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Proline treatment ameliorates water deficit induced oxidative damage in wheat seedlings

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Upregulation of antioxidant system provides protection against drought induced oxidative stress in plants. Proline, an important osmolyte, acts as ROS scavenger, membrane stabilizer and also as protectant of enzymes. In this study, we investigated the effect of proline pre-treatment on the inhibitory effects of water deficit stress in two contrasting wheat cultivars, i.e. PBW 644 (Drought tolerant) and PBW 621 (Drought sensitive). Seedling growth was adversely affected in both the cultivars grown under stress. Pre-treatment of seeds with 15 mM proline stimulated seedling growth of both wheat cultivars accompanied by increased activities of SOD, POX, APX and GR and the contents of glycine betaine, proline and total phenols in the tolerant cultivar. In PBW 621, CAT and APX enzymes were upregulated along with increased glycine betaine content. Proline pre-treatment enhanced the membrane stability of both wheat cultivars grown under stress, as revealed by the significantly reduced malondialdehyde content. The levels of endogenous H_2O_2 contents in proline treated stressed seedlings of PBW 644 were also markedly lower than those of the non-treated stressed seedlings. The results indicated that proline pre-treatment could improve drought tolerance by stimulating ROS detoxification pathways, especially in tolerant cultivars.

Keywords: Abiotic stress, Antioxidant system, Glycine betaine, Reactive oxygen species, Seedling growth, *Triticum aestivum*

Wheat (*Triticum aestivum* L.) is one of the most important crops of Poaceae family and has a vital role in ensuring world food security. Widely adapted to different soil and climatic conditions, it is grown in almost all parts of the world. Various abiotic stresses affect the crop productivity[1]. Plants, over the period, have evolved intricate mechanisms to detect environmental changes, allowing optimal responses to such adverse conditions[2]. Drought is considered as one of the most common abiotic stresses that limit crop production[3]. Water scarcity has grown to such an extent that around four billion people face absolute water shortage for atleast one month in a year[4]. Plants experience water stress either when plants are unable to uptake water through roots or when the transpiration rate ishigh. These two conditions often coincide in semi-arid and arid environments in which wheat is generally grown.

Plants exposed to water deficit environment face oxidative stress in terms of elevated levels of reactive oxygen species (ROS), which causes substantial damage to plant cell membrane, pigments, DNA and other vital macromolecules[5]. Plants overcome this oxidative stress by upregulating the activities of antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), peroxidase (POX) and increasing the contents of non-enzymatic anti-oxidants, such as ascorbic acid, glutathione and osmolytes like proline and glycine betaine[6]. The osmotic adjustment by proline has considerable significance as it allows additional water to be taken up from the environment, thus buffering the immediate effect of water shortage within the plant[7]. Zouari *et al*.[8] has demonstrated proline's importance in cell osmotic adjustment, membrane stabilization and in scavenging free radicals.

Over the last few decades, researchers have tried various traditional breeding, biotechnological and several other strategies to enhance the productivity and stress tolerance of plants. Exogenous application of eco-friendly molecules through seed priming, foliar spray or hydroponics may improve stress tolerance of various plants. Similarly, the exogenous use of osmolytes is well known to induce abiotic stress tolerance in different plant species[9]. Addition of proline to cell suspension culture also alleviated the adverse effects of salt stress in tobacco by increasing the activities of antioxidant enzymes[10]. Foliar spray of

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proline also enhanced the growth and maintained nutrient status by promoting the uptake of K^+, Ca^+, P and N in maize plants exposed to drought stress[11]. Pre-treatment with trehalose protected thylakoid membranes from heat damage, maintained membrane integrity and reduced ROS accumulation in wheat plants grown under stress[12].

Out of various strategies used, a simple seed priming technique has shown remarkable potential to combat the adverse effects of abiotic stresses[13]. Basically, priming is a controlled hydration process followed by redrying that allows metabolic activities to proceed before radical protrusion. Seed priming with different eco-friendly chemicals was reported to have profound effects on seed vigor, germination and growth[14]. The antioxidant defense system was also strengthened in wheat seedlings pre-treated with different phenolic acids[15-17]. However, there are no reports available of the potential of proline in alleviating the detrimental effects of ROS generated in response to water deficit stress in wheat. Therefore, here, we assessed the antioxidant protection offered by proline pre-treatment against water deficit stress in wheat seedlings, both drought tolerant and drought susceptible.

