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EVALUATING CHANGES IN WHEAT GENOTYPES CAUSED BY HYDROGEN PEROXIDE DURING SEED TREATMENT AND THEIR INVOLVEMENT IN SALT TOLERANCE

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ABSTRACT

Two wheat genotypes (Khirman and Inqalab) were used to evaluate the physiological and biochemical changes caused by presoaking of seeds with different levels of hydrogen peroxide (0, 20, 40, 60, 80 and 100 µ*M*) against two salinity levels (0 and 100 m*M* NaCl). The data were recorded on membrane stability index, relative leaf water content, stomatal conductance, photosystem II efficiency and antioxidant assay. The results showed that the membrane stability index was significantly influenced by genotypes and salinity, while it was non-significantly influenced by H_2O_2 seed soaking concentrations. The relative water content was not affected by treatment, hence no significant effects of treatment or salinity were observed. The H_2O_2 seed treatment seems to protect the salt sensitive genotype Inqalab against yield reductions caused by salinity. The most important observations were protective effects of H_2O_2 treatment on salinity stress for stomatal conductivity. Salinity tends to decrease conductivity, while H_2O_2 treatment increased it. Salinity did not significantly affect Fv/Fm, but there was a significant effect of H_2O_2. The H_2O_2 treatment might strongly enhanced photosynthesis via its beneficial effect on Fv/Fm. The H_2O_2 signals the establishment of antioxidant activity in seeds, which persisted in the seedlings to balance the ion-induced oxidative damage. The data on antioxidant assay showed that treatment tends to reduce plant H_2O_2 levels, which was a significant effect. However, the data did not show any clear evidence that salt stress had caused increased levels of H_2O_2. On the whole, beneficial effect of H_2O_2 was observed on both wheat genotypes.

Keywords: membrane stability index, reactive oxygen species, salinity, wheat

INTRODUCTION

Wheat (*Triticum aestivum* L.) is cultivated throughout the world (Sara *et al.,* 2015). Its high adaptation and diverse consumptions make it the most important cereal in the world (Farzi and Bigloo, 2010). Wheat yield is reduced by abiotic

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stresses such as salinity, drought and heat in the world's semi-arid and arid areas (Farooq *et al.,* 2014). Out of these abiotic stresses, thousand hectares of cultivated land are affected by salinity the problem is increasing year after year (Koyro, 2006). The major land degradation issues in the world today are mostly associated with salinization of land and water resources; and some of these problems are human induced and some are the result of natural occurrence (Rengasamy, 2006; Fernández-Cirelli *et al.,* 2009).

Salinity is a harmful agent that affects negatively and causes important modifications in plant growth (Hala *et al*., 2015). One of the most important modifications is the production and accretion of reactive oxygen species (ROS). Key significance of abiotic stresses is the enhancement in the cell/ular levels of ROS, which shows toxicity to the metabolic functions after conversion (Sairam and Tyagi, 2004). Hydrogen peroxide is a ROS and a stress signal molecule which is primarily associated with activation of anti-oxidants and was evaluated to produce the metabolic changes as seed treatment (Wahid *et al.,* 2007). It plays a role in enhancement of scavenging the generated ROS under saline conditions; as it is not a free radical, it is potentially reactive oxygen. By comparison with superoxide ($O^{2\bullet-}$) and hydroxyl radical ($OH^{\bullet}$), H_2O_2 is moderately safe in the absence of transition metals (such as Fe), because it is stable and non-reactive even at high concentrations (Hala *et al.*, 2015). The H_2O_2 are responsible for various stress-induced damages to macro-molecules and ultimately to cellular structure (Mittler, 2000; Ashraf, 2007). The effect of hydrogen peroxide resulted in accelerated germination in wheat (Rashid *et al*., 2006; Yari *et al.,* 2010; Murungu, 2011; Yari *et al.,* 2011). The pretreatment of seeds with hydrogen peroxide can be extremely beneficial and leads to breaking of seed dormancy, if used in the right concentrations and conditions (Williams, 2003).

In seed physiology ROS such as hydroxyl radical OH, O_2 and H_2O_2 are usually considered as toxic molecules (Koornneef *et al.*, 2002). However, exogenously applied H_2O_2 ameliorate seed germination in many plants (Bailly, 2004). The scavenging activity for H_2O_2 is sufficient, resulting in the production of O_2 for mitochondrial respiration (Bailly, 2004). In contrast, H_2O_2 promotes seed germination rather than O_2. At early seed aging it performs important role in the growth processes (Kibinza *et al.,* 2006). For better understanding of physiological aspects of salinity stress tolerance and to chalk out accurate screening techniques ultimately aiding to crop improvement in saline soils, the present study was carried out.

