrease in global wheat production. Delayed sowing of winter wheat in the Eastern Indo-Gangetic Plains due to a late harvest of the preceding crop results in exposure of the anthesis stage to high temperatures, and causes a significant reduction in yield (Gupta *et al*. 2015). Therefore, there is real need to unravel the mechanisms of heat tolerance in Eastern Indo-Gangetic wheat genotypes, particularly at the anthesis stage. High temperature stress in wheat induces intense alterations in morpho-physiological (Mondal *et al*. 2016), anatomical and biochemical characteristics (Farooq *et al*. 2011; Dwivedi *et al*. 2017). Heat stress in wheat shortens the plants' life cycle through premature ripening, which is unable to be compensated for by accelerated growth (Gupta *et al*. 2015). High temperature-induced inhibition of chlorophyll biosynthesis accelerates leaf senescence, and decreases sugar reserves in plants because sugar

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accumulation at the anthesis period has strong correlation with leaf area dynamics (Tovignan *et al*. 2016). Heat stress-induced defective source–sink mobilisation of starch leads to poor grain yield (Dwivedi *et al*. 2017; Hütsch *et al*. 2019). Heat stress also triggers alterations in the rate of photosynthesis, water relations and accumulation of compatible osmolytes in plants (Hamid *et al*. 1990; Hameed *et al*. 2012). Consequently, the water use efficiency (WUE) – a major factor in determining yield – is also severely affected by the heat stress (Hamid *et al*. 1990).

High temperatures (>30°C) during reproductive growth stage of wheat has a detrimental effect on pollen viability with fertilisation and kernel setting (Dwivedi *et al*. 2017; Hütsch *et al*. 2019). Heat stress during the anthesis can cause pollen sterility resulting in poor fertilisation (Dwivedi *et al*. 2018; Zhao *et al*. 2018*a*). Most of the microspores remain immature and do not accumulate starch under severe heat stress. Elevated temperature at the onset of meiosis also has a detrimental effect on the pollen viability, with consequent decrease in fertilisation and grain production (Zhao *et al*. 2018*b*). Therefore, pollen might be considered as a suitable biomarker for screening heat tolerant genotypes (Liu *et al*. 2006).

The eruption of reactive oxygen species (ROS), including hydrogen peroxide (H_2O_2), the hydroxide ion (OH^-), singlet oxygen ($^1O_2$), superoxide anion ($\bullet O_2^-$), is one of the key events for the plants' response to heat stress (Hameed *et al*. 2012). Metabolic abnormality caused by elevated temperature promotes the accumulation of ROS in both photosynthetically active and senescent plant cells. Leaf senescence caused by increased membrane lipid peroxidation employs decreased sugar reserve, as sugar accumulation at anthesis period has strong correlation with leaf area dynamics (Dhindsa *et al*. 1981; Tovignan *et al*. 2016). Recent studies have revealed the elevated temperature during anthesis stage to cause marked increase in the ROS level in developing anther resulting in loss of pollen viability (Zhao *et al*. 2018*a*, 2018*b*; Fábián *et al*. 2019).

Heat susceptibility in plants is mostly associated with ROS-scavenging through enzymatic antioxidant defence system (Sairam *et al*. 2000). The equilibrium between the ROS production and scavenging is commonly known as redox homeostasis. The ROS is scavenged by primary antioxidant enzymes, including superoxide dismutase (SOD; EC 1.15.1.1), guaiacol peroxidase (POX; EC 1.11.1.7), catalase (CAT; EC 1.11.1.6) and enzymes of the ascorbate-glutathione cycle, such as ascorbate peroxidase (APX; EC1.11.1.11) (Chen *et al*. 2017). Antioxidant enzyme mediated ROS detoxification in anther is related to pollen fertility under heat stress (Zhao *et al*. 2018*b*). Growth enhancers like proline and soluble sugars also promote plant growth and ameliorate stress by interfering in metabolic and photosynthetic processes through osmotic adjustment (Hameed *et al*. 2012). However, thermal susceptibility in wheat obviously varies with genotypic diversity and growing phases with reproductive stage being the most sensitive (Dhyani *et al*. 2013).

Though a significant body of research has been published on ROS production and antioxidant enzyme activities in flag leaves of wheat (e.g. Sairam *et al*. 2000; Hameed *et al*. 2012), the role of antioxidant enzyme-mediated redox homeostasis in developing anthers of wheat has never been studied. However, loss of pollen viability caused by ROS overproduction in male reproductive organ (anther) plays a key role in yield reduction under heat stress. Therefore, there is a significant gap in the knowledge required to improve thermo-tolerance in wheat. A detailed understanding of the role of improved antioxidant enzyme activities with starch mobilisation and pollen viability might bridge the knowledge gap and facilitate improvement of wheat genotypes that exhibit enhanced heat tolerance under field conditions. The present study elucidated the association of thermo-tolerance in five different genotypes of Indian bread wheat with antioxidant enzyme-mediated improved ROS-scavenging mechanism in both flag leaves and developing anthers, and also explained how redox homeostasis and balanced source–sink activities play a significant role for sustainable yield and heat tolerance in wheat.

Materials and methods

Plant materials and experimental design

The present study was conducted using five Indian bread wheat genotypes (GW 273, Kundan, NW 1014, Raj 3765 and WH 730) grown in the dry season of 2015–16 at the field of ICAR research complex for Eastern Region, Patna (25.59°N, 85.14°E, 53 m above sea level). The meteorological conditions of the experimental site during the study period were recorded. The field was prepared for the trial as described in our previous study (Dwivedi *et al*. 2017). In brief, the experiment was performed in $5 \times 4\text{-m}^2$ stretched plots divided in six rows separated by 23 cm spacings. The field was labelled with laser labeller before the trials and optimum moisture was maintained during the sowing time. Crop management was optimised with normal irrigation, application of fertiliser and pest control.

The sowing time of the wheat genotypes was optimised in such way that the reproductive phase of the very late sown crop could be exposed to the heat stress. Wheat genotypes sown at late-November and early-January were considered as the timely sown (TS) and the very late sown (VLS), respectively. The experimental layout is presented in Fig. 1. Details of sowing time and phenological stages of the considered wheat genotypes are presented in Table S1, available as Supplementary Material to this paper. Morpho-physiological, anatomical and biochemical analyses were performed at anthesis of almost 90% of the spikes, i.e. after the plants experiencing heat stress. The grain yield and yield attributes were measured after harvesting of the plants.

Sampling time/stage

Crop phenology was monitored according to Zadoks decimal code (Zadoks *et al*. 1974). Completion of anthesis (Z69, i.e. anthesis completed) was recorded when the anthers emerged from the main stem in more than 70% of the plants. Morpho-physiological (plant height, relative water content (RWC), leaf area index (LAI), canopy temperature depression (CTD) and gas-exchange parameters), bio-chemical (chlorophyll, proline, total soluble sugars, ROS, antioxidant defence and starch content) and anatomical studies (starch mobilisation and pollen viability) were performed at anthesis of the spikes (Z65, i.e. anthesis halfway). Grain yield and yield attributes (thousand grain weight (TGW)) were measured after harvesting of the fully matured plants (Z92, i.e. kernel hard).

