Physiol Mol Biol Plants (January 2021) 27(1):151–163
https://doi.org/10.1007/s12298-021-00937-z

RESEARCH ARTICLE

Hydrogen peroxide regulates antioxidant responses and redox related proteins in drought stressed wheat seedlings

Rachana D. Bhardwaj[1] · Navdeep Singh[1] · Anupam Sharma[1] · Robin Joshi[3] · Puja Srivastava[2]

Received: 22 May 2020 / Revised: 13 January 2021 / Accepted: 19 January 2021 / Published online: 1 February 2021

Abstract Hydrogen peroxide plays pivotal role as a potent regulator in signalling pathways when the plant is under stress. The current study appraised the potential of hydrogen peroxide through seed pre-treatment on the seedling growth and defense responses of three wheat cultivars i.e. PBW 644 (tolerant), PBW 621 and HD 2967 (sensitive) grown under drought stress. Imposition of drought stress reduced seedling growth of all the three wheat cultivars. Pre-treatment of seeds with 60 mM H_2O_2 alleviated water stress induced growth inhibition in all the three wheat cultivars. Further, it enhanced the drought tolerance of PBW 644 by upregulating SOD, POX, APX and GR enzymes accompanied by an increase in total phenols and ascorbate content. H_2O_2 treatment also protected the sensitive cultivars from drought stress by increasing CAT, POX, APX, MDHAR and GR enzymes. The contents of osmolytes were comparable or slightly higher as compared to stressed seedlings. The levels of MDA content were reduced in the treated seedlings of all the cultivars which further revealed the role of H_2O_2 pre-treatment in alleviating membrane damage. The comprehensive scrutiny of proteins differentially expressed in control, stressed and H_2O_2 primed stressed seedlings revealed that drought stress enhanced the expression of proteins involved in photosynthesis, protein biosynthesis and degradation, carbohydrate metabolism, fatty acid metabolism, nucleic acid metabolism, phytohormone response, defense and regulation, whereas H_2O_2 pre-treatment led to over expression of proteins which had functions in processes such as defense, redox homeostasis and photosynthesis.

Keywords Drought stress · Reactive oxygen species · Antioxidant enzymes · Wheat · Seedlings · Protein expression

Abbreviations

ROS	Reactive oxygen species
H_2O_2	Hydrogen peroxide
SOD	Superoxide dismutase
POX	Peroxidase
APX	Ascorbate peroxidase
GR	Glutathione reductase
MDHAR	Monodehydroascorbate reductase
MDA	Malondialdehyde

Introduction

Wheat (*Triticum aestivum* L.) is the second most important cereal crop of India and is largely adjusted to diverse soil and climatic environments. It is cultivated worldwide due to its excellent yield and nutritive value, thus ensuring food security around the globe. Approximately, 21% of world's food is contributed by wheat crop which covers 200 million hectares of farmland globally. Although, India is expected to harvest record wheat production during 2019–2020 but still its demand including domestic use and livestock industry exceeds the production (USDA FAS

Supplementary information The online version of this article (https://doi.org/10.1007/s12298-021-00937-z).

✉ Rachana D. Bhardwaj
rachana1981_gautam@pau.edu

[1] Department of Biochemistry, Punjab Agricultural University, Ludhiana 141004, India

[2] Department of Plant Breeding and Genetics, Punjab Agricultural, University, Ludhiana 141004, India

[3] Biotechnology Division, CSIR, IHBT, Palampur 176062, India

2020). Further, the oxidative damage triggered by the various reactive oxygen species (ROS) generated in response to various stresses continuously decreasing the productivity of wheat. Among various stresses, drought condition is the result of low precipitation and is one of the key factors restraining wheat yield by restricting most stages of crop growth in arid and semiarid areas (Shahzad et al. 2016; Zhang et al. 2018). Since seedling growth is the most affected stage by drought stress hence, there is a burning need to augment the seedling growth of high yielding wheat varieties to ensure global food security which continues to be a key task for agricultural researchers and plant breeders.

Drought stress is a serious threat to agricultural production of major crops since water is vital factor in plant development (Shahzad et al. 2016). The first and foremost effect of drought is impaired germination which further affects the levels of proteins, antioxidants, osmolytes, chlorophyll and hormones. Drought further influences root characteristics, opening and closing of stomata, cuticle thickness and processes of photosynthesis and transpiration (Suzuki et al. 2014). It adversely affects many metabolic reactions within the cells and result in production of ROS like superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), and singlet oxygen (O_2). A rise in the generation of ROS during stressed conditions also prompts peroxidation of membrane lipids (Kohli et al. 2019). To cope with and to survive under drought stress, plants have acquired intricate antioxidant defense machinery which is comprised of various enzymate, non-enzyme and non-specific antioxidants (Mittler 2017). Key ROS scavenging enzymes reported are superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), ascorbate peroxidase (APX) and glutathione reductase (GR) whereas, glutathione, ascorbic acid (vitamin C) and tocopherols are the vital non-enzymae antioxidants used by plants under stressful conditions to alleviate the adverse effects of ROS. In addition, some osmoprotectants like proline, glycine betaine and trehalose are also involved in imparting tolerance against drought stress mainly by balancing of turgor and protecting the specific cellular functions (Blum 2017).

Abiotic stress tolerance could be attained by fine regulation of the antioxidant responses in plants (Mittler 2017; Parveen et al. 2019). Sulfur-enriched leonardite and humic acid soil amendments enhanced drought tolerance in maize plants by improving the antioxidative defense system and photosynthetic efficiency (Kaya et al. 2020). Exogenously applied eco-friendly chemicals like proline and trehalose improved the drought tolerance of wheat and sunflower by fine regulating the activities of SOD, CAT, POX and key enzymes of ascorbate–glutathione pathway (Singh and Bhardwaj 2019; Koser et al. 2020). Farooq et al. (2020) used foliar spray of ascorbic acid to mitigate the damaging effects of drought stress in safflower plants. Thus such environmentally safe chemicals can be used to improve the crop productivity under drought stress which can do so either by promoting or regulating defense related responses. Hydrogen peroxide is comparatively a stable freely diffusible and relatively long lived molecule among different ROS and plays a vital role either by exacerbating damage or by signalling. In many plants, selective aquaporins were also reported to have the ability to channel H_2O_2 across the membranes (Bienert 2014). Goud and Kachole (2011) reported that higher levels of H_2O_2 become toxic to nearly all the cell components viz. pigments, proteins, carbohydrates and nucleic acids whereas at low concentrations, it regulates variety of physiological processes comprising photosynthesis, opening and closing of stomata, senescence, cell growth and development (Deng et al. 2012). In stressed maize plants, exogenous use of H_2O_2 and Silicon at low levels ameliorated oxidative membrane damage by significantly decreasing MDA content (Terzi et al. 2014; Parveen et al. 2019).

Plants respond to various abiotic stresses by reprogramming their proteome to create a new stable equilibrium state. Owing to the posttranslational modifications, some of the proteins remain undetected at mRNA level, thus transcriptomic data does not give enough information about the proteins. Thus, proteomic investigation becomes an influential technique to explicate stress tolerance mechanisms as reported by Skalak et al. (2016). Despite the importance of exogenous application of hydrogen peroxide in different crops, there is limited information about the protective effects of seed pre-treatment with H_2O_2 on drought induced oxidative stress responses and expression of proteins. Better understanding of the protective effects of H_2O_2 pre-treatment on wheat seedlings grown under drought stress will be an essential step to develop tolerant genotypes and crop management practices that can alleviate detrimental effects of drought stress.

Materials and methods

Plant material and seedling growth

The study was carried out with one drought tolerant (PBW 644) and two sensitive (PBW 621, HD 2967) cultivars which were procured from the Department of Plant Breeding and Genetics, PAU, Ludhiana. The seeds were washed with detergent and running tap water followed by rinsing with distilled water. Following this, seeds of all the cultivars were soaked in solution containing varying concentrations of hydrogen peroxide (10–200 mM) for 12 h. The seeds were then taken out and washed with tap water followed by removal of extra moisture by putting them in

different layers of filter paper. All the seeds were sown in plastic cups having volume of 250 cm^3. They were filled with 90% of their capacity having well irrigated and untreated soil which was found to have an electrical conductivity of 0.12 μS cm^{-1}. The soil contained 0.50% organic carbon. All the plants were uniformly irrigated for the initial three days. Then, regular watering was done only for control plants while water was withheld in the rest of plants. Soil moisture content was measured by weighing the soil from the cups with irrigated soil and the from the cups where irrigation was stopped. Then the soil was dried in an oven at 100 °C till the constant weight was observed. The values for soil moisture content were 9.29 and 3.09% in irrigated and drought affected soil. Three cups with 6 seeds in each were used for one treatment. Then the cups were kept in an incubator at 25 ± 1 °C in the dark. At 8th day after germination (DAG), seedling growth data was taken in terms of lengths of growing tissues and biomass of roots, shoots and endosperms. Dry weights of roots, shoots and endosperms were measured after keeping all the tissues at 60 °C until the continuous biomass was acquired.

