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Ankita Rawat
Department of Agronomy, College of Agriculture, G.B. Pant University of Agriculture and Technology, Pantnagar, Uttarakhand, India

H Punetha
Department of Biochemistry, College of Basic Sciences and Humanities

VC Dhyani
Department of Biochemistry, College of Basic Sciences and Humanities

Sumit Chaturvedi
Department of Biochemistry, College of Basic Sciences and Humanities

Correspondence
Ankita Rawat
Department of Agronomy, College of Agriculture, G.B. Pant University of Agriculture and Technology, Pantnagar, Uttarakhand, India

Biochemical investigation of *Triticum aestivum* L. genotypes under different growth stages exposed to terminal heat stress

Ankita Rawat, H Punetha, VC Dhyani and Sumit Chaturvedi

Abstract
In the present study, the effect of terminal heat stress on 10 wheat genotypes (viz., VL-832, HD-3065, WH-1124, WH-1100, VL-892, Halna, PBW-590, PBW-658, PCPGR- 7854, and DBW-90) was evaluated under timely and late sown conditions. Various Physio-biochemical parameters were ascertained in all the cultivars throughout the growth duration stages. Increasing temperature showed marked effect in various physiological and biochemical parameters tested in all genotypes under study. The genotypes differed significantly with respect to time of sowing (timely and late) having significant effect on plant height, LAI and Fv/Fm ratio. Decrease in H_2O_2 content was observed in PBW-590, PBW-658, PCPGR-7854, Halna and WH-1124 under terminal heat stress (showing their better tolerance). Lower level of MDA was observed in WH-1124, VL-832 and PCPGR-7854. Elevated CAT activity was observed in WH-1124, PBW-590, Halna and PCPGR-7854 under terminal heat stress. APX activity increased significantly in DBW-90, HD-3065 and PBW-590 whereas POD activity increased significantly in PBW-658, WH-1124 and WH-1100 under terminal heat stress. The present study revealed that terminal heat stress causes significant oxidative damage by production of reactive oxygen species, causing membrane damage via, lipid peroxidation and triggers the production of antioxidant enzymes in tolerant genotypes

Keywords: *Triticum aestivum*, terminal heat, H_2O_2, MDA, antioxidative enzymes

Introduction
Wheat (*Triticum aestivum* L.) is one of the most important staple food crops of the world. It occupies 17 % of the world's crop acreage, feeding about 40 % of the world population by providing 20 % of total food calories and protein in human nutrition [1]. In India, wheat sowing time varies from October to December with temperature range of 10 to 35 °C. Apart from genotypic variation, temperature fluctuation plays a crucial role in germination of wheat seeds. Stress affects essentially every aspect of plant growth and development. High temperature stress is one of the important yield limiting factors in wheat. Constant efforts are urgently required to enhance its production to keep the pace with ever increasing population.

Heat stress is defined as rise in temperature beyond a threshold level for a period of time sufficient to cause a series of morpho-anatomical, physiological and biochemical changes, which affect plant growth and development [2]. High temperature stress during reproductive development resulting in a reduction in both individual kernel weight and kernel number [3, 4]. Heat stress acts as a limiting growth factor in cool season plant species specially wheat [5] and significantly responsible for reduction of yield irrespective wheat lines and causes multifarious, and often adverse, alterations in development and other processes [6]**.** These include severe water and oxidative stress [7], heat-induced oxidative stress and expression of antioxidant enzymes [8]. However, cell and subcellular systems protect themselves from the cytotoxic effects of these active oxygen radicals using antioxidant enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione reductase (GR), catalase (CAT) and metabolites like glutathione, ascorbic acid, α-tocopherol and carotenoids [9]. Tolerances to high temperature stress in crop plants have been reported to be associated with an increase in antioxidant enzymes activity [10-12].

Increasing heat tolerance in wheat is consequently a challenge for wheat breeders as heat tolerance is a complex phenomenon and difficult to measure. Many selection criteria based on morpho-physiological traits were reported to be associated with performance under heat stress in wheat. A common method of selecting plants for heat stress tolerance has been to grow diverse breeding materials in a hot target production environment and identify individuals/lines with greater yield potential [13]. Hence, it is necessary for promising advanced lines of wheat to be tested under both normal and heat stress conditions.

