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Full Length Research Paper

Pretreatment of seed with H_2O_2 enhances drought tolerance of wheat (*Triticum aestivum* L.) seedlings

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Drought is an important environmental constraint limiting the productivity of many crops worldwide. Seedling tolerance to drought is crucial for crop growth and development through the whole season under water-limited condition. Experiments were conducted to investigate the effects of seed pretreatment by hydrogen peroxide (H_2O_2) on drought tolerance in wheat (*Triticum aestivum* L.) seedlings. H_2O_2-pretreated seeds exhibited 56% higher in germination rate than the water-pretreated seeds (control) under PGE-induced drought condition. Level of H_2O_2 in seedlings arising from H_2O_2-treated seeds grown under drought stress was markedly lower than the controls, indicating the operation of antioxidant system in them. These seedlings exhibited increased growth characteristics including higher net photosynthetic rate, leaf area and dry weight. Moreover, H_2O_2 treatment improved water use efficiency (WUE) and proline level. H_2O_2 pretreatment enhanced the membrane stability, as revealed from greatly reduced membrane damage rate (MDA) and malondialdehyde (MDA) content. The seedlings showed the higher expression of antioxidative enzyme such as catalase (CAT) and ascorbate peroxidase (APX). The present data suggest that H_2O_2, a stress signal, could trigger the activation of antioxidants in seeds, which persists in the seedlings to alleviate the oxidative damage, leading to improvements in physiological attributes for the seedling growth under drought.

Key words: wheat (*Triticum aestivum* L.), seed pretreatment, H_2O_2, signaling, drought tolerance, membrane permeability, antioxidative system.

INTRODUCTION

Water shortage is increasingly becoming a major constraint for crop productivity worldwide (Wollenweber et al., 2003). As one of the most important crops in the world, wheat (*Triticum aestivum* L.) has a significant role in world food security. Water stress/soil drought usually occurs during the whole growth season of wheat, leading to a big loss of both seed yield and quality, particularly in arid and semi-arid regions. Therefore, it is very needed to increase wheat drought tolerance by genetic improvements and cultivation techniques for wheat production.

Among various technique strategies, pre-sowing treatment and priming of plant seeds are easy, low cost, low risk and effective approaches to enhance plant tolerance to the stressful environments (Wahid and Shabbir, 2005; Ashraf and Foolad, 2005). Priming is a controlled hydration process followed by redrying that allows metabolic activities to proceed before radical protrusion (Sivritepe et al., 2003, 2005). A number of priming strategies include seed treatments with osmotica, inorganic salts, hormones or water, respectively. These seed pretreatments are reported to induce pre-germination changes, which usually have beneficial effects on seed germination rate and uniformity, seedling growth and development, specifically under stressful conditions (Parera and Cantliffe, 1991; Ashraf and Foolad, 2005).

Hydrogen peroxide (H_2O_2) is one of the main chemicals which are induced to elevate in plants by biotic and abiotic stresses. Higher levels of H_2O_2 usually result in toxicity to cellular membrane system and damages to plant cells (Sairam et al., 2002; Sairam and Tyagi, 2004; Kathiresan et al., 2006). However, the increased data evidence the biological activity of H_2O_2 as a stress signal molecule in plants (Dat et al., 2000; Overmyer et al.,

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2003; Hung et al., 2005). H_2O_2 can serve as a second messenger in signal transduction pathways, leading to stress acclimation. Available information suggest that H_2O_2 directly regulates the expression of numerous genes involved in plant defense and the related pathways such as antioxidant enzymes, defense proteins and transcription factors (Kovtun et al., 2000; Robert and David, 2004; Hung et al., 2005). Hence, H_2O_2 signaling functions importantly in plant growth and development and defense against environmental stresses.