MATERIALS AND METHODS

This pot experiment was conducted in the glasshouse of Lancaster Environment Centre University of Lancaster, Lancashire, United Kingdom. The seeds of two wheat genotypes Khirman and Inqalab were obtained from Nuclear Institute of Agriculture, Tandojam, (NIA) Pakistan. Healthy seeds were selected and sterilized with 5% sodium hypochlorite (NaClO) solution for three minutes and were transferred in different levels (0, 20, 40, 60, 80 and 100 µ*M*) of H_2O_2 for 8 hours. Then five seeds were sown in compost filled pots and distilled water was given according to field capacity. Extra plants were thinned out leaving two plants in each pot after 8 days. Both salinity treatments (distilled water and 100 m*M*

NaCl) were applied after 8th day of sowing in four splits of two sets of pots at every 5th day. Further water was applied according to requirement. The minimum day time temperature of the glasshouse was at 22˚C, and minimum night temperature was 18˚C with light intensity of 600 µmol M^{-2} S^{-1}. The membrane stability index, leaf relative water content, stomatal conductance, photosystem II (P-II) efficiency and antioxidant assay were recorded.

Membrane stability index (MSI)

Membrane stability index was assayed by estimating the electrolyte leakage from fresh leaf tissues into distilled water. The method was described by Siaram *et al.* (2002). Hundred milligram leaves were cut into small 2mm length tubes and placed in air tight glass tubes containing 10 ml de-ionized water at room temperature (25˚C). After 12 hours their conductivity (C_1) was measured using electrical conductivity metre (Twin compact meter by Horiba). Then glass tubes were placed in an autoclave in which the temperature was maintained at 100˚C for 10 minutes. After boiling the autoclave was turned off and allowed to cool to completely kill the tissues and release all the electrolytes. Samples were cooled to 25˚C and conductivity (C_2) was measured. The MSI was calculated using the formula:

$$MSI= [(1-C_2/C_2)] \times 100$$

Where, MSI= Membrane stability index, C_1 = conductivity at room temperature before boiling and C_2 conductivity after boiling.

Leaf relative water content (RWC)

Leaf relative water content (%) estimation was done. The leaf sample from each plant under analysis, was harvested to record the fresh weight (FW) followed by hydrating each sample to full turgor for 48 hours in distilled water. Each sample was blotted dried with tissue paper before re-weighing to determine fully turgid weight (TW). Samples were dehydrated at 60˚C for 24 hours to record dry weight (DW). Relative Water Content (RWC) was derived from the following equation:

$$RWC\ (\%)= [FW - DW] \times 100$$

Stomatal conductance (mmol m^{-2} s^{-1})

Stomatal conductance was measured on the 7th day of treatments using an automatic diffusion Porometer (AP_4). The data were collected from three top most fully expanded leaves per replica.

Photosystem II efficiency – Fv/Fm (µmole m^{-2} s^{-1})

Photosystem II efficiency- Fv/Fm is used to indicate the maximum quantum efficiency of Photosystem II and presented as a ratio of variable of fluorescence (fv) over the maximum fluorescence value (Fm). Chlorophyll fluorescence parameters (PSII maximum efficiency, Fv/Fm) were measured according to Woo *et al*. (2008) after the 7th day of the top most fully expanded leaves using Efficiency Analyzer of Hansatech, UK. The photosynthetically active radiation

was maintained at 1200 µmole m^{-2} s^{-1} and the level of CO_2 concentration in leaf chamber was stabilized and was equal to that at ambient level (360 µmol mol^{-1}).

Antioxidant assay (µmol g^{-1})
Hydrogen peroxide levels were assayed as proposed by Velikova *et al*. (2002). Leaf tissues were homogenized on ice with 0.1% (w/v) of trichloroethane (TCA). The homogenate was centrifuged at 1300 X g for 10 minutes and 0.5 ml of the supernatant was added to 0.5 ml 10 m*M* potassium phosphate buffer (pH 7.0) and 1 ml of 1*M* KI. The sample was incubated at room temperature for 15 minutes. The absorbency of supernatant was determined at 390 nm on spectrophotometer.

Statistical analysis
The statistical analysis was done through computerized software programme of 8.1 version. The LSD value for mean comparison was calculated only if the general treatment F test was significant at a probability 0.05 (Gomez and Gomez, 1984).

RESULTS
The studies were carried out to minimize the effect of salt stress, to estimate physiological biochemical changes and genotypic comparison during seed treatment with H_2O_2.