Materials and Methods

Plant material and growth conditions

The present investigation was carried out in two wheat cultivars, namely PBW 644 which is drought tolerant and PBW 621 which is drought susceptible cultivar. Seeds were thoroughly washed with teepol and were pre-treated with water (hydroprimed) as well as varying concentrations of proline (0.5-20 mM) for 12 h. After the treatment, seeds were washed and dried by placing them in incubator at 25±1ºC. Untreated and pre-treated seeds were germinated in plastic cups (250 cm^3) containing untreated and well irrigated soil having pH 8.0, electrical conductivity of 0.12 mmhos cm^{-1} and organic carbon content of 0.51%. Water deficit stress was provided by withholding of water. For each treatment, three cups with 6 seeds in each were used. Plastic cups containing seeds were then placed in an incubator at 25±1ºC in the dark.

Growth analyses

Lengths of roots and shoots and biomass of roots, shoots and endosperms were determined at 8^{th} day after germination (DAG). Dry weight of different tissues was determined after drying the tissues at 60°C till the constant weight was obtained. Based upon the improvement of seedling growth in both the cultivars, 15 mM was selected for studying stress alleviating effects of proline pre-treatment in wheat seedlings.

Extraction and assays of ROS scavenging enzymes

Catalase (CAT EC 1.11.1.6) was extracted with 50 mM sodium phosphate buffer (pH 7.5) containing 1% polyvinyl pyrrolidone and its activity was measured according to the method of Chance and Maehly[18]. The assay was done by monitoring the decrease of absorbance at 240 nm for 3 min caused by the decomposition of H_2O_2. The extraction buffer for ascorbate peroxidase (APX EC 1.11.1.1) was similar to that of CAT except that additionally it contained 1.0 mM ascorbate. After centrifugation at 10000×g for 20 min, the supernatant was collected for the measurement of APX activity according to the methods of Nakano and Asada[19].

Superoxide dismutase (SOD EC 1.15.1.1), peroxidase (POX EC 1.11.1.7) and glutathione reductase (GR EC 1.6.4.2) were extracted with 1.5 mL of ice cold 100 mM sodium phosphate buffer (pH 7.5) containing 1.0 mM EDTA, 1% PVP, 10 mM β-mercaptoethanol using pre-chilled pestle and mortar. Homogenate was centrifuged at 10000×g for 15 min at 4°C. Supernatant was used for assaying SOD, POX and GR according to the methods of Shannon *et al.*[20], Marklund & Marklund[21] and Esterbaur & Grill[22], respectively.

Estimation of contents of soluble protein, ascorbate, osmolytes and total phenols

The protein content of each sample was determined following the method of Lowry *et al.*[23] using bovine serum albumin (BSA) as a protein standard. The ascorbate and total phenolic contents were determined by the methods earlier standardized in the lab[24]. The proline and glycine betaine contents in the roots and shoots were determined by adopting the standard methods of Bates *et al.*[25] and Grieve & Grattan[26], respectively.

Determination of MDA and H_2O_2 contents

The level of lipid peroxidation was measured by estimating MDA using thiobarbituric acid (TBA) as the reactive material[15]. The content of MDA was calculated by an extinction coefficient of 155 mM^{-1} cm^{-1} and expressed as nmol/g of fresh weight. Hydrogen peroxide was determined according to the method described earlier[15].

Statistical analysis

Data was analyzed by applying one way analysis of variance (ANOVA) followed by post hoc analysis i.e. the least significant difference (LSD) test.

Results

Influence of proline pre-treatment on growth parameters

Seedling growth was severely affected in both the wheat cultivars grown under water deficit conditions. In comparison to well watered plants, imposition of water stress reduced the lengths of roots and shoots by 17 and 33%, respectively in PBW 644 whereas in sensitive cultivar root and shoot lengths reduced by 25 and 36%, respectively (Table 1). This decrease in the length of growing tissues was also accompanied by decline in fresh weight but the fresh and dry biomass of endosperm increased under stress. Under stressed conditions, hydroprimed seedlings of both the cultivars did not show much growth stimulatory effects (Table 1 and 2). However, pre-treatment of seeds with different concentrations (0.5-15 mM) of proline improved the seedling growth of both the wheat cultivars grown under stress. In comparison to stressed seedlings, pre-treatment with 15 mM of proline increased root and shoot lengths by 25 and 39%, respectively in PBW 644 and it was further correlated with the increase in fresh and dry biomass of growing tissues (Tables 1 and 2). Although, proline treatment also had growth stimulatory effects in sensitive cultivar but such effects were comparably less as compared to PBW 644. Out of different levels of proline used for pre-sowing treatment of wheat seeds, 15 mM proline was found to be most effective in promoting the growth of wheat plants grown under water deficit stress (Tables 1 and 2). Therefore, proline pre-treatment (0.5-15 mM) counteracted the adverse effects of low water availability on the growth of wheat cultivars and significantly improved