Exogenous application of H_2O_2 increases chilling tolerance by enhancing the glutathione level of mung bean seedlings (Murphy et al., 2002). Likely, addition of H_2O_2 to the nutrient solution induces salt tolerance by enhanced activities of antioxidants and reduced peroxidation of membrane lipids in leaves and roots of maize as an acclimation response (Azevedo Neto et al., 2005). To date, there is limited information on the use of H_2O_2, a stress signaling molecule for crop seed treatments. Role and mechanism(s) of H_2O_2 as seed pretreatment in inducing plant drought tolerance are still unclear. Here we investigate the effects of seed pretreatment with H_2O_2 on drought tolerance in winter wheat seedlings. We also examine the physiological and biochemical changes in the seedlings arising from H_2O_2-treated seeds. The present data provide novel insights into understanding the mechanism of H_2O_2 regulating plant stress responses and benefit for developing new effective ways to increase wheat drought tolerance.

MATERIALS AND METHODS

Seed treatment with H_2O_2, PEG-induced drought stress and growth conditions

Drought-tolerant wheat variety Jin 47 and drought-susceptible variety Shun 1718 were used in this experiment. Healthy seeds were surface sterilized with 75% ethanol for 2 min followed by repeated washings with ddH_2O. 200 clean-healthy seeds per replicate were transferred to H_2O_2 solution at different concentrations (20, 40, 60, 80, 100, 120 and 140 mM). After 6 h soaking, the seeds were washed with ddH_2O and then blot dried. The soaked seeds with ddH_2O were used as the control.

The H_2O_2–treated and water-soaked seeds were sown in pots containing silicon sands. The sand-filled pots irrigated with 1/4 strength MS nutrient solution were used as well-watered (WW) conditions. Drought stress (DS) was achieved by adding PEG-6000 (-0.5 MPa) solution. Pots were kept in a controlled-growth chamber set at 25/20°C day/night temperature, a 12 h photoperiod, 200 µmol $m^{-2}s^{-1}$ light intensity at leaf surface and 50 - 55% relative humidity (day/night). The experiments conducted three times in complete randomization replicated design.

Measurements of growth characteristics of wheat seedlings

Number of seedlings emerged was recorded daily and subsequently, germination rate and the time taken to germination were calculated. After complete expansion of the third leaf, leaves and shoots of the wheat seedlings (25 plants per treatment) were harvested for assay of the growth parameters.

Fresh weight of the above-ground parts was measured and after that plants were oven-dried over a period of 24 h at 90°C to a constant weight for the determination of dry weight. Leaf area (cm^2) was examined according to Tsonev and Sergiev (1993) using a planar scanner and image plot software. Water use efficiency (WUE) was calculated with the formula: WUE (%) = net photosynthetic rate (Pn)/transpiration rate (Tr) × 100. Pn, Tr and stomatal conductance (µmol $m^{-2}s^{-1}$) were measured by the Li-6400 photosynthesis system (Li-Cor Inc.) according to the manufacturer's instructions.

Biochemical analysis of the wheat seedlings H_2O_2

The fresh plant materials were immediately extracted and used for biochemical assays. Leaf membrane damage rate (MDR) was determined using Conductivity meter (DDS-1, YSI) following the method of Sairam (1994). MDR (%) = initial electrical conductivity / boiled electrical conductivity after boiled × 100. Lipid peroxidation was measured by the amount of malondialdehyde (MDA) whose concentration was estimated by the method of Kramer et al. (1991). Leaves were cut and placed in a beaker containing distilled water and after 3 h at room temperature the conductivity of the solution was measured. Free proline was extracted, derivatized with acid ninhydrin and absorbance read according to Bates et al. (1973) method. Hydrogen peroxide was measured spectrophotometrically at 390 nm after reaction with KI. The reaction mixture consisted 0.5 mL0.1% trichloroacetic acid (TCA) leaf extract supernatant, 0.5 mL of 100 mM K-phosphate buffer and 2 mL reagent [1 M KI (w/v) in fresh double-distilled water]. The amount of hydrogen peroxide was calculated using a standard curve prepared with known concentrations of H_2O_2.