Lipid peroxidation and H_2O_2 content

The method of Heath and Packer (1968) was used for measuring the lipid peroxidation in terms of MDA content whereas Alexieva et al. (2001) was used to estimate hydrogen peroxide from the growing tissues of seedlings.

Antioxidative enzymes

Superoxide dismutase (EC 1.15.1.1), peroxidase (EC 1.11.1.7) and glutathione reductase (EC 1.6.4.2) were isolated from 0.1 g each of roots and shoots with 1.5 ml of 100 mM phosphate buffer (pH 7.5) having 1% polyvinyl pyrrolidone (PVP), 10 mM β-mercaptoethanol and 1 mM EDTA. Homogenate was centrifuged at 10,000 × g for 15 min at 4 °C and assaying SOD, POX and GR (Singla et al. 2020). Catalase (EC 1.11.1.6) was extracted from 0.1 g each of the growing tissues with 2.0 ml of 50 mM sodium phosphate buffer (pH 7.5) having 1% PVP. Homogenate was centrifuged at 10,000 × g at 4 °C for 20 min and clear supernatant was used for enzyme assay (Chance and Maehly 1955). The ascorbate peroxidase (EC 1.11.1.1) was isolated with 50 mM phosphate buffer containing 1% PVP and 1 mM ascorbate. It was assayed according to the methods of Nakano and Asada (1981). Another important enzyme of ascorbate–glutathione pathway, monodehydroascorbate reductase (EC 1.6.5.4) was isolated from 0.2 g each of growing tissues with 2.0 ml of 100 mM phosphate buffer (pH 7.5) containing 1 mM EDTA, 12 mM ascorbate and 2% PVP. Assay mixture for MDHAR consisted of 50 mM Tris HCl buffer (pH 7.5), 2.5 mM ascorbate, 0.2 mM NADPH, 200 μl of enzyme isolated from 0.1 g each of growing tissues with 1.5 ml of 100 mM sodium phosphate buffer (pH 6.8) (Zauberman et al. 1991).

Protein, ascorbate, total phenols and osmolyte contents

For calculating specific activities of different enzymes, the soluble protein content was measured from respective enzyme extracts following the method of Lowry et al. (1951). The ascorbate content was estimated following the method of Singla et al. (2020). Standard protocol of Swain and Hillis (1959) was used for estimation of the total phenolic content from methanolic extracts. Osmolyte contents including glycine betaine and proline from the growing tissues of three cultivars were measured according to the standard protocols of Grieve and Grattan (1983) and Bates et al. (1973) respectively.

H_2O_2 induced changes in root and shoot proteome of drought affected wheat seedlings

The total protein from 0.5 g each of roots and shoots was extracted from control, stressed and treated stressed seedlings using 6 ml of 25 mM Tris buffer (pH 8.3) containing polyvinyl polypyrrolidone (PVPP) and β-mercaptoethanol. Proteins were precipitated out using TCA-acetone method. Then the proteins were purified by using 2-D clean up kit and purified precipitates were dissolved in the rehydration buffer containing 6 M urea, 2 M thiourea, 50 mM DTT and 4% (w/v) CHAPS. Protein was quantified by Bradford reagent using BIOMATE 3S (Thermoscientific). To appropriately diluted protein with rehydration buffer, 0.002% bromophenol and 0.2% ampholyte were added. 125 μg of protein in 125 μl was loaded onto a 7 cm IPG strip with a linear gradient of 4–7 (GE Healthcare, USA) and IPG strip was run on Ettan IPGphore 3 (GE Healthcare, USA). The IPG strips were equilibrated twice for 15 min in 10 ml of equilibration buffer using 1% Dithiothreitol in 1st step and 2.5% iodoacetamide in the other. Then the proteins were separated by SDS PAGE i.e. in the 2nd dimension according to their charge/mass ratio on 12% SDS–polyacrylamide gels (Bio-rad Mini-PROTEAN Tetra System). The gels were stained with Coomassie Brilliant Blue and scanned with Image Scanner III (GE Healthcare, USA). The protein spots across the gels were also matched manually to improve the analysis. Three biological

replicates were taken for 2-D gel electrophoresis and the gels showing reproducible spots were analyzed for differentially expressed proteins (DEPs). Only those differentially expressed proteins and proteins showing altered expression under different treatments were analyzed by MALDI-TOF MS analysis which depicted significant changes (at least 1.5 fold, $P < 0.05$). The protein spots were subjected to in-gel digestion according to Yang et al. (2010). Spots corresponding to differentially expressed proteins were identified by MALDI-TOF MS and peptide mass fingerprinting (PMF) data was submitted to data bases using in-house Mascot server. Database queries were limited to *Viridiplantae*.

Statistical analysis

The data on various biochemical parameters was statistically analysed by using one way analysis of variance (ANOVA) followed by post hoc analysis i.e. the least significant difference (LSD) test at probability ≤ 0.05.

Results and discussion

Influence of H_2O_2 pre-treatment on growth dynamics

In the current study, H_2O_2, a secondary messenger, was evaluated as seed treatment to produce metabolic changes, which could improve the drought tolerance of wheat. As compared to control, root and shoot lengths of all the three wheat cultivars were reduced by more than 17% under drought stress (Table 1). Reduction in the seedling growth under drought stress was accompanied by slight increase in weight of endosperm in PBW 644. Seedling growth of wheat was significantly affected on priming with different concentrations of H_2O_2. It induced variable effects on germination, growth and physiological characteristics of wheat cultivars on exposure to drought. In the present study, exogenous H_2O_2 (10–200 mM) has proved very effective for the survival of wheat seedlings under drought conditions, 60 mM of H_2O_2 being the most effective concentration to promote the seedling growth (Table 1). In PBW 644, pre-treatment with 60 mM H_2O_2 enhanced the lengths of roots and shoots by 15 and 35% respectively compared to stressed seedlings whereas in PBW 621, increase of more than 19% was observed. Fresh weights of roots and shoots also increased in the stressed seedlings pretreated with 60 mM of H_2O_2 which indicated better seedling growth as compared to drought affected seedlings. Sohag et al. (2020) also observed that reduction in seedling growth of rice plants can be overcome by exogenous use of H_2O_2. Moreover, exogenous applications of H_2O_2 at low concentration ameliorated membrane damage by reducing lipid peroxidation in maize plants under osmotic-stress (Terzi et al. 2014).

Influence of H_2O_2 pre-treatment on membrane damage

Elevated MDA content is regarded as an indicator of oxidative stress in plants subjected to various stresses including drought stress. Water stressed plants of all the three cultivars exhibited an increase in the levels of MDA content due to peroxidation of membrane lipids. The MDA content reduced by more than 10% in the shoots of all the three cultivars (Fig. 1a). As compared to drought stress, H_2O_2 pre-treatment led to significant decrease in MDA level. Similar results were obtained in rice plants on exposure to drought stress (Sohag et al. 2020). Also, it has been reported that H_2O_2 treatment at low concentrations enhanced stress tolerance by decreasing MDA contents in maize plants (Sarwar et al. 2017). Therefore, the data clearly revealed that H_2O_2 pre-treatment played a role in maintenance of MDA levels by activating defense responses and assisted them to cope up with the adverse effects of drought. H_2O_2 plays an important role in cell signalling at low concentrations. Drought stress reduced H_2O_2 in sensitive cultivars whereas in PBW 644, elevated levels of H_2O_2 were observed. Hossain et al. (2013) also reported upregulation in the endogenous levels of H_2O_2. Conversely, H_2O_2 treated seedlings of sensitive cultivars depicted higher H_2O_2 level while it declined in the shoots of PBW 644 (Fig. 1b). These results are in negative correlation with the findings of Liu et al. (2010). Accumulation of H_2O_2 content in sensitive cultivars after H_2O_2 pre-treatment may arise due to differences in NADPH oxidase, which generates O_2^- that are dismutated by superoxide dismutase. If we compare the H_2O_2 treated plants with the control and drought affected plants, it seems that H_2O_2 induced increase in its level was more in the roots of all the three cultivars which may be due to the fact that the roots are the first tissues to respond to stress.