Therefore, it is imperative to understand the underlying mechanism of plant exposed to heat stress. The present study focused to compare antioxidative potential of different *Triticum aestivum* L. germplasm under different growth stages exposed to terminal heat stress.

Materials and Methods

Plant material

Ten wheat (*Triticum aestivum* L.) genotypes (VL-832, VL-892, PCPGR-7854) procured from Pantnagar Centre of Plant Genetic resource, PCPGR, Pantnagar; other genotypes (WH-1124, PBW-590, DBW-90, WH-1100, HD-3065, PBW-658, Halna) procured from Department of Genetics and Plant Breeding, Pantnagar were grown in Crop research centre, Pantnagar for the present study. All these 10 genotypes were selected on the basis of their superiority in terms of grain yield under late sown condition.

Plant height

Plant height was determined 30, 60, and 90 days after planting, in both timely and late sown conditions in all the 10 selected wheat genotypes. Plant height was measured using a scale and the height was recorded from the base to the top of the plant for each genotypes. A total of three observations were taken to determine plant height at respective days for each genotype under timely and late sown conditions.

Leaf Area Index (LAI) and Chlorophyll Fluorescence

Chlorophyll a fluorescence in green plants reflects the efficiency of photosynthetic PS II system. A hand held plant efficiency analyzer (Handy PEA, Hansatech, UK) was used to evaluate the Fv/Fmax ratios in different wheat genotypes. Measurements were recorded in the forenoon hours (9-10 am) to avoid photo inhibition, due to excessive sunlight. The maximum fluorescence level (Fm) of closed PS II centre was determined by providing 1.5 sec. saturating pulse at 300 μ mol m^{-2} s^{-1} on dark adopted leaves (20 minutes). Leaf area index (LAI) was measured in each genotype by a leaf area meter from five flag leaves of five plants, and then averaged.

Malondialdehyde content

The procedure of Heath and Packer (1968) was followed for measuring the MDA content [14]. 0.2 g of leaf material was homogenized in 3 ml 0.1 % trichloroacetic acid (TCA), the homogenate was centrifuged at 10,000 x g for 10 min. 0.3 ml of crude extract was mixed with 1.2 ml of 0.50 % (w/v) 2-thiobarbituric acid (TBA) prepared in 20 % (w/v) trichloroacetic acid (TCA). The mixture was incubated at 95 °C for 30 min and was then centrifuged at 10000 x g for 10 min. Absorbance at 600 nm was subtracted from the absorbance at 532 nm for non-specific absorbance. The concentration of MDA was calculated by using an extinction coefficient of 155 mM^{-1} cm^{-1}.

Hydrogen Peroxide content

Hydrogen peroxide was measured spectrophotometrically after reaction with potassium iodide (KI) (15). 0.2 g of leaf was crushed in 1.0 ml of 0.1 % (w/v) trichloroacetic acid (TCA) and centrifuged at 10000 rpm for 30 min at 4 °C. The reaction mixture consisted of 0.5 ml of supernatant, 0.5 ml of 0.1M potassium phosphate buffer and 2 ml of 1 M KI reagent. The reaction was allowed to develop for 1 hour in dark and absorbance was measured at 390 nm.

Anti-oxidant enzymes

For protein and enzyme extractions, 0.3 g of leaf samples were homogenized with 50 mM sodium phosphate buffer (PH 7.0) containing 1 mM ethylenediamine tetra acetic acid (EDTA) and 2 % (w/v) polyvinylpyrolidone (PVP). The whole extraction procedure was carried out at 4 °C. The homogenate were centrifuged at 10,000 x g for 15 min at 4 °C and the supernatants were collected and used for the assays of enzyme activity. Protein content was measured by standard protocol using bovine serum albumin as a standard [16].

Catalase (CAT) Activity

Catalase (EC 1.11.1.6) activity was measured based on standard method with minor modifications [17]. The assay mixture consisted of 50 μL of the enzyme extract, 100 mM phosphate buffer (pH 7.0), 0.1 μM EDTA, and 20 mM H_2O_2 in a total volume of 1.5 ml. The decrease of H_2O_2 was monitored by reading the absorbance at 240 nm at the moment of H_2O_2 addition and 1 min later. The difference in absorbance ($\Delta A240$) was divided by the H_2O_2 molar extinction coefficient (36 M−1cm−1) and the enzyme activity expressed as μmol of H_2O_2 $min^{-1}mg^{-1}$ protein.