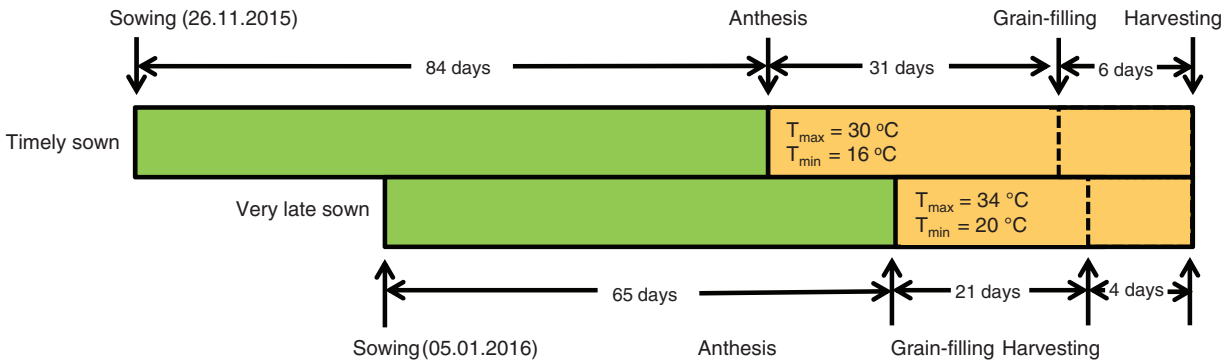

Fig. 1. Schematic representation of experimental layout for evaluating the impact of terminal heat stress on wheat performed during the wheat growing season of 2015–16 at the field of ICAR research complex for Eastern Region, Patna, India. The diagram portrays sowing time and phenological stages of the considered wheat genotypes sown under timely (TS) and very late (VLS) conditions. Vertical arrows represent the developmental stages of wheat. Days to anthesis, physiological maturity and harvest are expressed as the average of the five wheat genotypes ($n = 5$). T_{max} and T_{min} represent the average maximum and minimum temperature, respectively, during the anthesis period depicting the exertion of heat stress on VLS plants. Red arrows indicate the sampling stages (anthesis) for the TS and VLS plants.

Growth parameters

Plant height of all genotypes sown under different sowing time was measured at their respective anthesis stages with measuring tape. In this experiment, ambient temperature during the anthesis of the timely sown (TS) plants did not exceed the optimum temperature for anthesis of wheat and therefore, was considered as control (C). In contrast, the very late sown (VLS) plants were considered as heat stressed (HS), as the ambient temperature during the VLS plant anthesis reached beyond the optimum temperature. Relative growth for each genotype was calculated using the following formula.

$$\text{Relative growth} = (\text{C} - \text{HS}) \times 100/\text{C}. \quad (1)$$

Determination of canopy temperature depression (CTD) and leaf area index (LAI)

Canopy temperature was recorded using Infrared thermometer (AG-42, Telatemp Crop) during anthesis (Reynolds *et al*. 1998). Ambient temperature was also measured using a thermometer. The CTD was determined from the following formula:

$$CTD = \text{ambient temperature} - \text{canopy temperature} \quad (1a)$$

Leaf area was measured following the method of Gardner *et al*. (1985) using leaf area meter (LI-COR Biosciences). Leaf area index (LAI) was calculated by using the following formula.

$$\text{LAI} = \text{leaf area}/\text{ground area} \quad (1b)$$

Gaseous-exchange measurements

Gas-exchange parameters were measured on fully expanded flag leaves using portable Infrared Gas Analyser (IRGA, LI-6400Model, LI-COR Biosciences). Photosynthetic rate was determined at different growth stages between 1000 and 1130 hours by providing artificial light source with light intensity of 1000 µmol photon $m^{-2}s^{-1}$. The net photosynthetic rate (P_N) was expressed as µmol CO_2 m^{-2} s^{-1}. Stomatal conductance (g_s) and transpiration rate (E) was measured in the same way; g_s was expressed as mol H_2O m^{-2} s^{-1} and transpiration rate (E) was expressed as mmol H_2O m^{-2} s^{-1}. With these data, water use efficiency (WUE) (P_N/E) (µmol CO_2 mol^{-1} H_2O) was quantified (Hamid *et al*. 1990).

Quantification of leaf water status and total chlorophyll content

Relative water content (RWC) was determined according to the protocol of Weatherley (1950). Fully expanded topmost flag leaf was collected and weighed quickly omitting the mid-rib to record FW. The samples were hydrated to full turgidity by floating on de-ionised water for 4 h. After incubation the surface moisture of samples was removed and immediately weighed to obtain fully turgid weight. The dry weight of the samples was taken after proper drying in a hot air oven at 80°C for 24 h. RWC was calculated for each sample following the formula.

$$\text{RWC}(\%) = \frac{(\text{FW} - \text{DW})}{(TW - DW)} \times 100 \quad (1c)$$

Total chlorophyll (chl) content was estimated according to Lichtenthaler and Wellburn (1983). Samples (0.05 g) of flag leaves of each genotype were taken and 10 mL DMSO was added. The samples were incubated at 60°C for 4 h. Absorbance was recorded at 645 and 663 nm on a UV-VIS spectrophotometer (Model BIO 200, Thermo Fisher Scientific). Total chlorophyll content was calculated following the Arnon's (1949) equation:

$$\textit{Total chlorophyll} = \frac{(20.2 \times O.D645 + 8.02 \times O.D663) \times V}{1000} \times W \quad (1d)$$

Estimation of proline content

Proline was estimated according to Bates *et al*. (1973). Flag leaves were homogenised in 3% salicylic acid and filtered. The filtrate was mixed with 0.2% ninhydrin, glacial acetic acid and incubated at 100°C for 1 h. Then the samples were cooled on ice. Proline was extracted from the mixture with toluene and absorbance was recorded at 520 nm. Proline content was calculated from the standard.

Assay of ROS

Hydrogen peroxide (H_2O_2) was determined according to Sergiev *et al*. (1997) with some modifications. Briefly, H_2O_2 was extracted from 0.5 g plant tissue with 0.1% (w/v) TCA. After centrifuged at 12 000*g* for 15 min, the supernatant was incubated with 0.1 mM potassium phosphate buffer (pH 7.0) and 1 M potassium iodide solution and absorbance was measured at 390 nm. H_2O_2 content was obtained from a standard curve.

Superoxide anion ($\bullet O_2^-$) was estimated as described by Chaitanya and Naithani (1994) with minor modifications. Plant samples were homogenised in ice-cold 0.2 M phosphate buffer (pH 7.2) containing 10^{-3} M diethyldithiocarbamate to inhibit SOD activity. The homogenate was centrifuged at 10000 g for 10 min. The $\bullet O_2^-$ was measured in the supernatant by its capacity to reduce NBT (2.5×10^{-4} M). Change in absorbance due to formation of $\bullet O_2^-$ was measured at 540 nm for 1 min.

The H_2O_2 and $\bullet O_2^-$ in developing anthers were visually detected by 3,3-diaminobenzidine (DAB) and nitro blue tetrazolium (NBT) uptake method (Basu *et al*. 2017). Anthers from unopened flower buds were infiltrated with 0.5 mg ml^{-1} freshly prepared DAB/NBT staining solutions. Samples were decolorised by incubating in 90% ethanol. The H_2O_2 and $\bullet O_2^-$ were visualised as brown/ blue colour due to DAB/NBT polymerisation.

Measurement of lipid peroxidation

Lipid peroxidation was measured in terms of MDA content. The MDA content was determined by thiobarbituric acid (TBA) test according to Heath and Packer (1968). After extracting MDA from plant tissue with 0.5% TBA in 20% trichloroacetic acid (TCA) absorbance was measured at 532 nm. Values of non-specific absorption recorded at 600 nm were subtracted from the values recorded at 532 nm. MDA content was calculated according to its extinction coefficient $\varepsilon = 155\ mM^{-1}\ cm^{-1}$.