Antioxidant enzymes in H_2O_2 pre-treated drought stressed seedlings

Superoxide dismutase plays a crucial role in antioxidative defense machinery as it dismutates O_2^- to H_2O_2, thus preventing the generation of highly reactive toxic hydroxyl radicals. Superoxide dismutase activity decreased by more than 18% under stress in all the three cultivars except in the shoots of HD2967 (Fig. 2a). Kaya et al. (2020) also

Table 1 Effect of pre-treatment with varying concentrations of H_2O_2 on seedling growth of PBW 644, PBW 621 and HD2967 grown under water deficit stress

Treatments	Roots			Shoots			Endosperms	
	Length (cm)	FW (mg)	DW (mg)	Length (cm)	FW (mg)	DW (mg)	FW (mg)	DW (mg)
PBW 644								
Control	20.5 ± 0.3	131 ± 9.2	11.1 ± 3.0	16.9 ± 0.6	119 ± 4.5	10.3 ± 0.6	37.2 ± 5.7	7.3 ± 1.9
Stress	16.4 ± 2.4^{a}	94.2 ± 15.4^{a}	10.2 ± 3.0	12.5 ± 1.0^{a}	94.2 ± 10.0^{a}	9.5 ± 0.9	38.2 ± 5.6	10.9 ± 2.2
Hydroprimed + Stress	18.0 ± 0.5^{ab}	110 ± 4.2	11.5 ± 1.0	14.3 ± 0.8^{a}	110.0 ± 3.4	11.0 ± 0.3^{b}	37.5 ± 5.3	8.3 ± 1.5
10 mM + Stress	14.7 ± 1.4^{a}	95.4 ± 44.9	14.6 ± 2.3^{ab}	12.0 ± 2.0^{a}	83.8 ± 14.1^{a}	8.8 ± 0.4^{a}	37.9 ± 8.7	10.0 ± 2.0
20 mM + Stress	16.5 ± 1.2^{a}	90.9 ± 6.3^{a}	8.4 ± 1.5	15.7 ± 0.6^{b}	110 ± 17.7	11.7 ± 2.7^{ab}	38.9 ± 6.6	8.5 ± 1.4
60 mM + Stress	19.2 ± 0.1^{b}	148 ± 22.9^{b}	12.2 ± 1.2	19.4 ± 0.1^{ab}	152 ± 2.0^{ab}	14.3 ± 0.5^{ab}	39.2 ± 2.0	8.5 ± 0.2
80 mM + Stress	19.4 ± 0.1^{b}	141 ± 41.1^{b}	11.2 ± 0.2	17.0 ± 0.4^{b}	129 ± 20.1^{b}	12.0 ± 1.2^{ab}	40.6 ± 12.1	8.5 ± 1.5
100 mM + Stress	19.3 ± 1.0^{b}	150 ± 1.0^{b}	13.5 ± 1.0^{b}	16.7 ± 1.0^{b}	121 ± 1.0^{b}	12.8 ± 1.0^{ab}	42.3 ± 1.0	9.3 ± 1.0
LSD (5%)	2.3	36.8	3.1	2.1	21.3	1.7	NS	NS
PBW621								
Control	22.0 ± 1.2	121 ± 11.4	9.0 ± 1.0	19.0 ± 0.5	130 ± 5.5	12.3 ± 0.8	31.9 ± 4.0	5.0 ± 0.8
Stress	18.3 ± 1.0^{a}	72.2 ± 9.1^{a}	7.6 ± 0.8^{a}	14.0 ± 0.7^{a}	107 ± 10.3	10.6 ± 1.6	33.0 ± 2.3	5.8 ± 0.3
Hydroprimed + Stress	20.7 ± 1.0^{b}	100 ± 10.0^{ab}	10.1 ± 0.9^{b}	16.5 ± 0.9^{ab}	121 ± 13.7	15.1 ± 0.9	32.0 ± 1.3	5.0 ± 0.9
10 mM + Stress	20.9 ± 0.2^{b}	113 ± 8.8^{b}	9.0 ± 1.4	17.0 ± 0.9^{ab}	120 ± 34.7	12.6 ± 1.1	28.8 ± 7.4	6.0 ± 0.7
20 mM + Stress	20.5 ± 0.3^{ab}	119 ± 13.8^{b}	10.7 ± 2.2^{b}	17.9 ± 0.9^{ab}	115 ± 23.8	12.6 ± 1.1	28.7 ± 3.5	4.3 ± 0.8
60 mM + Stress	22.5 ± 1.6^{b}	121 ± 16.1^{b}	8.3 ± 2.5	18.3 ± 0.4^{b}	138 ± 14.4	11.4 ± 1.5	29.8 ± 4.0	5.0 ± 0.9
80 mM + Stress	22.0 ± 1.2^{b}	122 ± 6.7^{b}	8.9 ± 1.2	17.8 ± 0.7^{ab}	143 ± 4.1	11.6 ± 4.0	29.8 ± 5.3	6.2 ± 0.8
100 mM + Stress	21.5 ± 0.2^{b}	115 ± 12.4^{b}	7.7 ± 0.1	17.6 ± 0.7^{ab}	132 ± 4.1	13.3 ± 2.8	29.5 ± 5.5	3.8 ± 0.5
LSD (5%)	1.4	18.2	2.1	1.1	NS	NS	NS	NS
HD 2967								
Control	18.7 ± 2.9	103.4 ± 16.6	6.3 ± 0.6	17.9 ± 0.7	139 ± 9.8	10.6 ± 0.7	40.8 ± 6.8	7.9 ± 1.5
Stress	14.4 ± 0.8	80.7 ± 20.9	5.2 ± 0.4	12.6 ± 0.9	111 ± 9.0	8.5 ± 1.1	43.8 ± 7.8	9.0 ± 2.9
Hydroprimed + Stress	15.2 ± 0.8	82.7 ± 20.9	5.2 ± 0.4	13.9 ± 0.9	117 ± 6.8	9.9 ± 1.1	42.8 ± 7.8	8.9 ± 2.9
10 mM + Stress	15.4 ± 1.5	86.6 ± 13.8	5.2 ± 0.8	13.1 ± 0.7	123 ± 19.4	10.3 ± 1.3	41.7 ± 14.2	8.8 ± 1.3
20 mM + Stress	16.5 ± 2.2	89.0 ± 25.8	7.2 ± 0.6	15.0 ± 0.4	121 ± 10.7	11.1 ± 0.6	45.9 ± 15.1	8.0 ± 4.2
60 mM + Stress	15.9 ± 2.2	79.9 ± 17.5	4.7 ± 1.2	14.6 ± 1.6	126 ± 6.8	10.5 ± 1.5	46.7 ± 1.7	8.5 ± 0.7
80 mM + Stress	16.3 ± 1.4	87.1 ± 9.6	5.9 ± 1.3	14.9 ± 1.4	124 ± 13.4	9.7 ± 0.8	38.7 ± 7.3	6.5 ± 0.5
100 mM + Stress	16.2 ± 0.3	93.7 ± 1.4	5.3 ± 0.6	15.3 ± 1.3	123 ± 19.9	9.7 ± 0.9	36.6 ± 7.7	8.2 ± 5.5
LSD (5%)	NS	NS	NS	NS	NS	NS	NS	NS

Values are mean ± SD of three replicates. LSD ($P \leq 0.05$) Least significant difference at 5% probability level