Table 1 — Effect of pre-treatment with varying concentrations of proline on seedling growth of PBW 644 grown under water deficit stress at 8th DAG

Treatment	Roots			Shoots			Endosperms	
	Length (cm)	FW (mg)	DW (mg)	Length (cm)	FW (mg)	DW (mg)	FW (mg)	DW (mg)
Control	14.6±1.5	74.0±4.4	7.6±0.8	18.7±1.7	136.0±16.6	9.0±0.7	25.7±3.0	5.8±0.2
Water stress	12.0±0.9[a]	58.2±5.9[a]	5.8±0.6	12.5±1.2[a]	84.7±4.5[a]	7.6±0.8	33.4±2.7	7.7±0.6
Hydroprimed+stress	12.5±0.5[a]	60.0±2.2[a]	5.6±0.4	14.2±1.7[a]	89.3±15.6[a]	7.9±0.9	30.2±6.1	5.8±0.6
0.5 mM+ stress	12.3±1.2[a]	73.9±2.1[b]	8.4±2.0[b]	15.6±0.5[ab]	108.1±10.4[a]	11.8±2.1[b]	35.6±2.0	6.4±1.1
1 mM+ stress	13.5±0.9	74.7±9.2[b]	8.0±0.6	16.6±0.1[ab]	124.7±8.9[b]	12.1±1.6[b]	31.5±3.3	7.0±0.7
2 mM+ stress	14.6±2.1[b]	75.5±1.5[b]	9.2±1.0[b]	18.7±0.9[b]	151.2±26.5[b]	14.5±4.2[ab]	33.0±2.7	5.8±0.8
5 mM+ stress	13.4±1.3	72.0±8.0[b]	8.3±0.9	15.8±0.7[ab]	108.0±1.0[a]	12.7±4.0[b]	25.6±1.6	5.6±0.4
10 mM+ stress	15.5±0.1[b]	73.5±8.3[b]	8.8±0.6[b]	14.5±1.6[ab]	137.0±14.3[b]	10.7±1.5	34.2±1.2	5.5±0.8
15 mM+ stress	16.0±0.9[ab]	72.8±0.2[b]	10.5±1.2[ab]	20.6±1.4[ab]	192.4±21.6[ab]	11.7±0.3[b]	27.5±1.8	5.7±0.5
20 mM+ stress	12.0±1.0[a]	43.4±5.0[ab]	7.3±0.3	12.6±0.1[a]	69.0±6.4[a]	9.9±0.3	13.6±1.2[ab]	6.4±0.7
LSD (5%)	1.8	12.7	2.6	1.8	26.1	4.1	15.6	NS

[Values are Mean±S.D. of 18 seedlings. Least significant difference (LSD) at 5% probability level. a, significant differences with respect to control; and b, significant differences with respect to stress. DAG, Day after germination]

Table 2 — Effect of pre-treatment with varying concentrations of proline on seedling growth of PBW 621 grown under water deficit stress at 8th DAG

Treatments	Roots			Shoots			Endosperms	
	Length (cm)	FW (mg)	DW (mg)	Length (cm)	FW (mg)	DW (mg)	FW (mg)	DW (mg)
Control	15.3±0.5	58.2±10.5	5.5±0.9	15.7±0.4	114.0±10.3	9.0±0.5	20.5±1.0	5.5±0.2
Water stress	11.5±1.2[a]	44.8±9.4[a]	6.9±0.8	9.9±1.3[a]	81.7±9.3[a]	8.9±0.5	26.2±0.8	12.1±0.3
Hydroprimed+stress	13.3±1.7[a]	50.2±4.0	10.6±1.3	12.3±1.2[ab]	96.1±10.0	6.1±0.2	27.4±1.5	8.3±0.9
0.5 mM+ stress	12.8±1.0[a]	34.0±3.0[ab]	6.8±0.5	13.4±0.3[ab]	82.4±7.7[a]	5.7±0.5	25.3±2.2	6.8±0.51
1 mM+ stress	11.3±1.1[a]	38.7±2.8[a]	6.5±1.1	11±1.2[a]	64.7±6.4[a]	8.6±0.3	31.9±4.4	11.1±0.7
2 mM+ stress	13.6±0.3	37.0±2.8[a]	7.4±0.8	12.2±0.9[ab]	63.0±6.0[a]	11.1±1.5	27.4±2.7	6.2±0.3
5 mM+ stress	13.2±1.2[a]	40.0±6.0[a]	7.7±0.1	12.0±0.4[a]	73.3±4.7[a]	5.9±0.5	19.8±2.0	10.6±1.0
10 mM+ stress	12.4±1.5[a]	38.7±2.9[a]	5.3±0.5	11.8±1.5[a]	60.9±2.8[a]	8.9±0.4	19.3±2.6	5.7±0.2
15 mM+ stress	14.4±1.4[b]	44.5±4.6[a]	7.9±0.5	13.3±0.4[ab]	88.4±2.7[a]	7.9 ± 0.7	20.6±1.8	5.7±0.8
20 mM+ stress	12.9±0.7[a]	34.3±2.6[a]	5.2±0.2	11.8±1.4[a]	70.6±0.9[a]	7.0±1.1	19.9±1.1	5.2±0.7
LSD (5%)	1.9	8.3	NS	2.2	23.5	NS	NS	NS