Determination of antioxidant enzyme activities

Lyophilised plant tissue was homogenised with ice-cold phosphate buffer (pH 7.0) (Basu *et al*. 2017). Total protein was quantified in the plant tissue according to Bradford (1976). The absorbance of Coomassie Brilliant Blue after binding with the protein was recorded at 595 nm and was calculated from the BSA standard curve. The extracted protein was used to detect different antioxidant enzyme activities.

Superoxide dismutase (SOD) (EC1.15.1.1) activity was determined at 560 nm by its ability to reduce the formation of blue coloured formazone by NBT and $\bullet O_2^-$ radical (Dhindsa *et al*. 1981).

Catalase (CAT) (EC 1.11.1.6) activity estimation was performed according to Aebi (1983). Change in absorbance with addition of H_2O_2 was recorded at 240 nm for 1 min. Enzyme activity was expressed as unit min^{-1} mg^{-1} protein and a change in absorbance by 0.01 corresponded to 1 unit of enzyme activity.

Peroxidase (POX) (EC 1.11.1.7) activity was determined at 436 nm by its ability to convert guaiacol to tetraguaiacol (Polle *et al*. 1994). The increase in absorbance was recorded by the addition of H_2O_2 at 436 nm for 1 min.

Ascorbate peroxidase (APX) (EC 1.11.1.11) activity was estimated following the method of Nakano and Asada (1981). Change in absorbance with was recorded at 290 nm for 1 min after addition of ascorbate and H_2O_2.

Quantification of starch and total soluble sugar content

Starch was extracted from plant samples and was hydrolysed to glucose using dilute HCl. In hot acidic medium glucose was dehydrated to hydroxymethyl furfural, which formed green coloured product with anthrone reagent. The intensity of green colour was measured at 620 nm and starch was quantified from the glucose standard (McCready *et al*. 1950).

Total soluble sugar from flag leaves were extracted with 80% hot ethanol (Yemm and Willis 1954). Total sugar hydrolysed to glucose and was measured at 620 nm with anthrone reagent and was quantified from the glucose standard.

Study of starch mobilisation and pollen viability

Study of starch mobilisation through stem internode-I and pollen viability was performed following our previous study (Dwivedi *et al*. 2017). Cross-sections of internode and pollen grains were observed under a Motic K400 light microscope and photographed with a Nikon E995 digital camera.

Determination of yield attributes, grain yield and heat susceptibility index (HSI)

After harvesting of the fully matured plants (Z92), grain yield (GY) was measured (t ha^{-1}) and TGW, grain number $spike^{-1}$, spike length, tiller m^{-2} were also recorded.

Heat susceptibility index (HSI) for grain yield in each genotype was determined according to Dhyani *et al*. (2013). Heat tolerant genotypes were categorised as extremely heat tolerant ($s \leq 0.50$); heat tolerant (s = 0.51–0.75); moderately heat tolerant (s = 0.76–1.00); heat susceptible (s >1.00).

Statistical analyses

The data were statistically analysed for analysis of variance (ANOVA) as a two way factorial completely randomised design (physiological and biochemical parameters) and two way factorial randomised block design (yield attributes) using Statistics 8.1, USA software with three replications ($n = 3$). Treatments were compared by computing the *F*-test. The standard error of mean (± s.e.) and differences between the treatments was compared pair wise by critical difference (CD) at the 5% level of probability.

Results

Weather condition and heat stress imposition

Weather data during the experimental period (dry season of 2015–16) has been presented in Fig. 2. Mean weekly temperature is expressed in standard meteorological weeks (SMW). The crop season had similar average temperatures from the last week of November till January with T_{max} ranging between 21.3 and 28.4°C. The coolest temperature was observed at the end of January (average T_{min} 7.3°C). Gradually, the average temperatures started to increase and became higher during

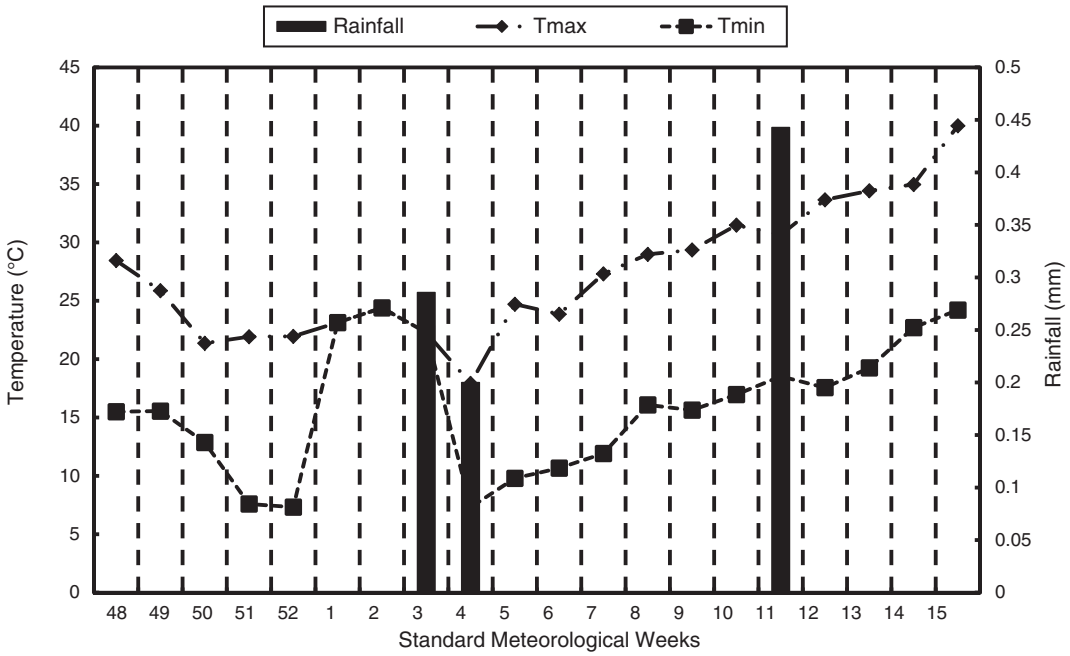

Fig. 2. Meteorological data for the 2015–16 wheat growing season (26 November 2015 to 15 April 2016) in ICAR-RCER, Patna, India. Air temperatures are represented in lines and precipitation levels in bars. T_{max} is maximum temperature; T_{min} is minimum temperature.

March and April (average T_{max} 33.5°C). Temperature during the anthesis stage of VLS genotypes (T_{max} 34.3°C, T_{min} 20°C during anthesis) reached beyond the optimum temperature for anthesis of wheat, exerting severe heat stress to the VLS plants. In contrast, the temperature during the anthesis of TS plants were lower and therefore, considered as the control condition (T_{max} 30°C, T_{min} 16°C during anthesis).