Ascorbate peroxidase (APX) Activity

Ascorbate peroxidase (APX, EC 1.11.1.11) activity was determined according to standard method (18). The reaction mixture contained 50 mM potassium phosphate (pH 7.0), 0.2 mM EDTA, 0.5 mM ascorbic acid, 2 % H_2O_2 and 0.1 ml enzyme extract in a final volume of 3 ml. The decrease in absorbance at 290 nm for 1 min was recorded and the amount of ascorbate oxidized was calculated using extinction coefficient (2.8 mM-1 cm-1). The enzyme activity expressed as μmol of H_2O_2 min^{-1} mg^{-1} protein.

Peroxidase (POD) Activity

Peroxidase (POD, EC 1.11.1.17) activity was determined based on standard method with minor modifications [19]. The reaction mixture contained 0.05 M sodium phosphate buffer (pH 5.5), 2 % H_2O_2, 0.05 M guaiacol and 0.1 ml enzyme extract in a final volume of 5 ml. The reaction was started by the addition of enzyme extract. The formation of tetraguaiacol was measured at 470 nm. One unit of enzyme was defined as the amount of enzyme to decompose1 μ mol of H_2O_2 per min at 25 °C.

Statistical analysis

Statistical analysis was performed using software SPSS 20 (IBM Corp). Mean value of all the parameters and their respective standard errors (n=3) were tabulated using M S excel. The graphs were plotted with the help of Origin Pro 8 (Origin Lab Corp).

Results and Discussion

Plant height: Plant height differed significantly ($p \leq 0.5$) from 30 to 90 DAS under both timely and late sown condition in all the genotypes under study. A decrease in plant height was observed during the late sown condition in all the genotypes under study at respective days after sowing i.e., 30, 60, 90 DAS. Greater decrease in plant height was observed at 30 DAS followed by 90 days and least at 60 days stage under the late sown condition as compared to timely sown condition in all the genotypes (Fig.1). Least reduction in plant height was found in Halna variety under late sown condition. Terminal heat stress showed a marked effect in plant height in all the tested genotypes resulting in short stature of plants. The significant reduction of plant height in PBW-590 and Halna under late sown condition may be due to the effect of terminal

heat stress on growth and development. Similar results have been reported by earlier workers [20, 21]

Leaf Area Index (LAI): LAI increased from 30 to 90 DAS under both timely and late sown condition in all the genotypes under study (Fig.1). A decrease in LAI was observed during the late sown condition with respect to timely sown condition in all the genotypes at respective days after sowing i.e., 30, 60, 90 DAS. Greater decrease in LAI was observed at 30 and 90 DAS under the late sown condition as compared to timely sown condition, with significant ($p \leq 0.5$) decrease in LAI observed at 90 DAS. Leaf area index decreased from timely to late sown condition at all the stages i.e. 30, 60 and 90 DAS however increase in LAI too was observed in some genotypes at earlier stages. Higher decrease of LAI in PBW-658, VL-892 and HD-3065 may be due to adverse effect of terminal heat stress. Similar results were reported earlier for wheat genotypes grown under late sown conditions [22]. The decrease in LAI at later growth stages under late sown condition might be ascribed by aging of leaves, leaf senescence and thermal stress at later growth stages [23].

Chlorophyll Fluorescence: Chlorophyll florescence (Fv/Fm) decreased from anthesis to 14 DAA under both timely and late sown condition in all genotypes under study (Fig.1). Significant ($p \leq 0.5$) decrease in Fv/Fm ratio was observed in Halna and VL-832 from anthesis to 14 DAA under late sown condition. In general greater decrease in chlorophyll florescence (Fv/Fm) was observed at anthesis under late sown condition with respect to timely sown condition in all the genotypes, there after gradual decrease in Fv/Fm was observed from 7 to 14 DAA. There was not a definite pattern observed in different genotypes with respect of Fv/Fm at different stages. Decrease of chlorophyll florescence (Fv/Fm) in VL-832, Halna, HD-3065, WH-1100, PBW-658, PCPGR-7854, and DBW-90 may be due to sensitivity of these genotypes towards terminal heat stress, whereas increase of Fv/Fm in WH-1124, VL-892, and PBW-590 during the same time period may be ascribed to tolerance towards terminal heat stress, thereby minimizing damage to the photosynthetic apparatus. Similar results were earlier reported [24]. High temperature stress reduced the Fv/Fm ratio, indicating that a structural and functional disorder of the photosynthetic apparatus and damage to the PS II had occurred [25].