Assays were carried out using the crude extract of the leaves as the enzyme source. Leaves were homogenized at 4°C in 100 mM K-phosphate buffer (pH 7.8), 10 mM $MgCl_2$, 0.2 mM EDTA. The homogenate was centrifuged at 17000 g for 30 min to yield a crude enzyme extract. Catalase (CAT) (EC 1.11.1.6) activity was evaluated by decomposition of H_2O_2 at 240 nm (Knorzer et al., 1996). The reaction mixture contained 25 mM potassium phosphate buffer (pH 7.0), 10 mM H_2O_2 and prepared enzyme extract in a final volume of 3 ml. Ascorbate peroxidase (APX) (EC 1.11.1.11) activity was determined by estimating the decreasing rate of ascorbate oxidation at 290 nm, according to Nakano and Asada (1981). The reaction mixture consisted of 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM ascorbate, 0.1 mM H_2O_2, 0.5 mM EDTA and extract in a final volume of 1 ml. Protein content was determined by dyebinding technique (Bradford 1976) using bovine serum albumin as a protein standard.

Data were statistically analyzed to determine the significance of variance with one-way ANOVA using SPSS 11.0 for Windows. Duncan's multiple range test was applied to find meaningful differences among treatments. In the results presented asterisks are used to identify the levels of significance: $^*P < 0.05$ and $^{**}P < 0.01$.

RESULTS

H_2O_2 pretreatment increases seed germination and seedling growth under water-stress conditions

In order to detect the optimized concentration of H_2O_2 for enhancing wheat seedling growth, the seeds were treated with different levels of H_2O_2 and subsequently, the characteristics of seedling growth were recorded. Figure 1 shows that 80 mM H_2O_2 treatment is the best in

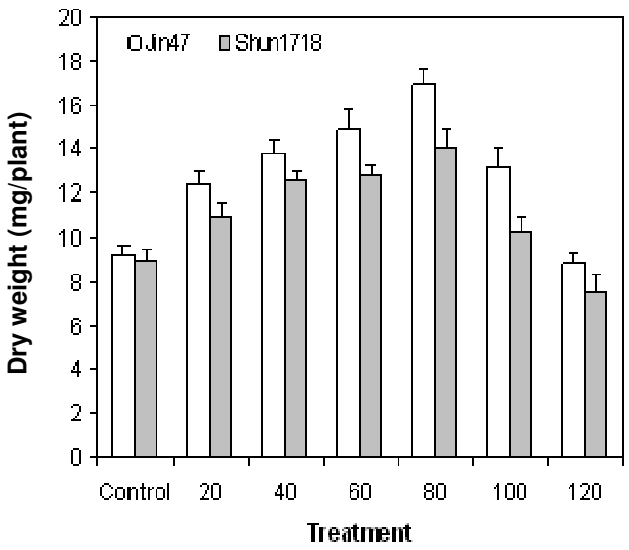

Figure 1. Effects of different levels of H_2O_2 on the shoot dry weight of the wheat seedlings under drought stress. Data bar represents mean value of shoot dry weight per plant for Jin47 and Shun1718 varieties. Vertical bars are standard errors (SE) of means. Control represents the seedlings arising from water-soaked seeds (0 mM H_2O_2 treatment).

promoting wheat seedling growth under drought stress. At lower dosage range (< 80 mM), dry weight of the seedling was elevated with the increasing of H_2O_2 level. However, the dry weight was dropped when H_2O_2 level was over 80 mM. Therefore, 80 mM H_2O_2 was selected as an optimized concentration to treat wheat seeds in the following experiments.

Table 1 summarizes the growth parameters of H_2O_2–pretreated seeds under water stress. For both genotypes, H_2O_2–pretreated seeds demonstrated slightly better in all the growth parameters than water-soaked seed control under the normal well-watered (WS) conditions, but these differences are not statistically significant ($P < 0.05$) (data not shown). Under drought (D) stress, the water-soaked seeds (control) displayed considerable difference ($P < 0.05$) between the two genotypes in the most of the growth characteristics except for stomatal conductance and germination traits. H_2O_2-pretreated seeds exhibited higher germination rate in spite of 0.7 days of delay for germination compared to the control for both varieties. Seed treatment with H_2O_2 greatly increased all the growth characteristics of wheat seedlings under drought stress. The elevation of dry weight and WUE were higher in the drought-tolerant variety than in the drought-susceptible one while the increased scales of the other characteristics (fresh weight, leaf area and stomatal conductance) showed no much different between these two genotypes ($P < 0.05$).