Effect of heat stress on growth and yield traits in wheat

Heat stress considerably altered the growth of the five wheat genotypes. Under TS condition, relative change in plant height ranged from 9.0 to 20.2% across the wheat genotypes, which was severely decreased under heat stress (Fig. 3*a*).The reduction in plant height was minimal under heat stress condition for NW 1014, GW 273, Raj 3765 and WH 730 (9.0, 10.2, 11.9 and 12.4% decrease respectively), whereas, Kundan showed the maximum decrease in plant height under VLS condition (20.2%). Heat stress during anthesis also caused decrease in the LAI, tiller numbers, grain yield and TGW across the wheat genotypes (Table 1). Elevated temperature resulted in significant decrease of LAI ($P \leq 0.05$) that had inter-genotypic variation, but the effect of heat stress was the same across the considered genotypes. The lowest reduction in LAI was noted in WH 730 (10.5% decrease), contrasting with GW 273 exhibiting the maximum decrease in LAI (44% decrease) under VLS condition. Raj 3765 and NW 1014 also had higher reduction in LAI due to heat stress (34.6 and 37.5% reduction respectively). High temperature during anthesis decreased the number of tillers across the wheat genotypes. WH 730 showed the lowest reduction in tiller numbers (9.8% decrease), contrasting with GW 273 and Kundan (33.4 and 27.2% decrease respectively). Raj 3765 and NW 1014 also maintained the number of tillers under VLS condition (11.6 and 14.7% decrease respectively). Grain number spike^{-1} was significantly decreased under heat stress in all genotypes as compared with the control plants ($P \leq 0.5$). It was observed that WH 730, Raj 3765 and NW 1014 had the lowest reduction in grain number per spike (average 11.5 ± 0.4% decrease) under heat stress contrasting with Kundan and GW 273 (31.6 and 25% decrease respectively). Moreover, total grain yield was also significantly reduced following heat stress at anthesis ($P \leq 0.05$). According to the result, WH 730 exhibited the lowest decrease in grain yield even under VLS condition (9.3% decrease). However, Kundan had the highest decrease in grain yield (21.4% decrease) under VLS condition, followed by GW 273 and NW 1014 (20.9 and 15.9% decrease respectively), whereas, Raj 3765 had comparatively less decrease (11.6% decrease). The HSI for grain yield was determined in the considered wheat genotypes (Fig. 3*b*). WH 730 exhibited potential heat tolerance followed by Raj 3765 (s = 0.51–0.75), whereas, NW 1014 showed moderate heat tolerance followed by GW 273 and Kundan (s = 0.76–1.00). None of the genotypes were heat susceptible (s > 1.00). Heat stress also resulted in significant reduction in TGW ($P \leq 0.05$). Following the present study, Kundan, GW 273 and NW 1014 showed greater decrease in TGW (15.5, 14.8 and 14.6% decrease respectively), whereas, WH 730 and Raj 3765 maintained the TGW after perceiving severe heat stress (9.5 and 12.4% decrease respectively).

Physiological traits affected by heat stress

Elevated temperature severely impeded the physiological activities of wheat plants. RWC of the flag-leaves in the

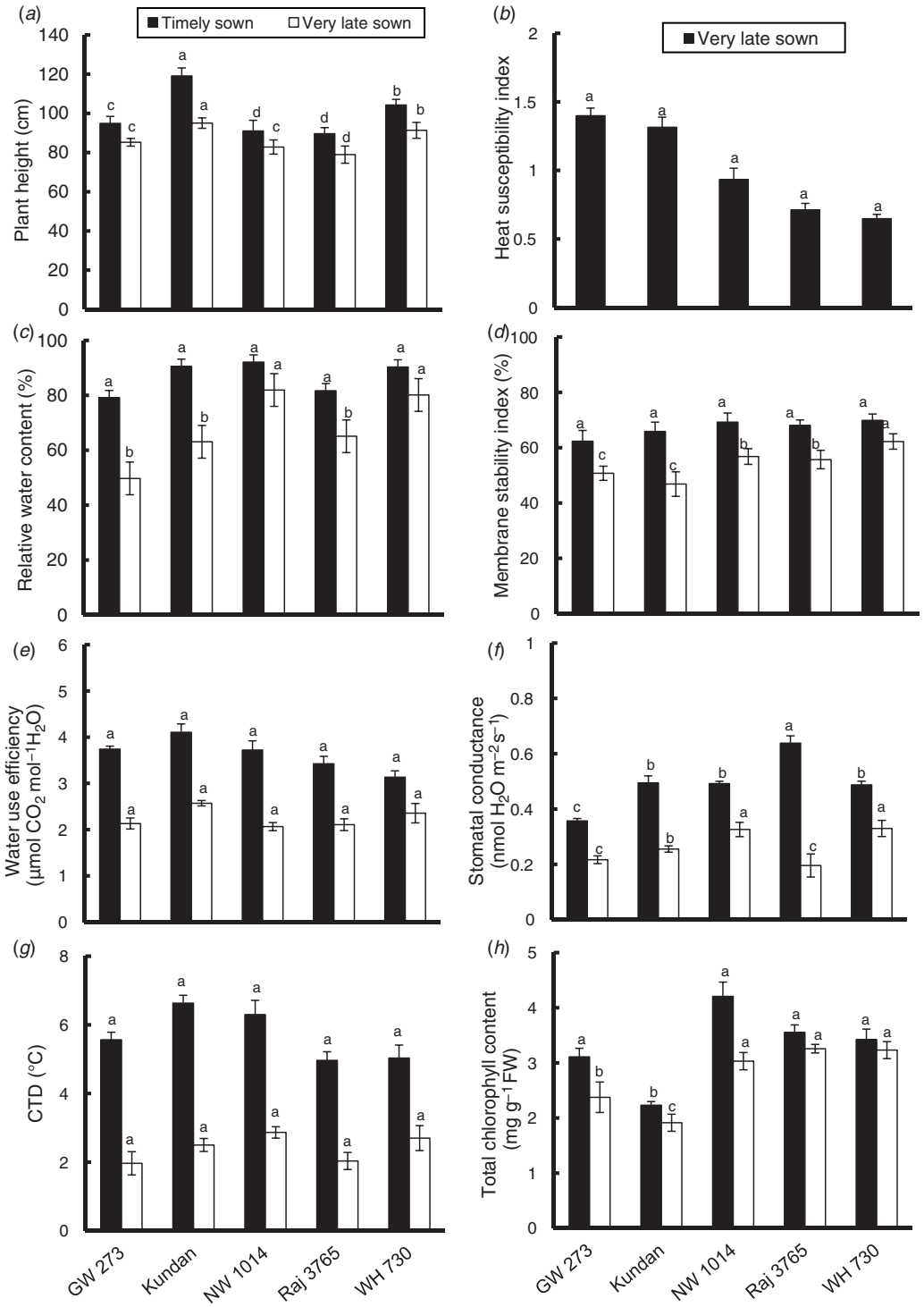

Fig. 3. Morpho-physiological studies of five wheat genotypes at anthesis stage sown in the dry season of 2015–16 with different sowing windows. The effect of heat stress on plant height (*a*), heat susceptibility index (*b*), relative water content (*c*), membrane stability index (*d*), water use efficiency (*e*), stomatal conductance (*f*), canopy temperature depression (*g*), total chlorophyll content and (*h*) of the considered wheat genotypes grown under timely sown (TS) and very late sown (VLS) conditions. Data represent means ± s.e. ($n = 3$). The level of significance was compared among the five wheat genotypes under TS and VLS conditions separately. Values with different letters are significantly different at $P \leq 0.05$.