[Values are Mean±S.D. of 18 seedlings. Least significant difference (LSD) at 5% probability level. a, significant differences with respect to control; and b, significant differences with respect to stress. DAG, Day after germination] morphological characters. However, application of 20 mM proline led to significant decrease in the lengths and fresh biomass of roots and shoots in both the wheat cultivars.

Influence of proline pre-treatment on ROS scavenging enzymes and ascorbate content

In comparison to well watered plants, SOD activity reduced under drought stress in both the cultivars. Hydroprimed seedlings showed an increase in SOD activity in the roots of both the cultivars whereas it remained unaffected in shoots (Fig. 1A). However, proline treatment upregulated the SOD acitivity in the roots of PBW 644 seedlings by more than 45%; whereas in PBW 621, only slight increase was observed. Under water deficit conditions, CAT activity decreased in shoots but increased in the roots of both cultivars (Fig. 1B). In Comparison to the stressed plants, hydroprimed seedlings showed an increase in CAT activity of sensitive cultivar whereas a decrease was observed in PBW 644. Under stressed conditions, proline treatment had no effect on CAT activity in PBW 644 whereas in PBW 621 it increased CAT activity by more than 19%. In comparison to control, POX activity showed a variable response under water deficit conditions. Hydroprimed seedlings showed an upregulation of POX activity in both the cultivars under stress (Fig. 1C). Proline treatment led to an increase in POX activity of roots of PBW 644 under water stress whereas in PBW 621 POX activity was upregulated only in the shoots.

Imposition of water deficit stress led to upregulation of APX activity in PBW 644 seedlings whereas in the roots and shoots of sensitive cultivar it decreased by 1.2 and 2 folds, respectively (Fig. 2A). Hydroprimed stressed seedlings of PBW 621 showed an increase in APX activity. Under water deficit stress, proline treatment led to considerable increase in APX activity of both the cultivars but increase was more in sensitive cultivar. In PBW 621, it increased by more than 2 folds in the growing tissues whereas in the roots and shoots of PBW 644 it increased by 1.7 and 1.3 folds, respectively. Glutathione reductase activity was reduced significantly in both the cultivars except the shoots of PBW 621 where an increase was observed. Hydroprimed stressed seedlings showed an increase in GR activity in both the cultivars except the shoots of PBW 621 where a decrease was reported (Fig. 2B). On the other hand when compared to stressed plants, proline treatment led to an increase in GR activity of PBW 644 seedlings grown under stress whereas it remained unaffected in PBW 621. Imposition of water deficit stress led to an increase in ascorbate content in the shoots of tolerant cultivar whereas a decline was observed in the roots as well as shoots of PBW 621. However, the proline treated