The images of the seedlings on Figure 2 further demonstrates that H_2O_2 pretreatment apparently improves the subsequent growth performance of the wheat seedlings of both genotypes under drought stress. Compared to the control, H_2O_2 pretreated seedlings grew better under the stressful condition.

H_2O_2 pretreatment reduces the damages on cellular membrane caused by drought stress

As shown in Figure 3A, the endogenous H_2O_2 level of shoots was statistically lower in the control seedlings of the tolerant genotype than in the control of the susceptible one ($P < 0.05$) under drought stress. Seed pretreatment with H_2O_2 caused a substantial reduction of the H_2O_2 content and this effect was much stronger in the susceptible variety ($P < 0.05$). Likely, MDA and MDR (Figures 3B and 3C), two indexes of membrane damage, were little less in the tolerant control than in the susceptible control upon the stress ($P < 0.05$). The H_2O_2 treatment greatly decreased MDA and MDR for both varieties with pretty effective in this regard for the tolerant variety ($P < 0.05$). Data revealed that seed pretreatments with H_2O_2 reduce cellular membrane damages by the stress.

H_2O_2 pretreatment improves the expression of antioxidant system upon drought stress

Antioxidant systems including proline, CAT and APX play crucial roles in plant defense against stresses. Under drought condition, the obvious higher CAT (Figure 4C) and APX (Figure 4A) activities ($P < 0.05$) were observed in the wheat seedlings raised from the water-soaked control seeds of the tolerant variety, whilst free proline content (Figure 4B) showed just a small difference in the control seedlings for both genotypes. A significant increase was detected for the three molecules in the H_2O_2-treated wheat seedlings. Moreover, this promotion was much effective in the tolerant genotype. Clearly, H_2O_2 treatment improved the antioxidant system in the wheat seedlings.

DISCUSSION

Various environmental stresses such as drought can inhibit plant growth and development, leading to crop reduction. For food security in the world and agricultural production, more efforts are needed to develop multiple effective strategies to improve crop stress tolerance. In this study, we investigated the effects of seed treatment with H_2O_2 on wheat growth characteristics and physiological-biochemical response to drought stress.

H_2O_2 is a strong oxidizing agent accumulated upon various stress. High level of H_2O_2 injures plant cells and damages photosynthesis when produced internally or applied externally (Bowler and Fluhr, 2000; Sairam et al.,

Table 1. Growth characteristics of H_2O_2-pretreated seeds under drought stress.

Growth characteristics	Drought-tolerant variety		Drought-susceptible variety	
	Control	H_2O_2 treatment	Control	H_2O_2 treatment
Germination rate (%)	56.24 ± 1.23a	95.84 ± 1.76b	55.81 ± 1.19a	96.71 ± 1.89b
Time for germination (day)	4.71 ± 0.11a	5.44 ± 0.23b	4.92 ± 0.16a	5.53 ± 0.27b
Fresh weight (mg/plant)	52.76 ± 2.6a	121.46 ± 5.2c	48.38 ± 2.1b	117.39 ± 4.9c
Dry weight (mg/plant)	9.72 ± 0.7a	17.81± 1.3c	7.43 ± 0.6b	13.64 ± 1.1d
Leaf area (cm^2/plant)	6.77 ± 0.35a	8.81± 0.40c	5.06 ± 0.32b	7.29 ± 0.39c
WUE (%)	51.47 ± 1.45a	67.93 ± 1.89c	47.53 ± 1.32b	60.18 ± 1.76d
Stomatal conduction (μmol $m^{-2}s^{-1}$)	0.14 ± 0.03a	0.27± 0.07c	0.15 ± 0.05a	0.29 ± 0.06c

See "Materials and methods" for details about the treatments.
The data are mean ± SE (n = 25). Means denoted by same alphabet differ non-significantly (P > 0.5).