Table 1. Grain yield and yield attributes of five wheat genotypes grown under timely sown (TS) and very late sown (VLS) conditions during the dry season of 2015–16

Abbreviation: CD, critical difference

Treatment		Leaf area index		No. of tiller m^{-2}		Grain no. $spike^{-1}$		Grain yield (t ha^{-1})		Thousand grain weight (g)	
Date of sowing											
TS		2.3		509.3		50.4		4.3		37.9	
VLS		1.6		408.5		41.1		3.7		32.6	
Genotypes (G)											
GW 273		2.0		410.5		47.5		3.8		33.0	
Kundan		1.9		508.7		41.5		3.7		38.8	
NW 1014		1.9		469.15		48.5		4.0		33.6	
Raj 3765		2.2		453.5		45.2		4.1		35.6	
WH 730		1.8		454.35		46.2		4.1		36.2	
Date of sowing × genotype											
Genotype		Date of sowing		Date of sowing		Date of sowing		Date of sowing		Date of sowing	
		TS	VLS	TS	VLS	TS	VLS	TS	VLS	TS	VLS
GW 273		2.5 ± 0.2	1.4 ± 0.2	492.7 ± 20.5	328.3 ± 17.6	54.3 ± 3.5	40.7 ± 1.2	4.3 ± 0.3	3.4 ± 0.2	35.7 ± 2.4	30.4 ± 1.1
Kundan		2.1 ± 0.2	1.8 ± 0.1	588.7 ± 11.8	428.7 ± 22.2	49.3 ± 5.1	33.7 ± 4.5	4.2 ± 0.2	3.3 ± 0.4	42.0 ± 2.6	35.5 ± 2.3
NW1014		2.4 ± 0.2	1.5 ± 0.2	506.3 ± 15.9	432.0 ± 15.3	51.3 ± 3.2	45.7 ± 3.1	4.4 ± 0.2	3.7 ± 0.5	36.2 ± 2.5	30.9 ± 2.1
Raj 3765		2.6 ± 0.2	1.7 ± 0.2	481.3 ± 13.9	425.7 ± 14.4	48.0 ± 5.0	42.3 ± 2.1	4.3 ± 0.2	3.8 ± 0.3	37.9 ± 2.5	33.2 ± 3.5
WH 730		1.9 ± 0.1	1.7 ± 0.1	477.7 ± 12.5	431.0 ± 15.1	49.0 ± 1.0	43.3 ± 4.0	4.3 ± 0.3	3.9 ± 0.3	38.0 ± 1.7	34.4 ± 2.7
CD ($P \leq 0.05$)	D	0.1 0.2 0.2		13.7		2.3		0.2		1.9	
	G			21.7		3.7		3.6		3.0	
	D × G			30.7		5.2		0.5		4.3	

considered wheat genotypes was also decreased by 11.0–37.2% under VLS conditions (Fig. 3*c*). GW 273 had the maximum decrease in RWC (37.2% decrease), whereas, NW 1014 and WH 730 exhibited almost similar decrease in RWC under heat stress condition (11.0 and 11.3% decrease respectively). Kundan also had severe decrease in RWC under VLS condition (30.3% decrease). Heat stress also resulted in decreased flag-leaf MSI in the considered wheat genotypes (Fig. 3*d*). WH 730 maintained comparatively higher MSI under VLS condition (62.3%), whereas Kundan had the lowest MSI (46.9%) under heat stress among the studied genotypes. Heat stress during anthesis had a significant negative effect on flag leaf net photosynthesis rate (P_N) of all wheat genotypes (data not shown). The P_N was markedly decreased in GW 273 and NW 1014 (43 and 44.5% decrease respectively), whereas WH 730 maintained higher P_N after receiving severe heat stress (24.8% decrease). Trend in transpiration rate (*E*) was similar to that of the P_N in flag leaves (data not shown). Consequently, the instantaneous WUE was significantly decreased ($P \leq 0.05$) under VLS condition across the wheat genotypes studied (Fig. 3*e*). However, WH 730 exhibited the minimum decrease (24.8% decrease) in WUE and sustained the WUE (2.36 ± 0.2 µmol H_2O mol^{-1} CO_2) under heat stress condition. Moreover, decreased P_N and *E* in VLS genotypes were accompanied by reduced stomatal conductance (g_s) (Fig. 3*f*); g_s was similar for the five wheat genotypes under TS condition, with an overall average of 0.49 ± 0.01 nmol H_2O m^{-2} s^{-1}, which was significantly decreased ($P \leq 0.05$) under VLS condition. WH 730 upheld the maximum stomatal conductance (0.33 ± 0.03 nmol H_2O m^{-2} s^{-1}) after perceiving heat stress (32.3% decrease). Heat stress caused successive decrease in CTD of the considered wheat genotypes during anthesis (Fig. 3*g*). Canopy temperatures of the genotypes increased markedly under VLS conditions but were less than the ambient temperature. Among the five wheat genotypes, NW 1014 and WH 730 (2.8 ± 0.51 and 2.7 ± 0.53°C respectively) showed the highest CTD contrasting with GW 273 (1.97 ± 0.42°C).

Total chlorophyll content in the flag leaves of the considered wheat genotypes was also significantly reduced under heat stress (Fig. 3*h*). The average chlorophyll content in the TS plants of all genotypes (3.3 ± 0.2 mg g^{-1} FW) was higher as compared with their VLS condition (2.8 ± 0.4 mg g^{-1} FW). The results showed that WH 730 and Raj 3765 maintained their flag-leaf chlorophyll content (5.5 and 8.3% decrease respectively) under VLS condition, whereas, GW 273 and Kundan had the lowest leaf chlorophyll content (23.6 and 14.3% decrease respectively) under the heat stressed condition.

Role of osmolytes and source-sink dynamics

Heat stress caused accumulation of osmolytes (proline) in the wheat genotypes (Fig. 4*a*). In the study, WH 730 showed the highest increase in proline accumulation under VLS condition (2.6-fold increase), followed by Raj 3765 and NW 1014 (1.9- and 1.5-fold increase respectively). Lowest increase in proline content was observed in Kundan and GW 273 (1.1- and 1.3-fold increase, respectively). Similar trend was observed in total soluble sugar (TSS) content (Fig. 4*b*). WH 730 exhibited the maximum increase in TSS under heat stress (1.5-fold increase) among the five wheat genotypes.

Impaired source-sink mobilisation of starch under heat stress also played a significant role in loss of pollen viability, consequently decreasing grain yield in wheat genotypes. Heat stress caused decrease in the flag leaf starch content of the