'a' represents significant differences with respect to control and 'b' represent significant differences with respect to stress

reported a decrease in SOD activity in the sunflower plants subjected to drought stress. As compared to drought stress, H_2O_2 pre-treatment enhanced SOD activity in the roots and shoots of PBW 644 by more than 54%, however, the effect was not noticeable in the sensitive cultivars. It has been reported that pre-sowing treatment of seeds with different concentrations of H_2O_2 induced a consistent increase in the SOD activity of drought stressed maize seedlings (Ashraf et al. 2014). In plants, the H_2O_2 detoxification involves the activities of CAT, POX, APX, GR, MDHAR and DHAR. Catalase activity reduced by more than 10% in the shoots of all the three cultivars on exposure to drought stress (Fig. 2b). However on H_2O_2 pre-treatment, it has been observed upregulated in the stressed shoots of PBW 621 and HD 2967 by more than 15%. Contrary to this, the roots of the tolerant cultivar displayed 1.9 times increase in CAT activity. This is in agreement with the earlier reports which stated that CAT activity increased under drought stress by seed pre-treatment with H_2O_2 in wheat seedlings (Terzi et al. 2014). Many other reports also depicted that exogenous application of various biomolecules resulted in an increase in SOD and CAT activities and membrane

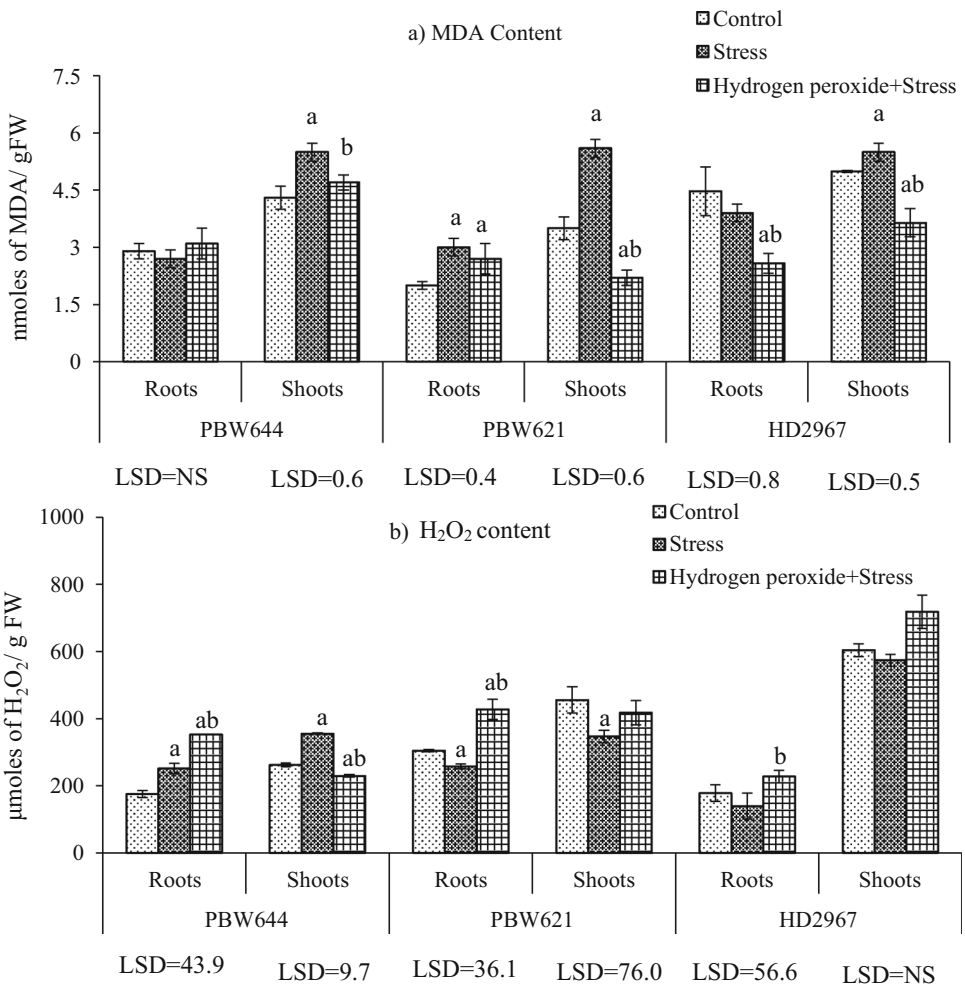

Fig. 1 Effect of H_2O_2 pre-treatment on MDA and H_2O_2 content of wheat seedlings grown under stress. Values are Mean ± S.D. of three replicates. LSD ($P \leq 0.05$) Least significant difference at 5% probability level. 'a' represents significant differences with respect to control and 'b' represent significant differences with respect to stress

integrity which may further be involved in the maintenance of photosynthetic pigments under drought affected conditions (Parveen et al. 2019; Kosar et al. 2020). Peroxidase also detoxifies H_2O_2 and thus, possesses a vital role in stress tolerance of plants. POX activity showed differential behavior in seedlings of three cultivars (Fig. 2c). As compared to stress, H_2O_2 pre-treatment caused an increase in POX activity of roots of PBW 644 and shoots of PBW 621. These results are in harmony with Hossain et al. (2013) who observed that seed pre-treatment with 140 mM H_2O_2 significantly enhanced the activities of POX and CAT under drought stress. Earlier Goud and Kachole (2011) reported an increase in POX activity and induction of oxidative stress tolerance by exogenous H_2O_2 in *Cajanus cajan*. Moreover, perusal of results indicated that under drought stress, H_2O_2 pre-treatment was able to maintain the POX activity of tolerant cultivar either higher or comparable to that of the control whereas activity was slightly less in PBW 621 (Fig. 2c).

Asada Halliwell pathway in H_2O_2 treated drought stressed wheat seedlings

Ascorbate peroxidase is among the most important enzymes which detoxify H_2O_2 in plant cells and also responsible for regulating the appropriate levels of H_2O_2. Thus, it works as signalling molecule. Imposition of drought stress upregulated APX activity in the roots and shoots of PBW 644 by more than 1.5 times but declined in the roots of sensitive cultivars (Fig. 3a). Raja et al. (2020) observed that combined heat and drought stress up-regulated APX activity in tomato plants by more than 2 times. As compared to drought stress, APX activity increased by about 25% in the roots of all the three wheat cultivars on treatment with H_2O_2. Similarly, H_2O_2 pre-treatment caused significant increase in APX activity of the shoots of PBW644 and PBW621 whereas slight increase was observed in shoots of HD2967 compared to stress. However, compared to control, the upregulation was much more in the treated PBW 644 plants subjected to drought stress. Therefore, H_2O_2 pre-treatment has the potential to keep