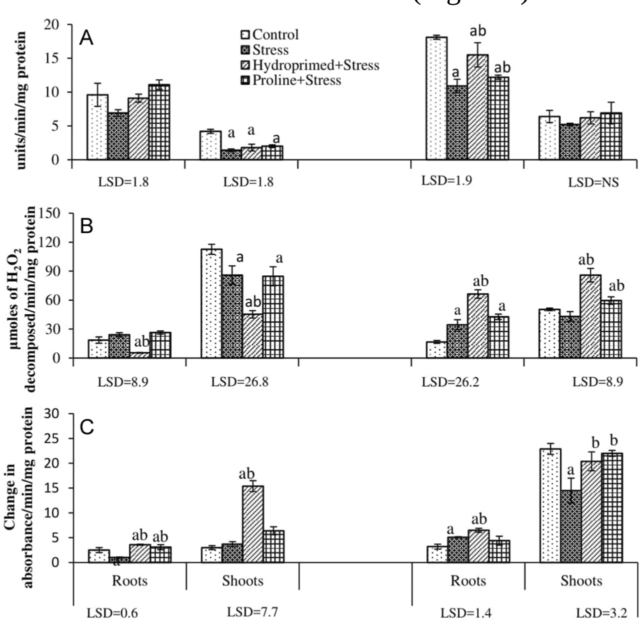

Fig. 1 — Effect of proline pre-treatment on (A) SOD, (B) CAT; and (C) POX activities of wheat seedlings grown under stress. [Values are Mean±SD of three triplicates. LSD ($P \leq 0.05$) Least significant difference at 5% probability level. a, significant differences with respect to control; and b, significant differences with respect to stress]

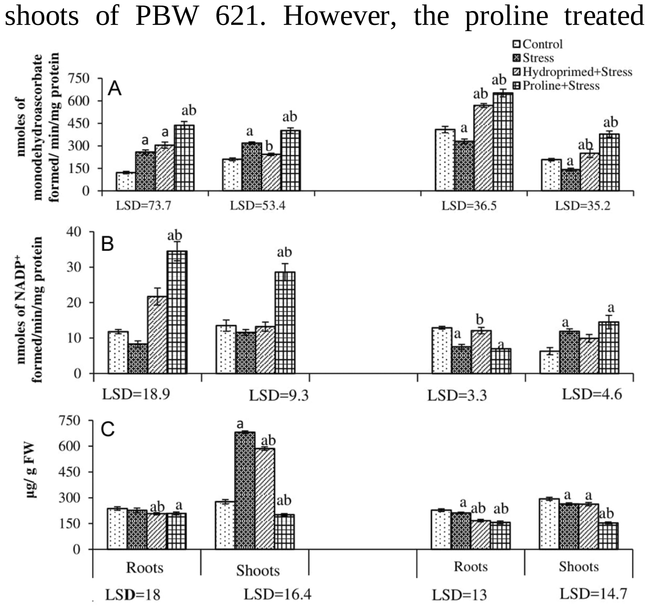

Fig. 2 — Effect of proline pre-treatment on (A) APX; and (B) GR activities and on ascorbic acid content of wheat seedlings grown under stress. [Values are Mean±SD of three triplicates. LSD ($P \leq 0.05$) Least significant difference at 5% probability level. a, significant differences with respect to control; and b, significant differences with respect to stress]

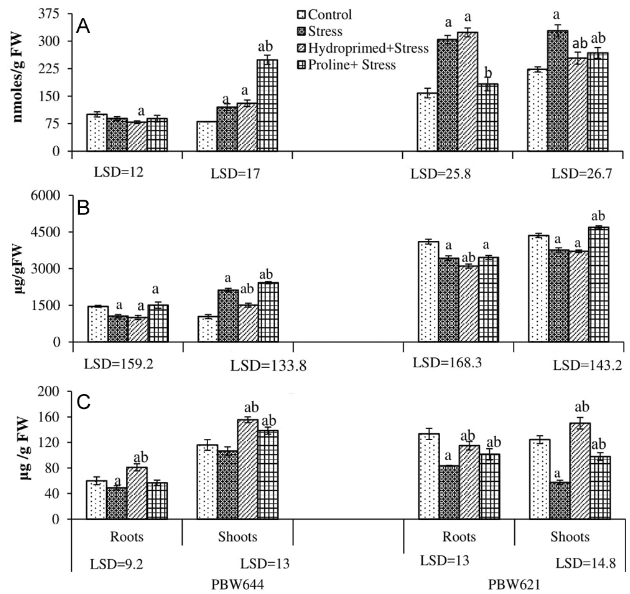

Fig. 3 — Effect of proline pre-treatment on (A) proline, (B) glycine betaine; and (C) total phenolic contents of wheat seedlings grown under stress. [Values are Mean±SD of three triplicates. LSD ($P \leq 0.05$) Least significant difference at 5% probability level. a, significant differences with respect to control; and b, significant differences with respect to stress]

stressed seedlings showed a decrease in ascorbate levels of both the cultivars (Fig. 2C).