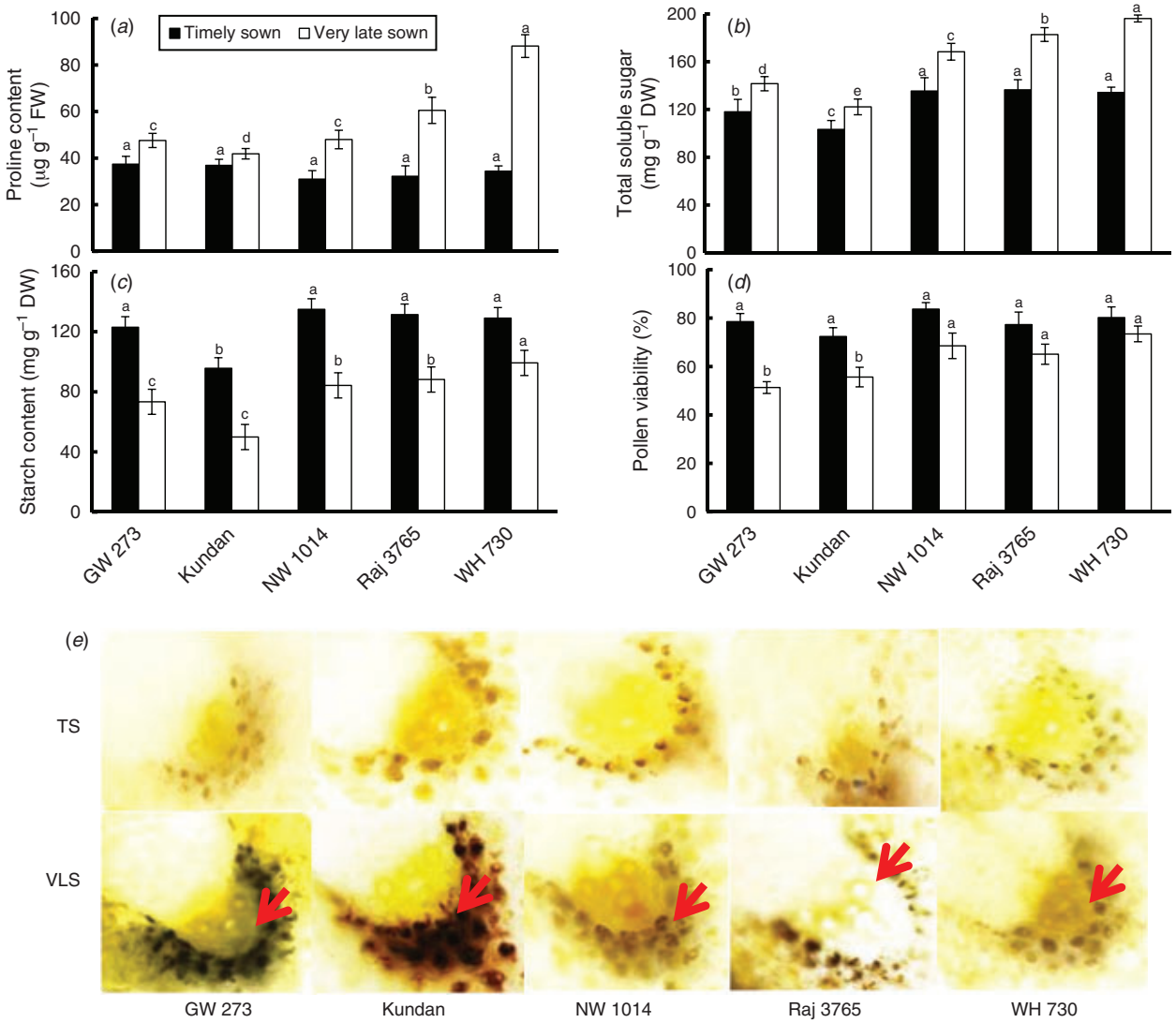

Fig. 4. Role of osmolytes and source-sink dynamics on wheat genotypes at anthesis stage under different sowing windows. The effect of heat stress on proline content (*a*), total soluble sugar content (*b*), flag leaf starch content (*c*), pollen viability (*d*) and starch mobilisation (solid arrows indicate starch deposition at internode-I from the top) (*e*). Data represent means ± s.e. ($n = 3$). The level of significance was compared among the five wheat genotypes under TS and VLS conditions separately. Values with different letters are significantly different at $P \leq 0.05$.

considered wheat genotypes (average 36.4% decrease) (Fig. 4*c*). Kundan exhibited the maximum reduction in starch content (47.9% decrease) among the five genotypes, whereas WH 730 to conserved the starch (23.1% decrease). Congested starch mobilisation in wheat under heat stress condition resulted in accumulation of starch at the internodal region (Fig. 4*e*). On application of KI-I_2 solution, intense starch staining was observed in the VLS genotypes, compared with their respective TS conditions. However, under VLS conditions, transport of assimilate was conspicuously higher in WH 730, followed by Raj 3765 and NW1014, whereas, it was markedly reduced in Kundan and GW 273. Heat stress-induced ROS accumulation and obstructed source-sink mobilisation were consequently accompanied by significant decrease in pollen viability (Fig. 4*d* and Fig. S1, available as Supplementary Material to this paper). In the present study, the loss of pollen viability was highest in GW 273 among the genotypes examined (27.2% decrease). The maximum pollen viability under VLS condition was observed in WH 730 (6.8% decrease), followed by Raj 3765 and NW 1014 (12.2 and 15.2% decrease respectively).

Heat stress induces differential oxidative stress

Heat stress during anthesis stage caused significant increase in H_2O_2 content, $\bullet O_2^-$ production rate and MDA accumulation in developing anthers of the considered wheat genotypes (Fig. 5). However, the enhanced ROS build-up (H_2O_2 content, $\bullet O_2^-$ production rate) and ROS-induced oxidative damage (MDA accumulation) under heat stress was largely variable, depending upon the thermo-tolerance of wheat genotypes. For instance, Kundan exhibited the maximum accumulation of $H_2O_2/\bullet O_2^-$ under VLS condition, as visualised by higher