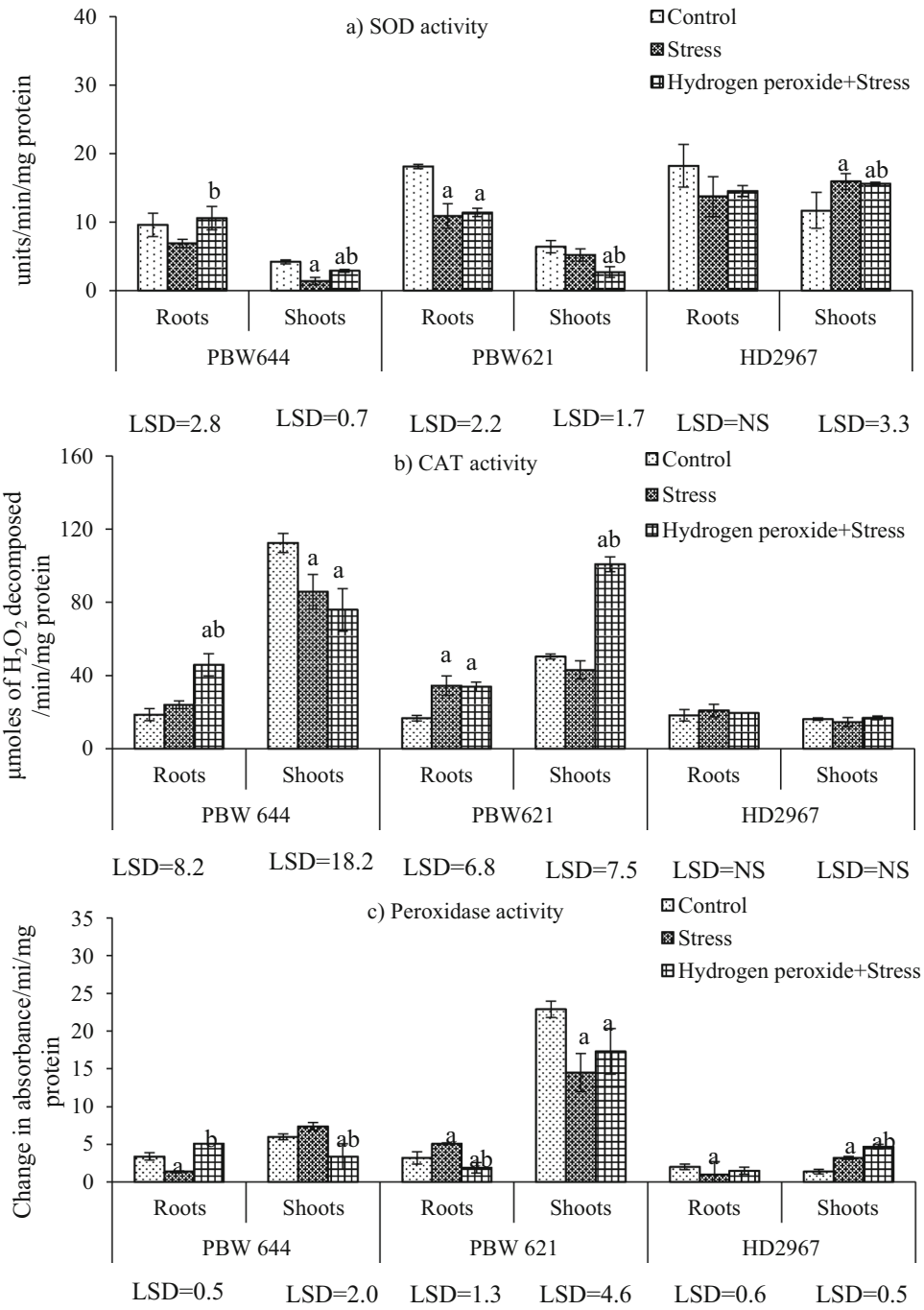

Fig. 2 Effect of H_2O_2 pre-treatment on SOD, CAT and POX activities of wheat seedlings grown under stress. Values are Mean ± S.D. of three replicates. LSD ($P \leq 0.05$) Least significant difference at 5% probability level. 'a' represents significant differences with respect to control and 'b' represent significant differences with respect to stress

APX activity high in the stressed seedlings of all the three cultivars. This is in agreement with the findings of He et al. (2009) and Li et al. (2011) which reported the increase in APX and CAT activities in H_2O_2 pre-treated drought and salt stressed seedlings. Monodehydroascorbate reductase is the vital enzyme involved in the regeneration of ascorbic acid. Drought stress declined MDHAR activity by more than 18% in the roots as well as the shoots of all the three cultivars (Fig. 3b). Similar observations were made by Raja et al. (2020). As compared to stress, H_2O_2 treatment upregulated MDHAR activity in stressed seedlings of sensitive cultivars. An increase was observed in MDHAR activity under heat stress as well as in H_2O_2 treated cucumber leaves (Gao et al. 2010). Contrary to this, MDHAR activity remained unaffected in H_2O_2 treated seedlings of PBW644. Glutathione reductase is the key enzyme of AsA-GSH cycle which mainly reduces GSSG utilizing NADPH as reducing equivalent donor. Drought stress downregulated GR activity in PBW644 while variable response was observed in the growing tissues of

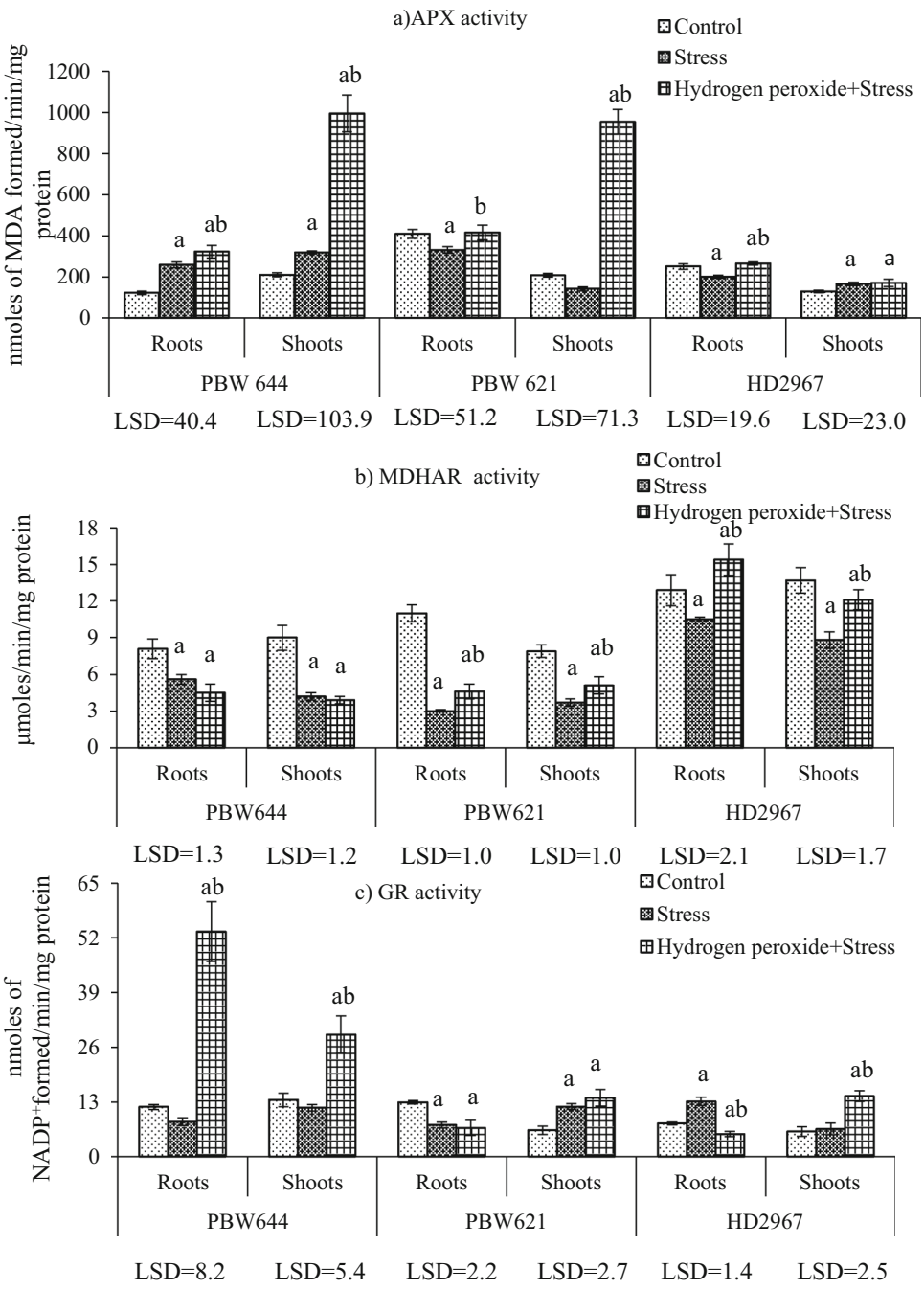

Fig. 3 Effect of H_2O_2 pre-treatment on APX, MDHAR and GR activities of wheat seedlings grown under stress. Values are Mean ± S.D. of three replicates. LSD ($P \leq 0.05$) Least significant difference at 5% probability level. 'a' represents significant differences with respect to control and 'b' represent significant differences with respect to stress

sensitive cultivars (Fig. 3c). Earlier, decrease in GR activity of mustard plants growing under drought was reported (Hossain et al. 2013). These results are in accordance with Wang et al. (2010) who observed upregulation of GR activity during chilling stress in manila grass. As compared to stress, H_2O_2 pre-treatment significantly enhanced GR activity by more than 2.5 times in the stressed seedlings of PBW644 while in sensitive cultivars, lesser increase was noticed in the shoots only. Increased GR activity in the H_2O_2 pre-treated stressed seedlings might be contributing to the maintenance of higher GSH/GSSG ratio.

On exposure to drought stress, shoots of PBW 621 seedlings displayed lower ascorbate contents. However, ascorbate level showed an increase in the roots of tolerant cultivar while decrease in the roots of HD2967. Decrease in the ascorbic acid content might be due to the increased activity of APX which uses ascorbic acid as a substrate for reduction of H_2O_2 to H_2O. Under drought stress, H_2O_2 pre-treatment enhanced the ascorbic acid content in the shoots of PBW644 while it remained unaffected in PBW 621.