Proline induced changes in osmolyte and total phenolic contents

Under water deficit stress, proline content increased by 1.4 folds in the roots and shoots of sensitive cultivar whereas in the tolerant cultivar it increased only in shoots (Fig. 3A). Proline content was not affected in the hydroprimed seedlings of tolerant cultivar whereas it decreased in the shoots of sensitive cultivar. In comparison to water deficit stress, proline treated seedlings of sensitive cultivar showed reduced levels of proline content whereas it increased in the shoots of tolerant cultivar. Under stressed conditions, glycine betaine content was reduced in both the cultivars except the shoots of tolerant cultivar. Under water deficit stress the roots of hydroprimed seedlings of PBW 621 showed reduced GB content whereas in PBW 644 it reduced only in the shoots. Proline treatment increased the GB levels in both the cultivars (Fig. 3B). It was increased by more than 8% in the growing tissues of tolerant cultivar whereas it was increased by 20% in the shoots of sensitive cultivar. In general, the levels of total phenols were reduced in both the cultivars grown under stress whereas hydroprimed and proline treated stressed seedlings showed an increase in the total phenolic content as compared to the stressed seedlings. Proline treated plants showed an increase in phenolic content by more than 21% in PBW 621 and 17% in PBW 644 (Fig. 3C).

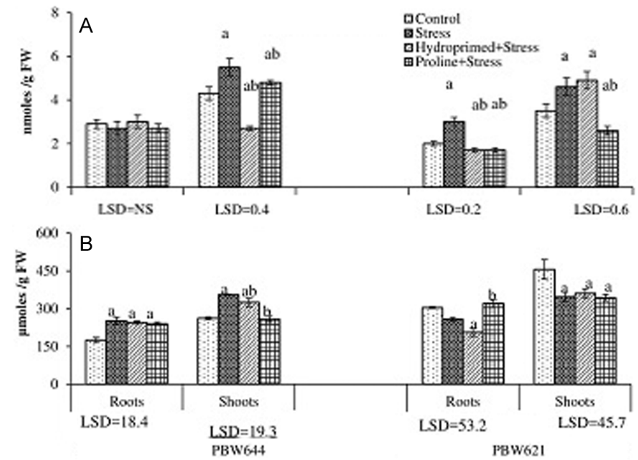

Fig. 4 — Effect of proline pre-treatment on (A) MDA; and (B) and H_2O_2 contents of wheat seedlings grown under stress. [Values are Mean±SD of three triplicates. LSD ($P \leq 0.05$) Least significant difference at 5% probability level. a, significant differences with respect to control; and b, significant differences with respect to stress]

Influence of proline pre-treatment on lipid peroxidation and H_2O_2 content

Drought stress led to an increase in the MDA levels of both wheat cultivars with the exception of the roots of tolerant cultivar where it was comparable to well watered plants (Fig. 4A). Hydroprimed stressed seedlings showed decreased MDA content in the shoots of PBW 644 and roots of PBW 621. Pre-treatment of seeds with proline further decreased MDA content in the growing tissues of sensitive cultivar whereas in tolerant cultivar it decreased only in the shoots. In general, the H_2O_2 content increased under stress in the tolerant cultivar whereas in the sensitive cultivar a decrease was observed. Hydropriming had no significant effects on H_2O_2 content in both the cultivars (Fig. 4B). The proline treated plants showed a decrease in H_2O_2 content in the shoots of tolerant cultivar whereas in the sensitive cultivar an increase was observed in the roots.

Discussion

In the present study, drought stress caused a significant reduction in the seedling growth of both wheat cultivars. Similar results were observed in pearl millet[27]. The decreased fresh weight (FW) of growing tissues under stressed conditions might be the reason for suppression of cell expansion and cell growth due to the low turgor pressure. Under stress, the increase in fresh and dry biomass of endosperm might be due to lesser mobilization of nutrients to growing tissues. In comparison to water deficit stress, pre-treatment with 15 mM proline enhanced the root and shoot lengths in both the cultivars. These results are in hormony with Ali *et al.*,[28] who reported that exogenous application of proline improved growth of maize plants under water stress. This increase in root and shoot lengths was further correlated with the increase in fresh and dry biomass of these tissues. The decreased endosperm weight in proline treated stressed seedlings of PBW 644 indicate enhanced biomass partitioning. The increase in plant biomass due to proline treatment may be attributed to an active role of proline in osmotic adjustment, which, in turn, enhanced water uptake and improved the plant growth. In various plant species grown under stress, proline induced positive effects on plant growth might be due to its role as a nutrient, as well as its role as an osmoprotectant[29]. Dawood *et al.*[30] reported that application of proline significantly affected growth parameters in faba bean under water stress. However, application of 20 mM proline led to significant decrease in the lengths and biomass of roots and shoots. High concentrations of proline may be harmful to plants, including inhibitory effects on growth or deleterious effects on cellular metabolism.