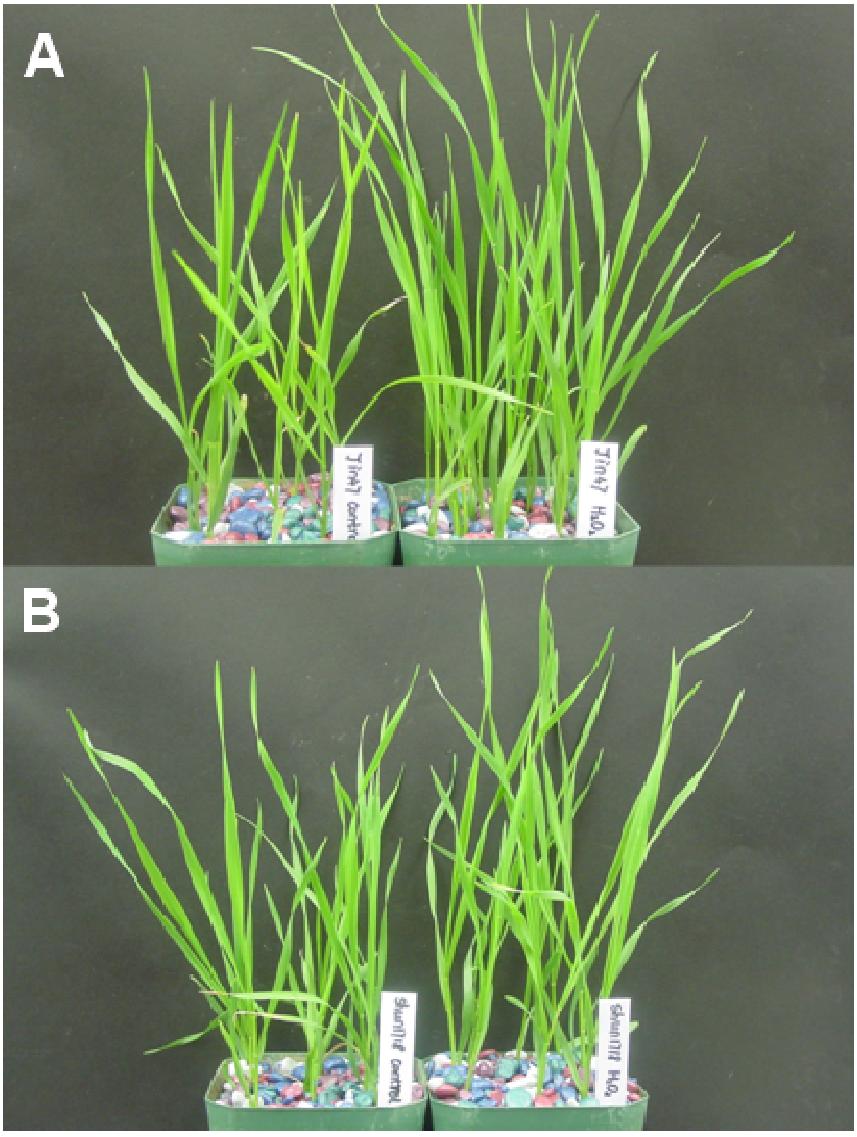

Figure 2. Wheat seedlings arising from H_2O_2-treated and control seeds under drought stress. (A) Wheat variety Jin47; (B) Wheat variety Shun1718. The left seedlings come from the control while the right ones come from the H_2O_2 treatment.