H_2O_2 pre-treated seedlings of HD 2967 depicted lower ascorbate content. Ascorbic acid content decreased in mustard seedlings under drought stress as reported by Hossain et al. (2013). However, Liu et al. (2010) described that ascorbate content increased with H_2O_2 treatment in cucumber plants grown under drought. It indicates that modulation of ascorbate levels varies from species to species under drought stress on H_2O_2 pre-treatment. Higher ascorbate contents in the shoots of PBW 644 demonstrated their superior tolerance mechanisms in terms of H_2O_2 scavenging compared with the sensitive cultivars.

Influence of H_2O_2 pre-treatment on PPO activity in relation to total phenols

Polyphenoloxidases are ubiquitious copper containing enzymes which function as phenol oxidase in various plant species. They oxidize phenolic compounds which have been associated with antioxidant activity. On exposure to drought stress, PPO activity displayed decrease in the roots of the tolerant cultivar (Fig. 4b). However, stressed roots of sensitive cultivars depicted an increase which was further increased by H_2O_2 pre-treatment. Earlier, it has been reported that exogenous H_2O_2 may improve stress tolerance by upregulating the POX and PPO enzymes under stressed conditions (Liu et al. 2010). PPO activity was upregulated by exogenous H_2O_2 treatment in tomato plants grown under sand ponic culture (Orabi et al 2015). In the present study, total phenolic content reduced in all the three wheat cultivars grown under drought except the roots of HD 2967 where it increased. In the seedlings of PBW 621, it reduced by more than 37%. As compared to drought, H_2O_2 pre-treatment increased total phenolic content by more than 31% in the roots and shoots of PBW 644 (Fig. 4c). However in PBW 621 seedlings, the total phenolic content was upregulated in shoots but remained unaffected in the roots of PBW 621 and in the growing tissues of HD 2967 seedlings (Fig. 3c). The correlation between the total phenolic content and PPO activity clearly indicated that this increase in total phenols may be attributed to the enhanced activity of enzymes responsible for their biosynthesis.

Status of osmolytes in H_2O_2 pre-treated drought stressed seedlings

Proline is an amino acid which gets accumulated in response to various stresses and acts as a scavenger of ROS (Mittler 2017). In general, drought stress increased the proline levels by more than 47% in wheat seedlings except the roots of PBW 644 (Fig. 5a). Previous studies revealed an increase in free proline content of maize grown under drought stress (Parveen et al. 2019). When compared to drought stress, shoots of H_2O_2 pre-treated plants of PBW 644 depicted an increase of 26% in proline content whereas roots of PBW 621 showed an increase of 22%. HD 2967 depicted more than 23% increase in the roots as well as shoots. Sohag et al. (2020) also reported an increase in proline content in rice plants on exogenous application of H_2O_2. Thus, it can be inferred that under drought, H_2O_2 pre-treatment differentially modulated proline content in the different parts of the tolerant and sensitive cultivars. Accumulation of proline in the stressed wheat seedlings may help in the scavenging of ROS, osmotic adjustment and maintenance of membrane integrity to confer drought tolerance. Glycine betaine acts as an osmoprotectant and thus plays a very important role in the growth and development of plants subjected to different stresses (Cha-um et al. 2013). On exposure to stress, it decreased in all the three cultivars except the shoots of PBW 644 (Fig. 5b). Conversely, H_2O_2 pre-treatment decreased glycine betaine content in the shoots of PBW644 and roots of PBW621.

Root and shoot proteomes of H_2O_2 pre-treated drought stressed seedlings

Seedling growth is considered as critical phase for growth under drought stress and roots were regarded to be the chief drought-responsive organs, as they exhibited the major changes in protein abundance in response to this stress. Exploration of plant reaction to stress conditions at protein level can offer an effective tool to decipher the mechanisms of underlying stress tolerance in plants (Skalak et al. 2016). Therefore, the root and shoot proteomes of control, stressed and treated wheat seedlings were analysed by 2-D gel electrophoresis and the differentially expressed proteins and proteins showing altered expression with H_2O_2 pre-treatment were analyzed by MALDI-TOF MS analysis. Proteins changing in abundance as a result of drought stress and H_2O_2 pre-treatment in PBW 644 are indicated by arrows and their number is also depicted in Fig. 6. The comprehensive analysis of DEPs in control and stressed seedlings revealed that imposition of drought stress in PBW 644 enhanced the expression of proteins involved in photosynthesis, protein degradation, carbohydrate metabolism, fatty acid metabolism. Analysis of these pathways suggested increased energy production that is required by the defense processes (Yan et al. 2006). Indeed in this case also, proteins involved in defense and regulation were also upregulated whereas in sensitive cultivars grown under stress, the proteins related with protein biosynthesis, fatty acid metabolism, nucleic acid metabolism and photosynthesis are upregulated. In comparison to drought stress,

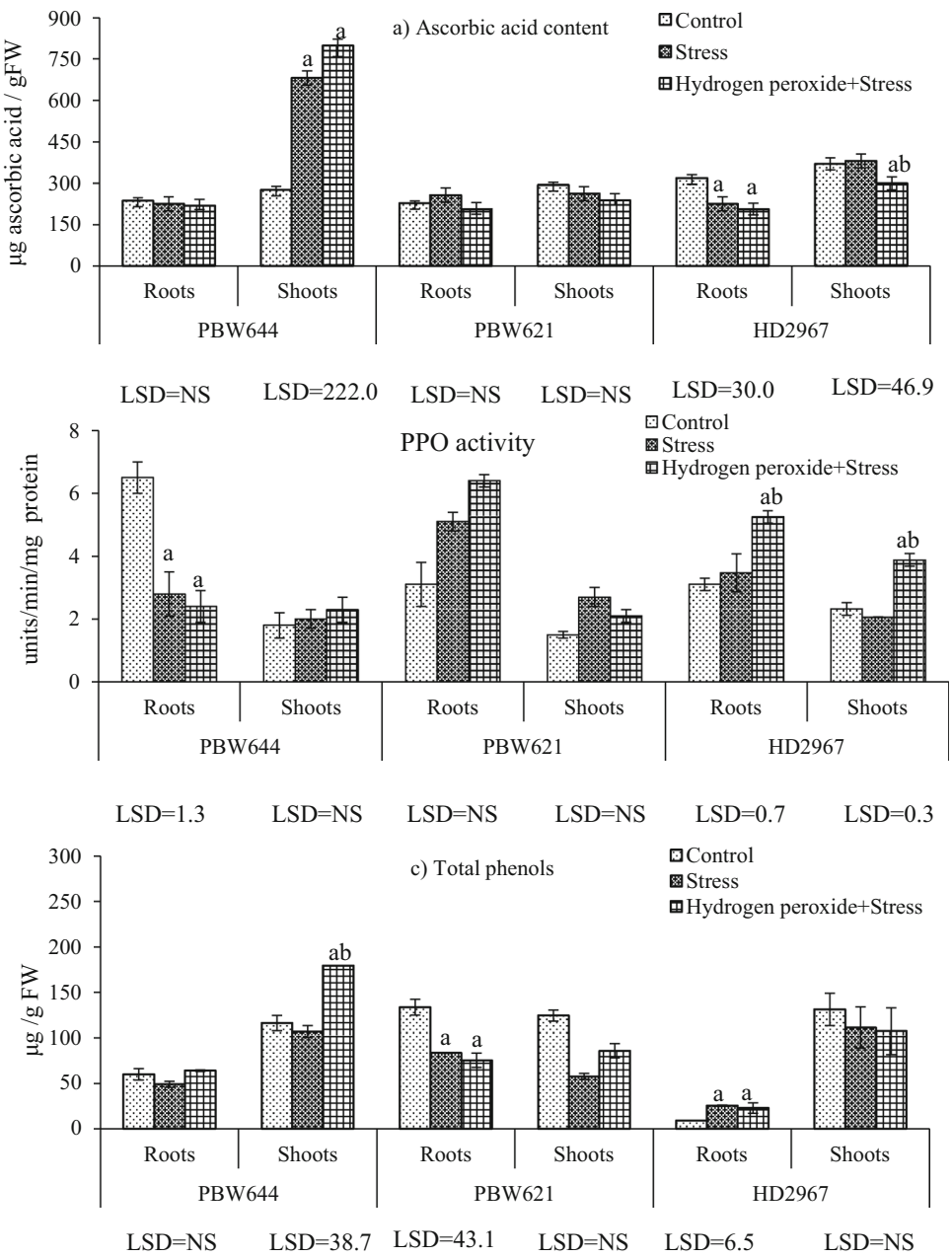

Fig. 4 Effect of H_2O_2 pre-treatment on ascorbate content, PPO activity and total phenol content of wheat seedlings grown under stress. Values are Mean ± S.D. of three replicates. LSD ($P \leq 0.05$) Least significant difference at 5% probability level. 'a' represents significant differences with respect to control and 'b' represent significant differences with respect to stress

H_2O_2 pre-treatment led to over-expression of proteins which had functions in processes such as defense, redox homeostasis and photosynthesis in all the three cultivars. This can also be visualized from the biochemical analysis since some key antioxidant enzymes are up-regulated under drought stress and are further enhanced by hydrogen peroxide treatment.