The antioxidant system plays a major role in scavenging of ROS produced in the cell when the plants undergo stressed conditions. Superoxide dismutase is the first line of defense that removes $O^{\cdot}_2{}^{-}$ by catalyzing its dismutation i.e. one $O^{\cdot}_2{}^{-}$ being reduced to H_2O_2 and another oxidized to O_2. In our results, water stressed plants of both the cultivars showed reduced SOD activity as compared to control plants. However, stressed seedlings of both cultivars treated with 15 mM of proline showed an increase in SOD activity as compared to non-treated plants grown under stress. Earlier, Anjum *et al.*,[31] reported an increase in SOD activity of maize plants grown under drought stress by exogenous use of glycine betaine. Proline induced SOD activity could scavenge more O_2^- and decrease the degree of lipid peroxidation. The dismutation of O_2^- leads to excess H_2O_2 production which is kept in control by the activities of CAT, POX and enzymes of ascorbate-glutathione cycle.

The metabolism of H_2O_2 is dependent on two functionally interrelated antioxidants *viz.* CAT and POX. Under water deficit stress, CAT activity increased in the roots of both cultivars but reduced in the shoots. Peroxidase activity reduced in the roots of PBW 644 and shoots of PBW 621 seedlings. In contrast Nojavan & Khorshidi[32] reported increased CAT and POX activities in maize plants which could limit cellular damage by eliminating H_2O_2 from stressed cells. However, Singh *et al.*[33] also reported differential response of POX activity in sensitive and tolerant varieties of wheat in response to salt stress. Among proline treated stressed plants, CAT activity was upregulated in PBW 621 cultivar, whereas POX increased in both the cultivars. These results are further in agreement with those of Demiral & Turkan[34], who observed that exogenous osmolytes help in enhancing antioxidant response by upregulating CAT activity in rice plants under salt stress. The upregulation of SOD and POX activities in proline treated stressed seedlings of PBW 644, indicates a more efficient quenching of ROS.

Ascorbate peroxidase is the first enzyme of ascorbate-glutathione cycle which maintains the ascorbate pool in its reduced form. Water deficit stress led to an increase in APX activity of PBW 644 seedlings whereas in PBW 621 it declined significantly. Under water deficit stress, upregulation of APX activity was also reported earlier in the roots and leaves of drought tolerant wheat cultivar[35]. Proline pre-treatment upregulated APX activity in both the cultivars grown under stress but the increase was more in sensitive cultivar. Earlier, Kaur *et al.*[15] also reported that exogenous phenolic acids enhanced salt tolerance in wheat plants by upregulating APX enzyme. Similar observation was recorded in perennial rye grass on exogenous application of glycine betaine[36]. Gharache *et al.*[37] mentioned that increased SOD and APX activities promote the removal of reactive oxygen species (ROS), thus conferring higher resistance to plants.

Ascorbic acid is a naturally occurring antioxidant in plants and acts as an electron donor for APX catalysed detoxification of H_2O_2. During drought stress, the levels of ascorbic acid were lowered down in the sensitive cultivar whereas stressed shoots of tolerant cultivar showed higher ascorbate content. Even in proline treated stressed seedlings, the levels of ascorbic acid were lowered in both the cultivars. Similar results were reported by Hoque *et al.*[10] in tobacco bright yellow-2 suspension cells under salt stress and proline stressed seedlings. This decrease of ascorbate content in differently treated stressed seedlings might be attributed to the increase in the

APX activity which utilizes ascorbate as a substrate in reducing H_2O_2 to H_2O as explained by Anjum *et al.*[38]. It was also reported earlier that ascorbate synthesis under stress is lower than ascorbate catabolism[10].

Glutathione reductase catalyses the rate limiting step of ascorbate-glutathione cycle and its high activity maintains glutathione in reduced state. Water deficit stress reduced GR activity in both cultivars with the exception of shoots of PBW 621 where an increase was observed. Increased GR activity had been reported earlier in drought stressed wheat seedlings[35]. In general, proline treated stressed seedlings showed an increase in GR activity except the roots of PBW 621 where it remained unaffected. Our results are in accordance with the findings of Patade *et al.*[39] which stated that exogenous proline and glycine betaine upregulated GR activity in salt stressed sugarcane plants. Therefore, proline treatment was more effective in enhancing APX activity in sensitive cultivar whereas GR activity was upregulated to greater extent in the tolerant cultivar. Proline induced GR activity not only ensures the availability of $NADP^+$ to accept electrons from the electron transport chain leading to reduced formation of O_2^- but also maintains a high ratio of GSH/GSSG which is required for the activation of chloroplastic CO_2 fixing enzymes.