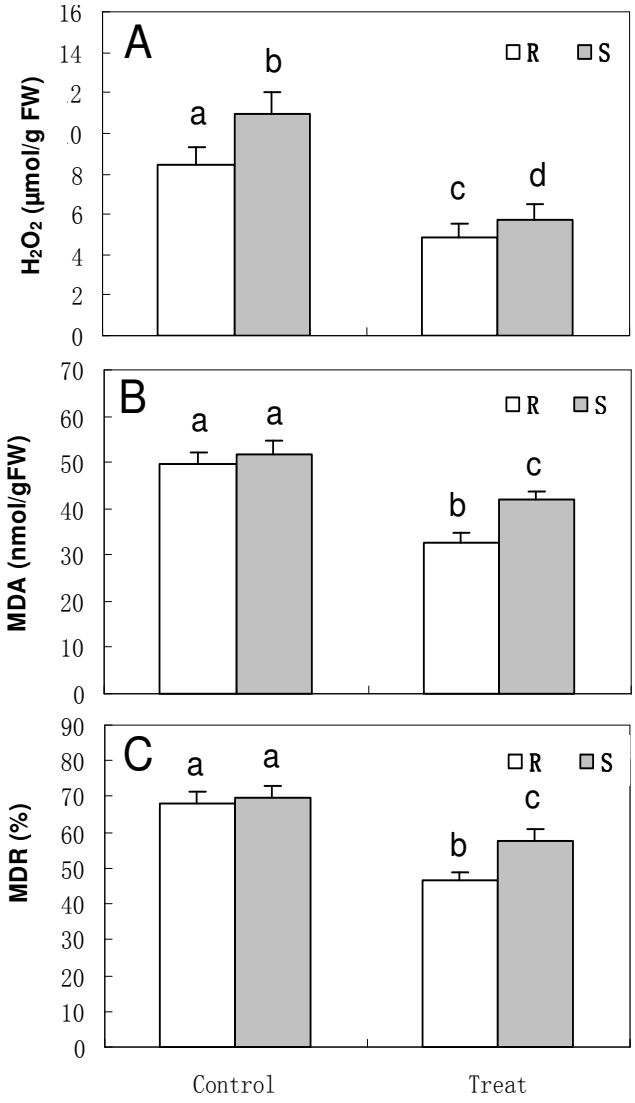

Figure 3. Effects of H_2O_2 pretreatment on endogenous H_2O_2 level and cellular membrane of wheat seedlings under drought stress. Control represents the seedlings arising from water-soaked seeds. Treat represents the seedlings generating from H_2O_2-treated seeds. Data bars (means) denoted by same alphabets differ non-significantly ($P > 0.05$). Vertical bars are standard errors (SE) of means.

2002; Alexieva et al., 2003), but also acts as signal molecules in plant response to stress in low concentrations (Gong et al., 2001; Aroca et al., 2003; Desikan et al., 2004). The present data further confirmed that H_2O_2 can effectively modulate the related gene expression and subsequently lead to the enhancement of plant tolerance to the stress (Brosch´e and Strid, 2003; 2002; Hung et al., 2005; Azevedo Neto et al., 2005).

It is vital for plant to adjust the enzymatic and non-enzymatic antioxidant system to control the level of reactive oxygen species (ROS) including H_2O_2 for avoiding oxidative stress (Allen, 1995; Kathiresan et al., 2006). Proline is such an antioxidant which accumulates in response to biotic and abiotic stresses, including water stress (Zhang et al., 1995), salt stress (Fedina et al., 2006), extreme temperatures (Ruiz et al., 2002) and heavy metal toxicity (Chen et al., 2001). Similarly, the activities of catalase (CAT) and Ascorbate peroxidase (APX), two H_2O_2-scavenging enzymes, are also increased in plant response to stresses (Yang et al., 2007). It is known that CAT reacts with H_2O_2 to produce water and oxygen. APX uses ascorbic acid as a substrate to detoxify H_2O_2. Our data (Figure 4) showed that seed pretreatment with H_2O_2 significantly enhanced free proline content and the activity of the two enzymes in wheat seedlings under drought stress, indicating that this seed pretreatment could induce the expression of plant antioxidant system upon stress condition.

The change in stability of biological membranes is a key indicator of cellular damage. Drought and other stresses always results in cellular membrane injures including the increase of membrane permeability and MDA content due to membrane lipid peroxidation (Agrawal and Rathore, 2007; Farooq and Azam, 2006). Compared to the control, the seedlings arising from the H_2O_2-treated seeds exhibited an obvious reduction of MDA and MDR under drought stress (Figures 3B and 3C). Moreover, cellular H_2O_2 level was also largely lower in the wheat seedlings than in the control plants (Figure 3A) although H_2O_2 level in the treated seeds was increased after the soaking of seeds in H_2O_2 for 5 - 6 h (data not shown). The initial absorption by seeds and later scavenging of H_2O_2 in the seedlings, together with decrease of MDA and MDR, provide evidence that seed treatment with H_2O_2 could greatly alleviate the deleterious effects of drought on the membrane integrity and stability in the wheat seedlings.