It is well known that hydrogen peroxide plays a vital role and acts as a potent regulator in signaling pathways whenever the plant is subjected to any kind of stress. The exogenous use of H_2O_2 had been reported to offer stress tolerance in different crops (Sohag et al. 2020). In the present study, hydrogen peroxide pre-treatment showed the potential of stimulating the seedling growth in wheat under drought stress. However, at high concentrations, H_2O_2 treatment displayed growth inhibitory effects. H_2O_2 pre-treatment alleviated drought stress induced effects in PBW 644 via stimulating SOD, APX and GR activities which participate in the scavenging of ROS produced under stressed conditions. It further enhanced ascorbate levels which may impart stress tolerance via acting as a radical scavenger or as a modulator for the expression of genes involved in the synthesis of antioxidant enzymes, thereby limiting the drought induced oxidative stress. In sensitive cultivars, H_2O_2 pre-treatment induced protective effects against drought stress were associated with the

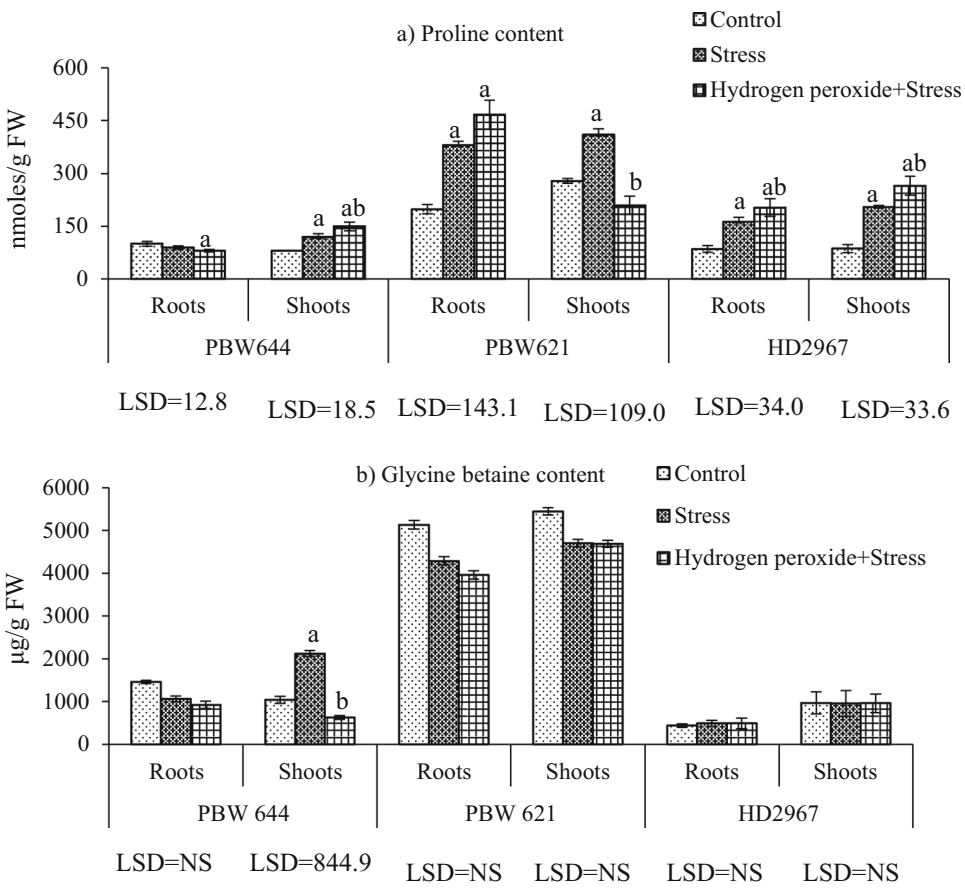

Fig. 5 Effect of H_2O_2 pre-treatment on proline and glycine betaine contents of wheat seedlings grown under stress. Values are Mean ± S.D. of three replicates. LSD ($P \leq 0.05$) Least significant difference at 5% probability level. 'a' represents significant differences with respect to control and 'b' represent significant differences with respect to stress

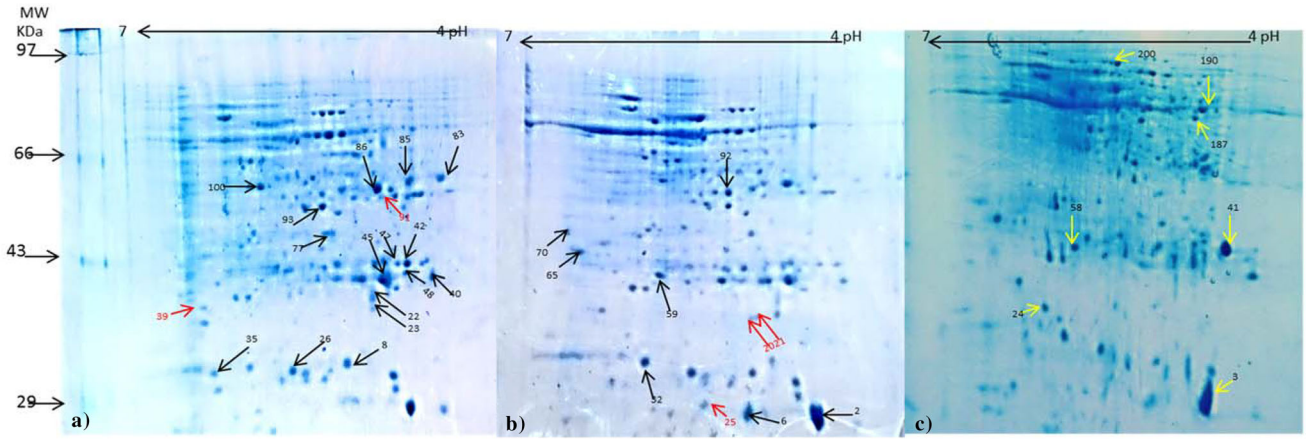

Fig. 6 Representative 2-D gel electrophoresis in the range of pH 4–7. Shoots of control (**a**), water deficit stressed (**b**), and H_2O_2 treated stressed (**c**) seedlings of PBW 644. Proteins changing in abundance as a result of water deficit stress and those altered by treatment under stress are indicated by black and yellow arrows respectively and their number is also depicted whereas, the newly synthesized proteins were demarcated by red arrows

upregulation of CAT, POX, APX, and MDHAR enzymes, membrane stability and osmotic adjustment. Indeed, these effects were corroborated from the proteomic studies where H_2O_2 pre-treatment upregulated the proteins involved in different processes like defense, redox balance and photosynthesis in all the three cultivars when compared to drought stress.