Under stress conditions such as drought and salinity plant cells accumulate osmolytes in order to reduce the osmotic potential which increases the water absorption capacity, maintains turgor pressure and protects the cell growth[6,]. Water deficit stress increased endogenous proline content of wheat seedlings as reported earlier[40]. In comparison to drought, proline treated plants showed significant decrease in proline content of PBW 621 whereas in PBW 644 an increase was observed in shoots. Increased proline content in stressed plants of tolerant cultivar may be an adaptation to compensate for the energy for growth and survival and thereby help the plant to tolerate stress, as reported in spinach leaves[41]. Therefore, proline treatment seems to have a protective role in the tolerant cultivar against drought stress which is verified by significant increase in the lengths and biomass of its shoots. Therefore, increased proline content in the treated shoots of PBW 644 seedlings grown under stress might be one another character responsible for its tolerance behaviour. Under water deficit conditions, glycine betaine content reduced in both the cultivars except the shoots of PBW 644. In comparison to stressed plants, proline treated stressed plants exhibited an increase in glycine betaine content in both the cultivars. Earlier, Raza *et al.*[42] observed that exogenous glycine betaine improved salt tolerance in wheat by enhancing endogenous glycine betaine levels.

Higher plants manifest a unique capability to synthesise non-enzymatic secondary metabolites, such as phenolics, which have a key role in scavenging ROS. Under water deficit conditions, the total phenolic content decreased in the growing tissues of both the cultivars. The decrease in total phenolic contents of wheat seedlings under drought stress was also reported by Ali *et al.*[43]. Proline treated plants further showed an increase in phenolic content by more than 21% in PBW 621 whereas in PBW 644 it increased by more than 17%. Phenolic compounds may protect cells from potential oxidative damage and increase the stability of cell membranes. Ali & Ashraf[44] revealed that foliar-applied compatible solutes such as glycinebetaine could enhance the levels of phenolic compound in maize under water deficit conditions.

Malondialdehyde is an indicator of membrane damage and is produced during peroxidation of membrane lipids. Under water stress, the levels of MDA increased in wheat seedlings except the roots of tolerant cultivar where it was comparable to control. The increased MDA content under stressed condition is a biomarker of oxidative damage to membrane lipids as reported earlier[45]. Proline treatment reduced the MDA content in growing tissues of PBW 621 and in the shoots of PBW 644 grown under stress which may result in enhanced membrane stability as observed by Bhardwaj *et al.*[17]. Proline-mediated reduction of MDA content revealed an efficient free radical scavenging system and provided an evidence that proline-treatment substantially ameliorate the detrimental effects of drought on integrity and stability of membranes in the wheat plants.

Hydrogen peroxide is the most stable reactive oxygen species. It is the only ROS which can cross the cell membranes and initiate oxidative damage at a site far from the site of its production. In general, H_2O_2 content declined under stress in PBW 621 seedlings whereas it increased in the tolerant cultivar. However, proline treatment reduced H_2O_2 content in PBW 644 which might be due to increase in the activities of CAT and POX and key enzymes of ascorbate glutathione cycle *viz*. APX and GR. Earlier, Bhardwaj *et al.*[17] also observed that pre-treatment with different phenolic acids resulted in a decrease of H_2O_2 content. Hydrogen peroxide content increased in roots of PBW 621 which may be due to reduced activity of POX but in shoots it remained unaffected. Thus even in the shoots of sensitive cultivar, H_2O_2 content was kept under control. This reduction in H_2O_2 and MDA contents could be attributed to the putative role of osmolytes in alleviating the deleterious effects of stress on the structure of cell membranes and activities of different enzymes as well as in reducing the generation of highly destructive free radicals.

Conclusion

There is a strong correlation between stress tolerance and accumulation of proline, thus pre-treatment of seeds with this molecule may play an important role in enhancing the stress tolerance of wheat plants. In comparison to water deficit stress, proline treatment enhanced the seedling growth of both wheat cultivars. In PBW 644, it resulted in the upregulation of SOD, POX, APX and GR enzymes alongwith an increase in glycine betaine, proline and total phenolic contents which was accompanied by decrease in H_2O_2 and MDA content. However, in PBW 621, proline treatment upregulated CAT and APX enzymes along with an increase in glycine betaine content which was followed by a significant decrease in MDA content. The levels of ascorbic acid under water deficit stress were lower in the treated seedlings of both wheat cultivars which were ascribed to the enhanced APX activity. Therefore, it seems that under water deficit stress the protective effects of proline treatment are related to the upregulation of ROS scavenging enzymes.

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Conflict of Interest

Authors report no potential conflict of interest.

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