Hydrogen peroxide (H_2O_2) content: The H_2O_2 content varied significantly from anthesis to post-anthesis under timely and late sown condition in the genotypes under study (Fig.2). Significant ($p \leq 0.5$) decrease in H_2O_2 content was observed in PBW-590 and PBW-658 during anthesis under late sown condition with respect to timely sown condition. In Halna and WH-1124 a significant decrease and in PBW-590 and PCPGR-7854 a corresponding increase in H_2O_2 content was observed from anthesis to post-anthesis under late sown condition. Decrease in H_2O_2 content was also observed in genotypes viz. Halna, WH-1100, HD 3065 and VL-892 under late sown condition compared to timely sown condition at anthesis stage. However no such trend was observed at post anthesis stage. A decrease in the concentration of the hydrogen peroxide observed in PBW-590 and PBW-658 during anthesis and Halna and WH-1124 during post-anthesis under late sown condition may be due to the high activity of peroxide scavenging antioxidant isoenzymes [20].Thus these genotypes may have adaptive advantage towards heat stress while increase in concentration of hydrogen peroxide in PBW-590 and PCPGR-7854 during post-anthesis under late sown condition may be ascribed to lower activity of antioxidant enzymes in these genotypes.

Malondialdehyde (MDA) content: MDA content varied significantly ($p \leq 0.5$) from anthesis to post-anthesis under timely and late sown condition(Fig.2).WH-1124 showed a significant decrease in MDA content during anthesis under late sown condition with respect to timely sown condition. VL-832 and PCPGR-7854 showed a significant decrease in MDA content during post-anthesis under late sown condition, whereas a corresponding increase was observed in HD-3065 during the corresponding period. The MDA content decreased significantly with rising temperature in WH-1124 and Halna during anthesis and in VL-832 and PCPGR-7854 during post-anthesis under late sown condition which may be due to lower levels of reactive oxygen species as a result of higher activity of antioxidant enzymes in these genotypes. While increased MDA content in PBW-658 and VL-892, may be ascribed to lower activity of antioxidant enzymes in these genotypes under terminal heat stress. Malondialdehyde (MDA) a product of lipid peroxidation due to oxidative damage and is often used as an indicator of increased damage [26, 27].

Antioxidative enzyme activities

Catalase (CAT) activity: A significant ($p \leq 0.5$) increase in CAT activity was observed in WH-1124 and PBW-590 during anthesis under late sown condition, whereas a corresponding decrease was observed in VL-892(Fig. 3). At post-anthesis an increase in CAT activity was observed in Halna and PCPGR-7854, whereas VL-832 and DBW-90 showed a corresponding decrease during the same time period. CAT activity increased in WH-1124 and PBW-590 during anthesis and in Halna and PCPGR-7854 during post-anthesis under late sown condition, thus having higher H_2O_2 scavenging potential. An increase in the amount of H_2O_2 is associated with oxidative damage [28]. Strategies for abiotic stress tolerance in plants often employ enzymatic antioxidants especially CAT [29], as higher activity of CAT decrease H_2O_2level in cells. The decrease in catalase activity in VL-832 during anthesis and in VL-832 and DBW-90 during post-anthesis may be ascribed to their low H_2O_2 scavenging potential and thereby sensitivity of these genotypes towards terminal heat stress.