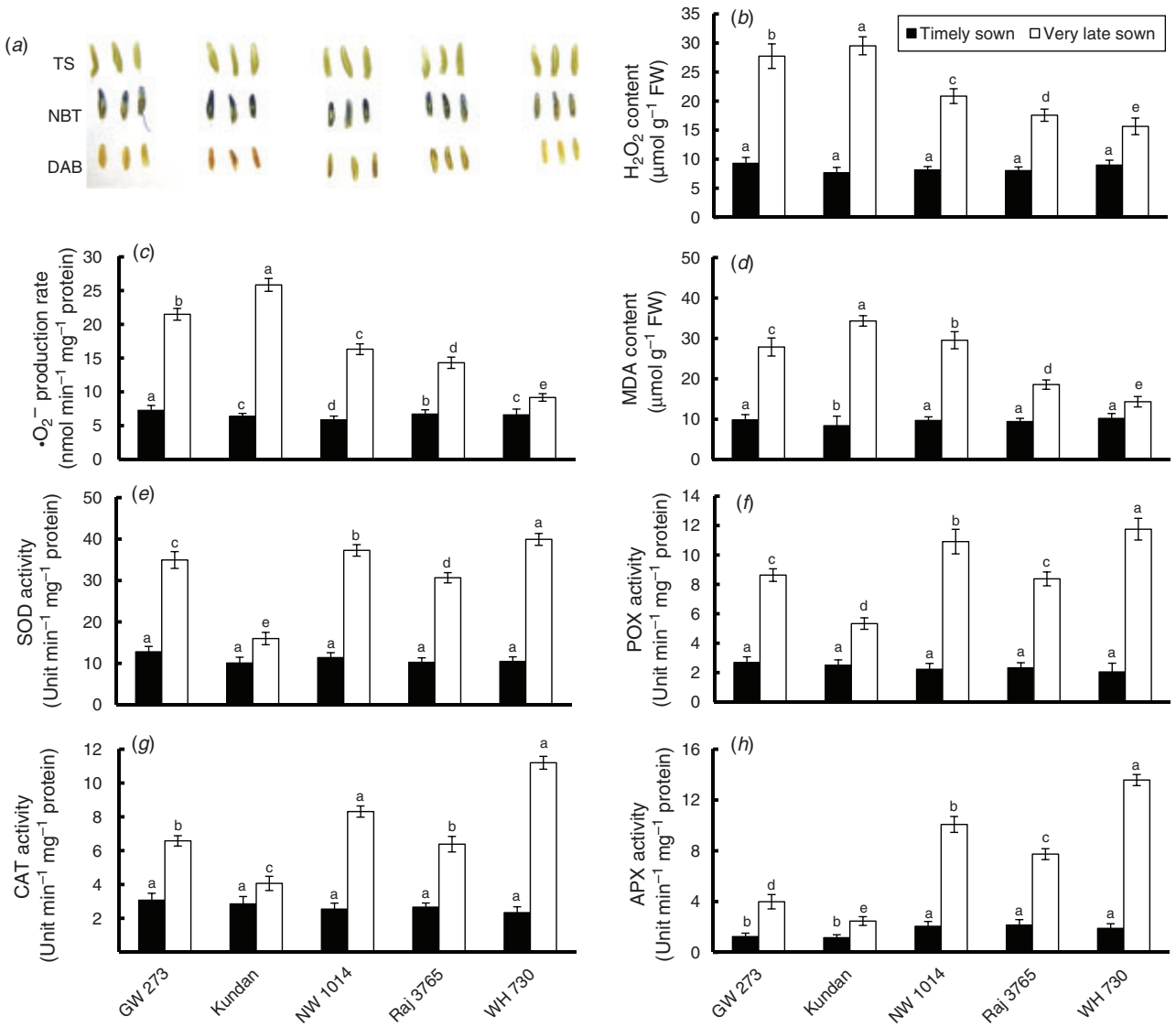

Fig. 5. Study of redox homeostasis in the developing anthers of five wheat genotypes at anthesis stage grown under timely sown (TS) and very late sown (VLS) conditions. Effect of heat stress on ROS accumulation (blue and brown colour represents $•O_2^-$ and H_2O_2 accumulation respectively) (*a*), H_2O_2 content (*b*), $•O_2^-$ production rate (*c*), MDA content (*d*), SOD activity (*e*), POX activity (*f*), CAT activity (*g*) and APX activity (*h*). Data represent means ± s.e. ($n = 3$). The level of significance was compared among the five wheat genotypes under TS and VLS conditions separately. Values with different letters are significantly different at $P \leq 0.05$.

accumulation of dark brown/blue colour with DAB/NBT staining (Fig. 5*a*). In contrast, WH 730 showed reduced ROS (H_2O_2 and $•O_2^-$) accumulation as depicted by the less intense DAB/NBT colouration under heat stress. The histochemical localisation of H_2O_2 and $•O_2^-$ was in agreement with the data obtained from biochemical analysis. The H_2O_2 content and rate of $•O_2^-$ production in developing anthers were significantly increased ($P \leq 0.5$) in all genotypes (Fig. 5*b*, *c*). WH 730 had the minimal increase in H_2O_2 (1.7-fold increase) and the smallest $•O_2^-$ production rate (9.2 nmol min^{-1} mg^{-1} protein) under heat stressed condition, contrasting with Kundan (3.9-fold increase in H_2O_2; $•O_2^-$ production rate 25.9 nmol min^{-1} mg^{-1} protein). ROS accumulation in developing anther led to oxidative damage through marked increase of MDA content (Fig. 5*d*). The minimum MDA content was observed in WH 730 (14.3 μmol g^{-1} FW) experiencing heat stress, whereas, Kundan had the highest MDA content (34.4 μmol g^{-1} FW).

Antioxidant enzyme activities contribute to heat stress tolerance

Antioxidant enzyme-mediated redox homeostasis was used as a marker to estimate the deleterious effect of ROS in developing anther. Several key antioxidant enzyme activities, including SOD, CAT, POX and APX were measured in under heat stress (Figs 5*e–h*, S2). Heat stress exposure caused a significant increase ($P \leq 0.5$) in the antioxidant enzyme activities in anther of all wheat genotypes. The effect of heat

stress on antioxidant enzyme activities in wheat anthers followed the similar trend as in flag leaves but was less pronounced. Elevated temperature during anthesis had huge impact on SOD and POX activities in developing anthers of wheat. Followed by heat stress, SOD activity was increased by 73.8 and 66.5% in WH 730 and NW 1012, whereas that of Kundan increased by 36.9% (Fig. 5*e*). Similarly, WH 730 also exhibited the maximum increase in POX activity (82.6% increase) under VLS condition contrasting with Kundan (53.3% increase) (Fig. 5*f*). Heat stress enhanced CAT and APX activities by 79.2 and 86.1%, respectively, in WH 730, whereas it did not cause any significant increase ($P \leq 0.05$) in Kundan (53.1 and 29.9% respective increase) (Fig. 5*g*, *h*).

Discussion

Heat stress severely affects the anthesis stage of wheat leading to dramatic yield losses. Global warming has been predicted to cause frequent heat stress impeding the anthesis and grain filling stages of wheat (Gourdji *et al*. 2013), therefore, establishing a promising heat tolerant wheat genotype represents an urgent need for sustainable food security. Thermo-tolerate in wheat involve morpho-physiological and molecular alterations in plants that obviously vary with genotypes (Dwivedi *et al*. 2017). Unravelling the significant differences among contrasting genotypes may help in understanding the mechanisms of heat tolerance.

Heat stress induces the production of ROS, which leads to oxidative damage of cellular membrane (Fig. 3*d*), thereby inhibiting the plant growth (Suzuki *et al*. 2012). In the present study, Kundan had the maximum decrease in plant growth under heat stress (Fig. 3*a*). In contrast, WH 730 maintained the relative growth rate showing potential heat tolerance. Heat stress also affects the membrane stability and impairs the rate of photosynthesis. Alternatively, the decrease in P_N may be attributed to the decreased stomatal conductance (Fig. 3*f*; Misson *et al*. 2010). Moreover, ROS-induced damage of chlorophyll may also be responsible for the reduction of the P_N (Fig. 3*h*; Tovignan *et al*. 2016). Decrease in E and P_N under heat stress successively causes the reduction in WUE, which is considered as a major yield determinant (Hamid *et al*. 1990). The study showed WH 730 to sustain the WUE and stomatal conductance under the heat stress and thus considered as the potential thermo tolerant wheat genotype (Fig. 3*e*, *f*). Plant water status is also indicated by the RWC, which was decreased in all the VLS genotypes, but showing maximum decrease in Kundan than the tolerant genotype WH 730 (Fig. 3*c*).

High temperatures disturb the process of starch synthesis due to reduction in the P_N, as observed in the VLS plants. Starch accumulates in the pollen grains, which provides energy for pollination (Zhang *et al*. 2010). Therefore, decrease in starch content impedes pollen maturity resulting in loss of pollen viability under heat stress, but exhibited maximum pollen mortality in Kundan contrasting with the potential tolerant genotype WH 730 (Fig. 4*c*, *d*; Datta *et al*. 2002). Prolonged heat stress leads to defective source–sink mobilisation of starch and depletes food reserves in maturing pollen grains, causing pollen mortality under heat stress (Dwivedi *et al*. 2017; Hütsch *et al*. 2019). Moreover, impaired mobilisation of stem reserve during the grain filling period also caused truncated grain in the VLS plants leading to the decrease in TGW (Table 1; Wardlaw et al. 2002). The TS plants showed weak starch staining, confirming the normal starch flow through the internode (Fig. 4*e*). In contrast, congestion in starch flow due to heat stress resulted in starch accumulation at the internode, endorsed by the strong starch staining of the VLS plants, but being less in the potential tolerant genotype WH 730 (Wang *et al*. 2012). Additionally, excessive ROS burst in developing anthers may also be responsible for the loss of pollen viability, consequently decreasing the grain yield (Fig. 5*a*–*c*; Table 1; Zhao *et al*. 2018*b*).