Acknowledgements Corresponding author is highly grateful to DST, New Delhi through SERB scheme vide letter no. SERB/F/3281/2014-15 for funding this research.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

Alexieva V, Sergiev I, Mapelli S, Karanov E (2001) The effect of drought and ultraviolet radiation on growth and stress markers in pea and wheat. Plant Cell Environ 24:1337–1344

Ashraf MA, Rasheed R, Hussain I, Iqbal M, Haider MZ, Parveen S, Sajid MA (2014) Hydrogen peroxide modulates antioxidant system and nutrient relation in maize (*Zea mays* L.) under water-deficit conditions. Arch Agron Soil Sci. https://doi.org/10.1080/03650340.2014.938644

Bates LS, Waldan RP, Teare LD (1973) Rapid determination of free proline under water stress studies. Plant Soil 39:205–207

Bienert GP, Chaumont F (2014) Aquaporin-facilitated transmembrane diffusion of hydrogen peroxide. Biochim Biophys Acta 1840:1596–1604

Blum A (2017) Osmotic adjustment is a prime drought stress adaptive engine in support of plant production. Plant Cell Environ 40:4–10

Chance B, Maehly AC (1955) Effects of saline stress and calcium on lipid composition in bean roots. Phytochemistry 32:1131–1136

Cha-um S, Samphumphuang T, Kirdmanee C (2013) Glycine betaine alleviates water deficit stress in indica rice using proline accumulation, photosynthetic efficiencies, growth performances and yield attributes. Aust J Crop Sci 7:213–218

Deng XP, Cheng YJ, Wu XB, Kwak SS, Chen W, Eneji AE (2012) Exogenous hydrogen peroxide positively influences root growth and metabolism in leaves of sweet potato seedlings. Aust J Crop Sci 6:1572–1578

Farooq A, Bukhari SA, Akram NA, Ashraf M, Wijaya L, Alyemeni MN, Ahmad P (2020) Exogenously applied ascorbic acid-mediated changes in osmoprotection and oxidative defense system enhanced water stress tolerance in different cultivars of safflower (*Carthamus tinctorious* L.). Plants 9:104

Gao Y, Guo YK, Lin SH, Fang YY, Bai JG (2010) Hydrogen peroxide pretreatment alters the activity of antioxidant enzymes and protects chloroplast ultrastructure in heat-stressed cucumber leaves. Sci Hortic 126:20–26

Goud PB, Kachole MS (2011) Effect of exogenous hydrogen peroxide on peroxidase and polyphenol oxidase in *Cajanuscajan* (L.) Millsp detached leaves. Int J Curr Res 3:61–65

Grieve CM, Grattan SR (1983) Rapid assay for the determination of water soluble quaternary ammonium compounds. Plant Soil 70:303–307

He L, Gao Z, Li R (2009) Pre-treatment of seed with H_2O_2 enhances drought tolerance of wheat (*Triticum aestivum* L.) seedlings. Afr J Biotech 8:6151–6157

Heath R, Packer L (1968) Photoperoxidation in isolated chloroplast I. Kinetics and stoichiometry of fatty acid peroxidation. Arch Biochem Biophys 125:189–198

Hossain MA, Mostofa MG, Fujita M (2013) Cross protection by cold-shock to salinity and drought stress-induced oxidative stress in mustard (*Brassica campestris* L.) seedlings. Mol Plant Breed 4:50–70

Hung KT, Kao CH (2005) Nitric oxide counteracts the senescence of rice leaves induced by hydrogen peroxide. Bot Bull Acad Sin 46:21–28

Kaya C, Şenbayram M, Akram NA, Ashraf M, Alyemeni MN, Ahmad P (2020) Sulfur-enriched leonardite and humic acid soil amendments enhance tolerance to drought and phosphorus deficiency stress in maize (*Zea mays* L.). Sci Rep 10:1–3

Kohli SK, Khanna K, Bhardwaj R, Abd_Allah EF, Ahmad P, Corpas FJ (2019) Assessment of subcellular ROS and NO metabolism in higher plants: multifunctional signaling molecules. Antioxidants 8:641

Kosar F, Akram NA, Ashraf M, Ahmad A, Alyemeni MN, Ahmad P (2020) Impact of exogenously applied trehalose on leaf biochemistry, achene yield and oil composition of sunflower under drought stress. Physiol Plant. https://doi.org/10.1111/ppl.13155

Li JT, Qiu ZB, Zhang XW, Wang LS (2011) Exogenous hydrogen peroxide can enhance tolerance of wheat seedlings to salt stress. Acta Physiol Plant 30:835–842

Liu ZJ, Guo YK, Bai JG (2010) Exogenous hydrogen peroxide changes antioxidant enzyme activity and protects ultrastructure in leaves of two cucumber ecotypes under osmotic stress. J Plant Growth Regul 29:171–183

Lowry OH, Rosebrough NT, Farr AL, Randall RJ (1951) Protein measurement with folin phenol reagent. J Biol Chem 193:265–275

Mittler R (2017) ROS are good. Trends Plant Sci 22:11–19

Nakano Y, Asada K (1981) Hydrogen peroxide is scavenged by ascorbate specific peroxidase in spinach chloroplasts. Plant Cell Physiol 22:867–880

Orabi SA, Dawood MG, Salman SR (2015) Comparative study between the physiological role of hydrogen peroxide and salicylic acid in alleviating the harmful effect of low temperature on tomato plants grown under sand-ponic culture. Sci Agri 9:49–59

Parveen A, Liu W, Hussain S, Asghar J, Perveen S, Xiong Y (2019) Silicon priming regulates morpho-physiological growth and oxidative metabolism in maize under drought stress. Plants 8:431

Raja V, Qadir SU, Alyemeni MN, Ahmad P (2020) Impact of drought and heat stress individually and in combination on physio-biochemical parameters, antioxidant responses, and gene expression in *Solanum lycopersicum*. 3 Biotech 10:1–8

Sarwar M, Saleem MF, Najeeb U, Shakeel A, Ali S, Bilal MF (2017) Hydrogen peroxide reduced heat induced yield losses in cotton (*Gossypium hirsutum* L.) by protecting cellular damage. J Agron Crop Sci. https://doi.org/10.1111/jac.12203

Shahzad MA, Jan SU, Afzal F, Khalid M, Gul A, Sharma I, Sofo A, Ahmad P (2016) Water stress and crop plants: a sustainable approach. Wiley, New Jersey

Singh N, Bhardwaj RD (2019) Proline treatment ameliorates water deficit induced oxidative damage in wheat seedlings. Indian J Exp Biol 57:399–407

Singla P, Bhardwaj RD, Kaur S, Kaur J (2020) Stripe rust induced defence mechanisms in the leaves of contrasting barley genotypes (*Hordeum vulgare* L.) at the seedling stage. Protoplasma 257:169–181

Skalak J, Cerny M, Jedelsky P, Dobra J, Ge E, Novak J, Hronkova M, Dobrev P, Vankova R, Brzobohanty B (2016) Stimulation of *ipt* overexpression as a tool to elucidate the role of cytokinins in high temperature responses of *Arabidopsis thaliana*. J Exp Bot 67:2861–2873

Sohag AA, Tahjib-Ul-Arif M, Brestic M, Afrin S, Sakil MA, Hossain MT, Hossain MA, Hossain MA (2020) Exogenous salicylic acid and hydrogen peroxide attenuate drought stress in rice. Plant Soil Environ 66:7–13

Suzuki N, Rivero RM, Shulaev V, Blumwald E, Mittler R (2014) Abiotic and biotic stress combinations. New Phytol 203:32–43

Swain T, Hillis WE (1959) The phenolic constituents of *Prunus domestica*. I. The quantitative analysis of phenolic constituents. J Sci Food Agric 10:63–68

Terzi R, Kadioglua A, Kalaycioglua E, Saglamb A (2014) Hydrogen peroxide pretreatment induces osmotic stress tolerance by influencing osmolyte and abscisic acid levels in maize leaves. J Plant Interactions 9:559–565

USDA FAS (2020) Global agricultural information book. https://apps.fas.usda.gov/newgainapi/api/report/downloadreportbyfilename?filename=Grain%20and%20Feed%20Annual_New%20Delhi_India_3-29-2019.pdf

Wang Y, Li J, Wang J, Li Z (2010) Exogenous H_2O_2 improves the chilling tolerance of manila grass and mascarene grass by activating the antioxidative system. Plant Growth Regul 61:195–204

Yan SP, Zhang QY, Tang ZC, Su WA, Sun WN (2006) Comparative proteomic analysis provides new insights into chilling stress response in rice. Mol Cell Proteom 5:484–496

Yang F, Jensen JD, Spliid NH, Svensson B, Jacobsen S, Jorgensen LN, Jorgensen DHJL, Collinge DB, Finnie C (2010) Investigation of the effect of nitrogen on severity of fusarium head blight in barley. J Proteom 73:743–752

Zauberman G, Ronen R, Akerman M, Weksler A, Rot I, Fuchs Y (1991) Postharvest retention of the red color of litchi fruit pericarp. Sci Hort 47:89–97

Zhang J, Zhang S, Cheng M, Jiang H, Zhang X, Peng C, Lu X, Zhang M, Jin J (2018) Effect of drought on agronomic traits of rice and wheat: a meta-analysis. Intl J Environ Res Pub Health 15:839