Ascorbate peroxidase (APX) activity: A significant ($p \leq 0.5$) increase in APX activity was observed in DBW-90 during anthesis under late sown condition, whereas a corresponding decrease was observed in VL-832 (Fig.3). During post-anthesis HD-3065 and PBW-590 showed significant increase in APX activity under late sown condition whereas PCPGR-7854 and VL-832 showed a corresponding decrease during the same time period. Higher APX activity in DBW-90 during anthesis and HD-3065, PBW-590 during post anthesis may be ascribed to the enhanced H_2O_2scavenging system in chloroplast [30]. Whereas decrease in APX activity in VL-832 during anthesis and in VL-832 and PCPGR-7854 during post-anthesis under late sown condition may be ascribed to weaker H_2O_2 scavenging system in chloroplast. In the present study the genotypes differed significantly with respect to each other at anthesis and post anthesis for APX activity under timely and late sown condition, moreover there was significant genotype×environment (terminal heat stress) interaction both at anthesis and post-anthesis. Similar finding was reported regarding up-regulation of APX by terminal heat stress during late sown condition [31]

Peroxidase (POD) activity: A significant ($p \leq 0.5$) increase in POD activity was observed in PBW-658, WH-1124 and WH-1100 during anthesis under late sown condition, whereas PCPGR-7854 and VL-832 showed a corresponding decrease during same time period (Fig.3). PBW-658, WH-1124 showed an increase in POD activity during post-anthesis under late sown condition, whereas VL-832 showed a corresponding decrease during the same time period. POD activity increased in PBW-658, WH-1124 and WH-1100 during anthesis and in PBW-658 and WH-1124 during post-anthesis under late sown condition, thus these genotypes may have adaptive advantage under terminal heat stress, having high potential to sustain elevated H_2O_2 concentration, thereby minimizing deleterious effect at cellular levels under terminal heat stress. As increased POD activity during terminal heat stress (late sown) may play a role in H_2O_2 detoxification [32]. While decline in POD activity in PCPGR-7854 and VL-832 during anthesis and in VL-832 during post-anthesis may be ascribed to sensitivity of these genotypes towards terminal heat stress.