Heat stress tolerance in plants is associated with maintenance of cellular redox homeostasis (Fig. 6; Foyer and Noctor 2005). Antioxidant defence system, mainly comprising antioxidant enzymes, such as SOD, CAT, POX and APX plays a crucial role in this background (Chen *et al*. 2017). Enhanced antioxidant enzyme activities in developing anthers under heat stress helps in eliminating oxidative damage through successful scavenging of ROS (Zhao *et al*. 2018*a*; Fábián *et al*. 2019). The present study also illustrated that increased antioxidant capacities effectively detoxified ROS and attenuated heat stress-induced oxidative membrane damage in both flag leaves and developing anther, upholding pollen viability and grain yield under heat stress in potential tolerant wheat genotype WH 730 (Figs 5*e*–*h*, S2; Table 1; Zhao *et al*. 2018*b*). Accumulation of osmo-protectants is anticipated to be another approach adopted for heat stress tolerance in plants (Hameed *et al*. 2012). Consistent with the previous reports, in this study, remarkable increase in proline content was observed in WH 730 that showed the highest heat tolerance among the considered genotypes (Fig. 4*a*; Hameed *et al*. 2012). Thus, proline maintained leaf RWC by acting as an osmoprotectant, as well as, a ROS scavenger (Hasanuzzaman *et al*. 2014). Moreover, increased accumulation of soluble sugar was also noted in the heat tolerant genotype, which might be responsible for increased carbon availability and osmo-protection during stress adaption (Fig. 4*b*; Kaushal *et al*. 2013).

Exploration of potential heat tolerant wheat genotype included the morpho-physiological, biochemical and anatomical evaluation of five wheat genotypes at anthesis stage. Association of thermal tolerance with redox homeostasis in developing anthers was critically analysed (Fig. 6). Following the study, WH 730 showed potential heat tolerance by sustaining pollen viability and grain yield through effective ROS-scavenging facilitated by enhanced antioxidant enzyme activities in flag leaves as well as developing anthers (Figs 5*e*–*h*, S2). Furthermore, it maintained the stomatal conductance by protecting foliar cells from ROS-induced oxidative damage (Fig. 3*f*; Hamid *et al*. 1990; Sairam *et al*. 2000). Consequently, it sustained the transpiration rate and successfully escaped heat stress through evaporative cooling of canopy (Fig. 3*g*). P_N and WUE were also maintained by this particular genotype (Fig. 3e). Exceptional osmotic adjustment (Fig. 4*a*, *b*) in WH 730 helped in sustainable RWC and protected the flag leaves from premature senescence (Fig. 3*c*). Higher starch content in flag leaves and effective source to sink starch mobilisation in WH 730 under heat stress maintained the pollen viability and grain yield (Fig. 4*c*–*e*; Table 1; Hütsch *et al*. 2019). Moreover, WH 730 also exhibited the minimum heat susceptibility index ($s \leq 0.50$) among the five wheat

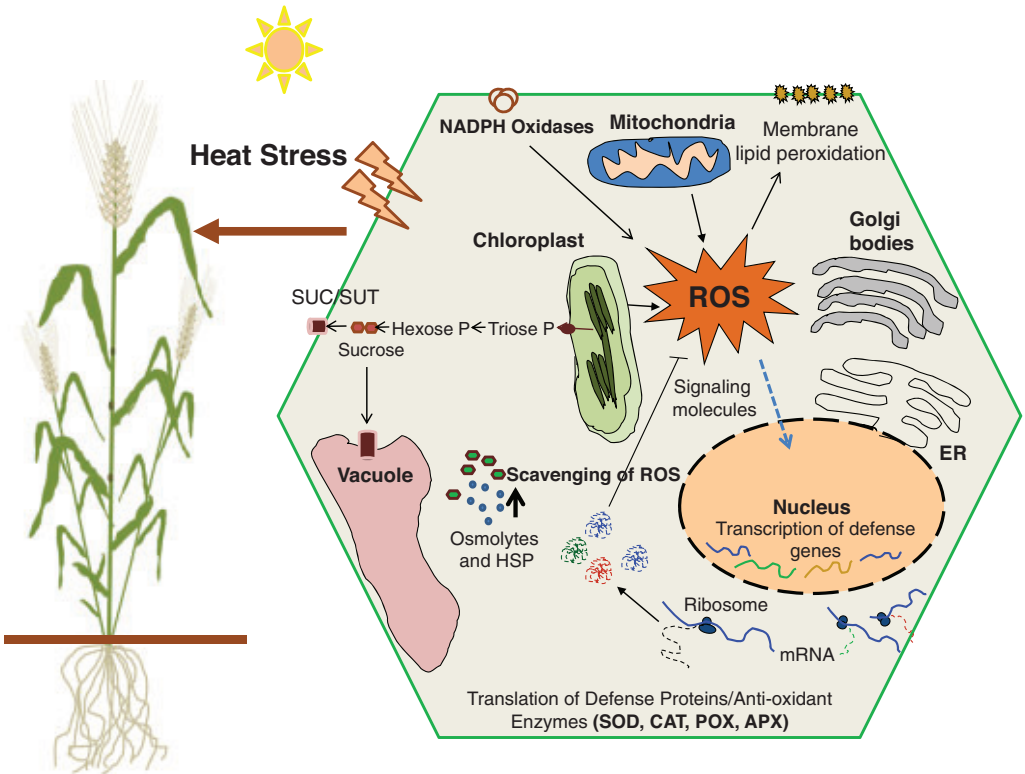

Fig. 6. Schematic diagram depicting the mechanism of redox homeostasis responsible for heat stress tolerance in wheat. Heat stress causes generation of ROS in plant cells through NADPH-oxidase pathway and leakage of electrons from the electron transport chain in chloroplast and mitochondria to molecular oxygen. The ROS signalling pathway triggers gene expression for several antioxidant enzymes (SOD, CAT, POX, APX) that contribute in scavenging of ROS. Heat stress also triggers accumulation of compatible osmolytes (proline) in plants that act as ROS scavenger and protect cells from stress-induced damage. Heat stress disturbs the redox equilibrium, leading to cellular and sub-cellular membrane damage through membrane lipid peroxidation. Heat stress-induced disturbance in ROS-homeostasis hinders the source-sink mobilisation of photosynthate leading to pollen mortality and ultimately resulting in poor grain yield.

genotypes (Fig. 3*b*; Dhyani *et al*. 2013). Considering the entire study, WH 730 might be considered as the potential heat tolerant wheat genotype for late sowing in the particular region.

Conclusions

The present study demonstrated the negative effect of heat stress on five Indian bread wheat genotypes at anthesis stage. Heat stress-induced intensive ROS accumulation led to disintegration of cell membrane. Accumulation of ROS in developing anthers and poor assimilates mobilisation lead to higher pollen mortality, severely decreasing grain yield. Excessive ROS burst in the leaves affected the gaseous exchange in plants, thereby decreasing the photosynthetic rate. Thermo-tolerance in wheat exhibited strong association with antioxidant enzyme-mediated detoxification of ROS. Increased level of proline and total soluble sugar also played a significant role in osmotic adjustment and ROS scavenging under heat stress. In the present study, wheat genotype WH 730 showed extreme heat tolerance by maintaining pollen viability thus grain yield through enhanced redox homeostasis in developing anthers and flag leaves. Therefore, WH 730 may act as a potential material for late sown heat stress condition in the Eastern Indo-Gangetic Plains to harvest better yield. Overall, our work provides a comprehensive understanding of heat stress tolerance mechanism in wheat through enhanced redox homeostasis in developing anthers as well as the flag leaves.

Conflicts of interest

The authors declare no conflicts of interest.

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