To address whether these effects of H_2O_2 treatment on cellule membrane and antioxidant system can lead to improvement of drought tolerance in the wheat seedlings, we further examined the seedling growth performance in term of germination, photosynthesis, fresh and dry weight (Table 1). The time taken to germination was approx 1 day more for H_2O_2-treated seeds, which might be partly because of higher H_2O_2 level in the treated seeds. However, germination rate was higher in the treated seeds than in the control, indicating that H_2O_2-mediated response did act at that stage. This is consistent with that ROS-activated accumulation of stress related genes transcripts is spread over 2 days of H_2O_2 application (Uchida et al., 2002). Furthermore, this positive effect of H_2O_2 treatment was much expressed in the consequent growth stage of the wheat seedlings, evidenced by a significant increase of the growth parameters measured here (Table 1). Higher WUE and stomatal conduction in the seedlings generated from H_2O_2-mediated seeds mean that H_2O_2 treatment enhanced net photosynthetic rate and reduced transpiration rate under the stress condition.

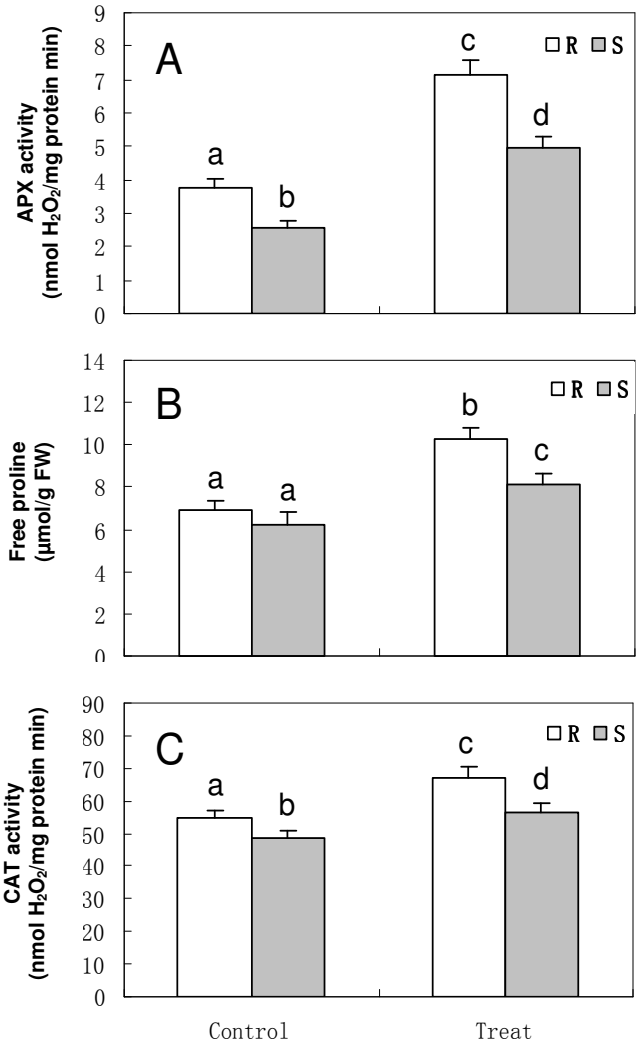

Figure 4. Effects of H_2O_2 pretreatment on the expression of antioxidant system in wheat seedlings upon drought stress. Control represents the seedlings arising from water-soaked seeds. Treat represents the seedlings generating from H_2O_2-treated seeds. Data bars (means) denoted by same alphabets differ non-significantly ($P > 0.05$). Vertical bars are standard errors (SE) of means.

Paralleling with the improved photosynthesis upon drought, the seedling growth and dry matter production largely increased. Notably, the tolerance improvement in the wheat seedlings by seed H_2O_2 treatment was much stronger in the tolerant genotype than in the susceptible variety (Figure 1 and 2).

In conclusion, seed pretreatment with H_2O_2 can induce the expression of antioxidant system and reduce the oxidative damage of cellular membranes in the wheat seed and seedlings upon drought through the H_2O_2 signaling pathway. And consequently, the treatment increases wheat tolerance against the stress by improving photosynthesis, WUE and growth in seedlings. Such responses are much profound in the tolerant genotype. The data are of considerable value in understanding the mechanisms of plant stress tolerances and in developing effective methods for crop protection against environmental stresses.

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