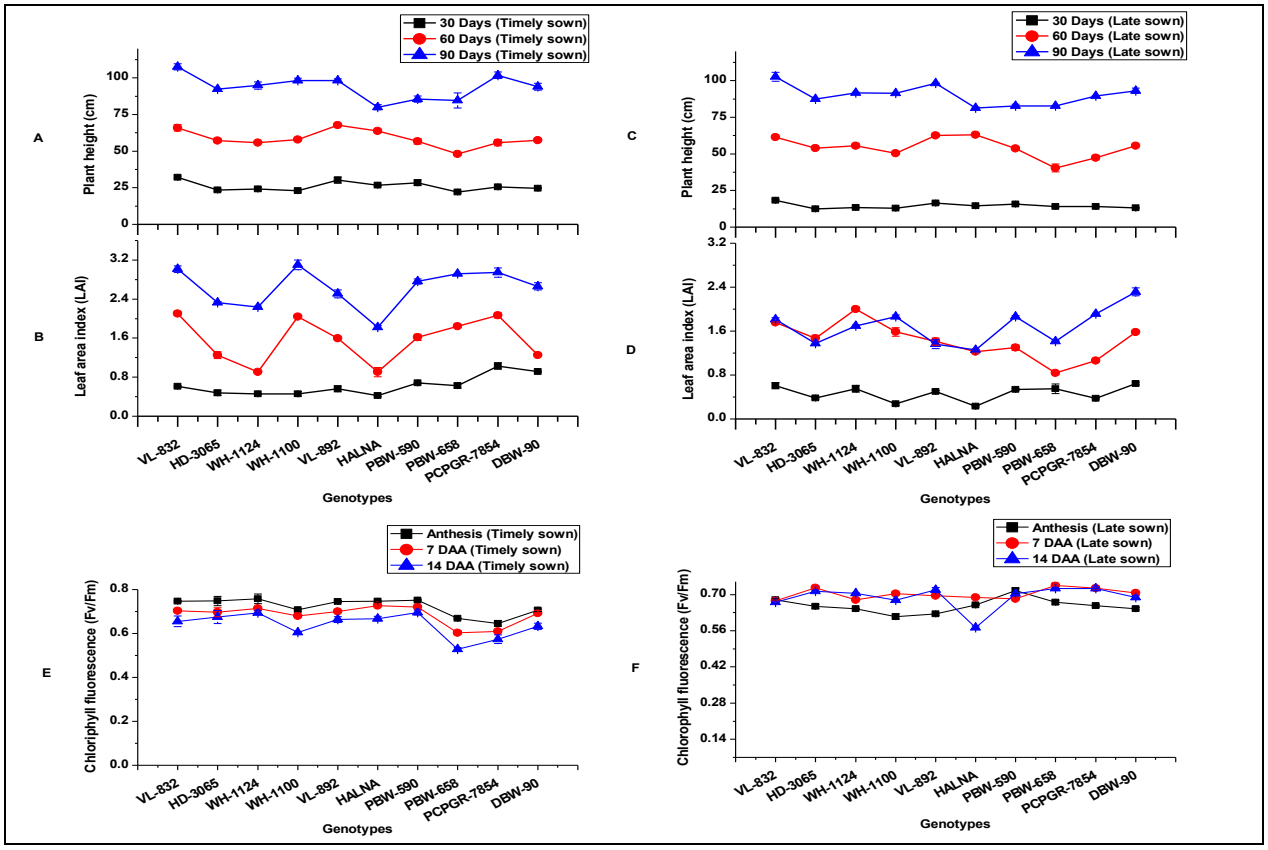

Fig 1: Plant height (A;C), LAI (B;D) at 30, 60, 90 DAS under timely and late sown conditions, Fv/Fm (E;F) at anthesis, 7 and 14 DAA under timely and late sown condition.

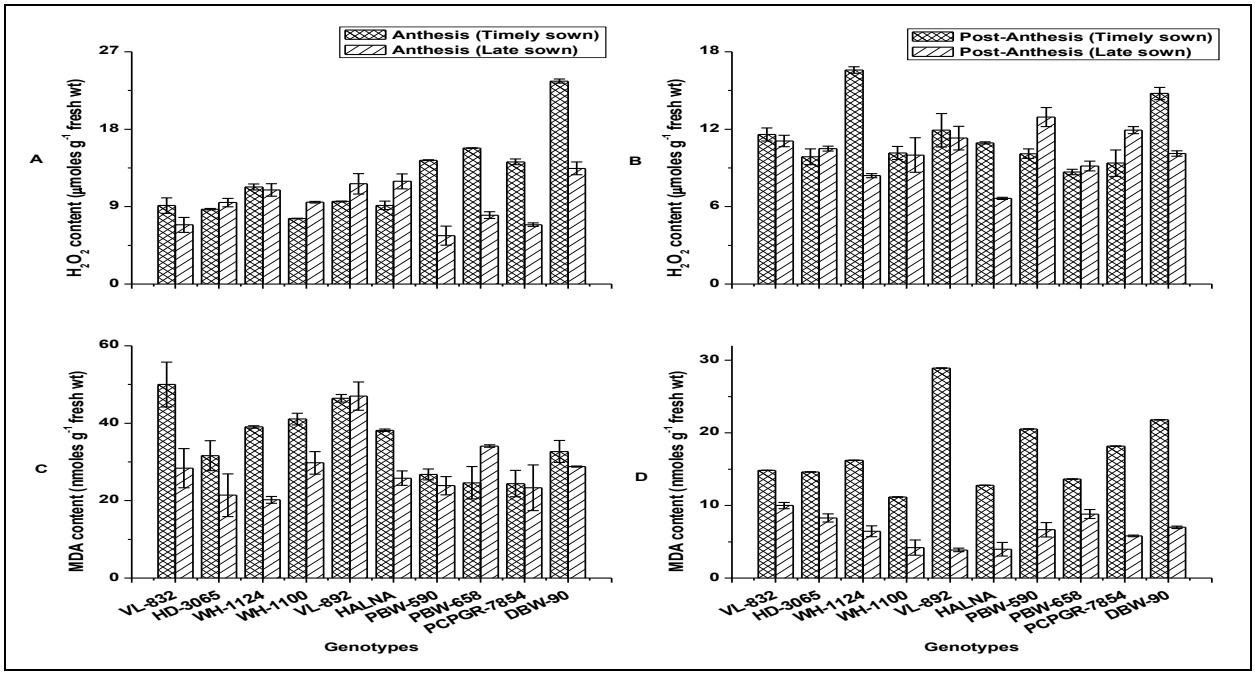

Fig 2: H_2O_2 and MDA content during anthesis and post-anthesis respectively, in different genotypes under timely and late sown condition. Data shown are mean values of three replicates.

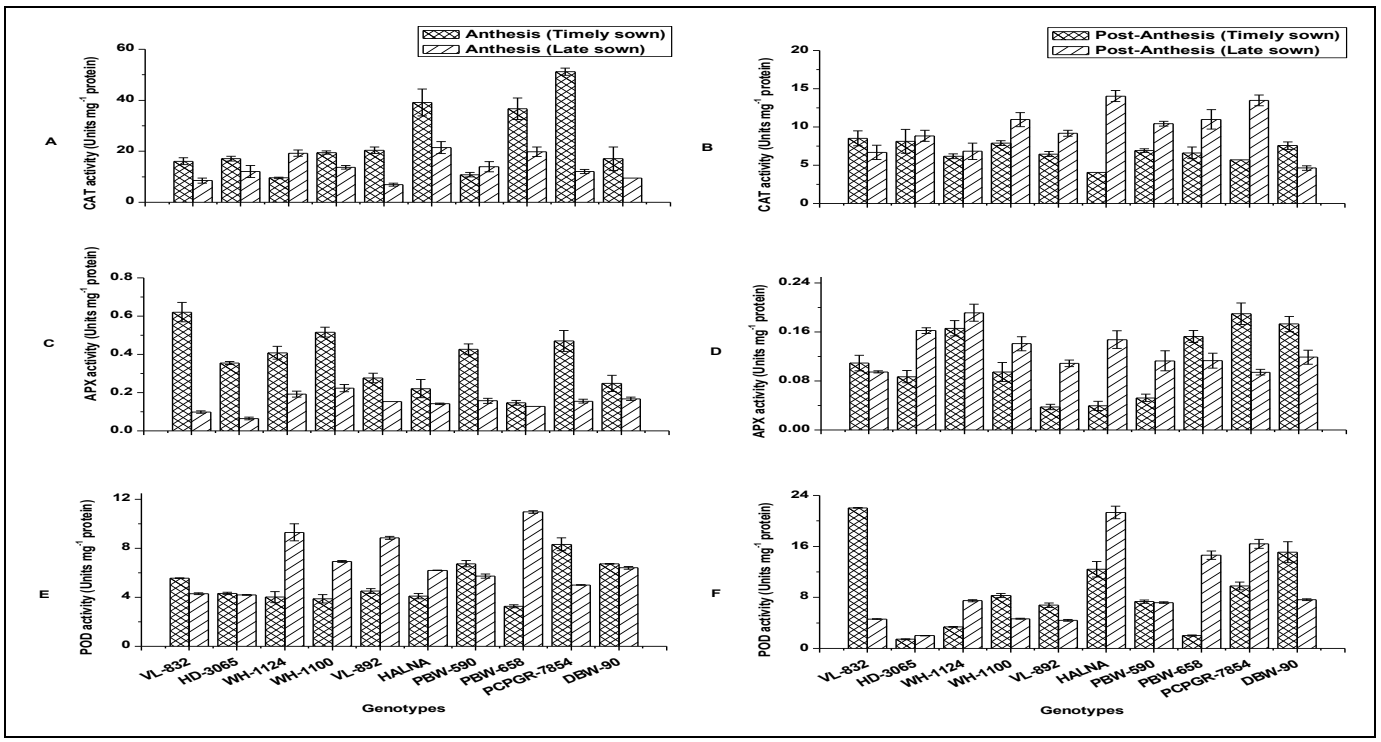

Fig 3: APX (A;D), POD (B;E) and CAT (C;F) activity (Units mg^{-1} protein) during anthesis and post-anthesis respectively, in different genotypes under timely and late sown condition. Data shown are mean values of three replicates.

Conclusion

In the present study, the genotype showed marked effect of terminal heat stress on different physio-biochemical parameters. Thus exploring the inter- and intra-species variability in wheat under high temperature stress would be valuable for plant breeders and molecular biologist. Moreover, understanding the response of wheat to high temperature at biochemical and molecular levels will accumulate knowledge, making the genetic manipulation easier which could lead to more important research on the mechanisms of high temperature stress tolerance in plants in the future. Present study revealed differential adoptive machinery as better photosynthetic machinery reflected by increased chlorophyll fluorescence (Fv/Fm) and improved oxidative controls due to increased activity of CAT, APX, and POD etc to be major adaptation mechanism for terminal heat stress

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