MSI (%)
The MSI of wheat under the effect of H_2O_2 seed soaking at various concentrations and salinity levels (H_2O and NaCl) is depicted in Figure 1. The MSI was significantly influenced by genotypes and salinity, while it was non-significantly affected by H_2O_2 seed soaking concentrations. The overall average indicated that MSI of genotypes Inqalab and Khirman was higher under H_2O treatment as compared to NaCl treatment. Maximum membrane stability index of genotype Inqalab under H_2O and NaCl treatment was recorded when seed was soaked with H_2O_2 at 20 µ*M* and 60 µ*M* concentration against MSI in control. The genotype Khirman under H_2O and NaCl treatment resulted in maximum MSI when seed was soaked with H_2O_2 at 60 µ*M* and 80 µ*M* against membrane stability index in control. The membrane stability index revealed an uneven variation under saline and non-saline conditions and H_2O_2 concentration for seed soaking. However, water (H_2O) treatment resulted in relatively higher MSI than salt (NaCl) treatment; while genotype Inqalab showed relatively higher MSI than Khirman.

RWC (%)
The results showed that RWC of genotypes Inqalab and Khirman was equally higher under H_2O treatment as compared to NaCl treatment (Figure 2). Maximum RWC of genotype Inqalab under H_2O and NaCl treatment was recorded when seed was soaked with H_2O_2 at 40 and 100 µ*M* concentrations against RWC in control (0 µ*M*). The genotype Khirman under H_2O and NaCl treatment resulted in maximum RWC when seed was soaked with H_2O_2 at 40 and 100 µ*M* against RWC in control (0 µ*M*). The leaf relative water content did not follow a linear

trend under the effect of H_2O_2 concentrations, genotypes and salinity. However, H_2O treatment resulted in relatively higher RWC as compared to NaCl treatment; while no difference was found in genotypes.

Stomatal conductance (mmol m^2 s^{-1})

The stomatal conductance in wheat genotypes Inqalab and Khirman was higher under H_2O treatment than NaCl treatment (Figure 3). The effect of H_2O_2 (µ*M*) concentrations indicated that maximum stomatal conductance in genotype Inqalab under H_2O and NaCl treatment was observed when seed was soaked with H_2O_2 at 40 µ*M* and 80 µ*M* concentrations against stomatal conductance in control; while genotype Khirman under H_2O and NaCl treatment resulted in maximum stomatal conductance when seed was soaked with H_2O_2 at 80 µ*M* against stomatal conductance in control, respectively. The stomatal conductance followed linear trend and increasing H_2O_2 (µ*M*) concentration resulted in a simultaneous increase in the stomatal conductance regardless of the genotypes and salinity. However, stomatal conductance was higher in H_2O irrigated plants than NaCl (m*M*) plants; while Khirman showed relatively higher stomatal conductance than Inqalab.

Photosynthesis II efficiency (µmole m^{-2} s^{-1})

The data in Figure 4 indicated that photosynthesis II efficiency of genotypes Inqalab and Khirman was higher under H_2O treatment as compared to NaCl (m*M*) treatment. Maximum photosynthesis II efficiency of genotype Inqalab under H_2O and NaCl treatment was equally recorded when seeds were soaked with H_2O_2 at 100 (µ*M*) concentrations against photosynthesis II efficiency in control. The genotype Khirman under H_2O and NaCl (m*M*) treatment resulted in maximum photosynthesis II efficiency when seed was soaked with H_2O_2 at 100 µ*M* against photosynthesis II efficiency in control (µ*M*). The photosynthesis II efficiency was directly proportional to H_2O_2 (µ*M*) concentration for seed soaking and regardless of the genotypes and salinity, at highest H_2O_2 (µ*M*) concentration, photosynthesis II efficiency reached its highest level in a simultaneous manner. However, H_2O treatment resulted in higher photosynthesis II efficiency as compared to NaCl treatment. The variety Inqalab showed higher photosynthesis II efficiency than Khirman.

Antioxidant assay (µmol g^{-1})

Antioxidant assay determined in Inqalab seemed to be higher under H_2O treatment than NaCl (m*M*) treatment, while in Khirman the antioxidant assay was higher under NaCl treatment than H_2O treatment (Figure 5). The effect of H_2O_2 (µ*M*) concentrations indicated that maximum antioxidant assay in genotype Inqalab under H_2O and NaCl treatment was recorded when seed was soaked with H_2O_2 at 80 µ*M* and 100 µ*M* concentrations against in control. The genotype Khirman resulted maximum antioxidant assay when seed was soaked with H_2O_2 at 100 µ*M* against control (0 µ*M*). However, non-linear trend of differences in antioxidant assay under saline and non-saline conditions was noted; Khirman showed relatively higher antioxidant assay than Inqalab.

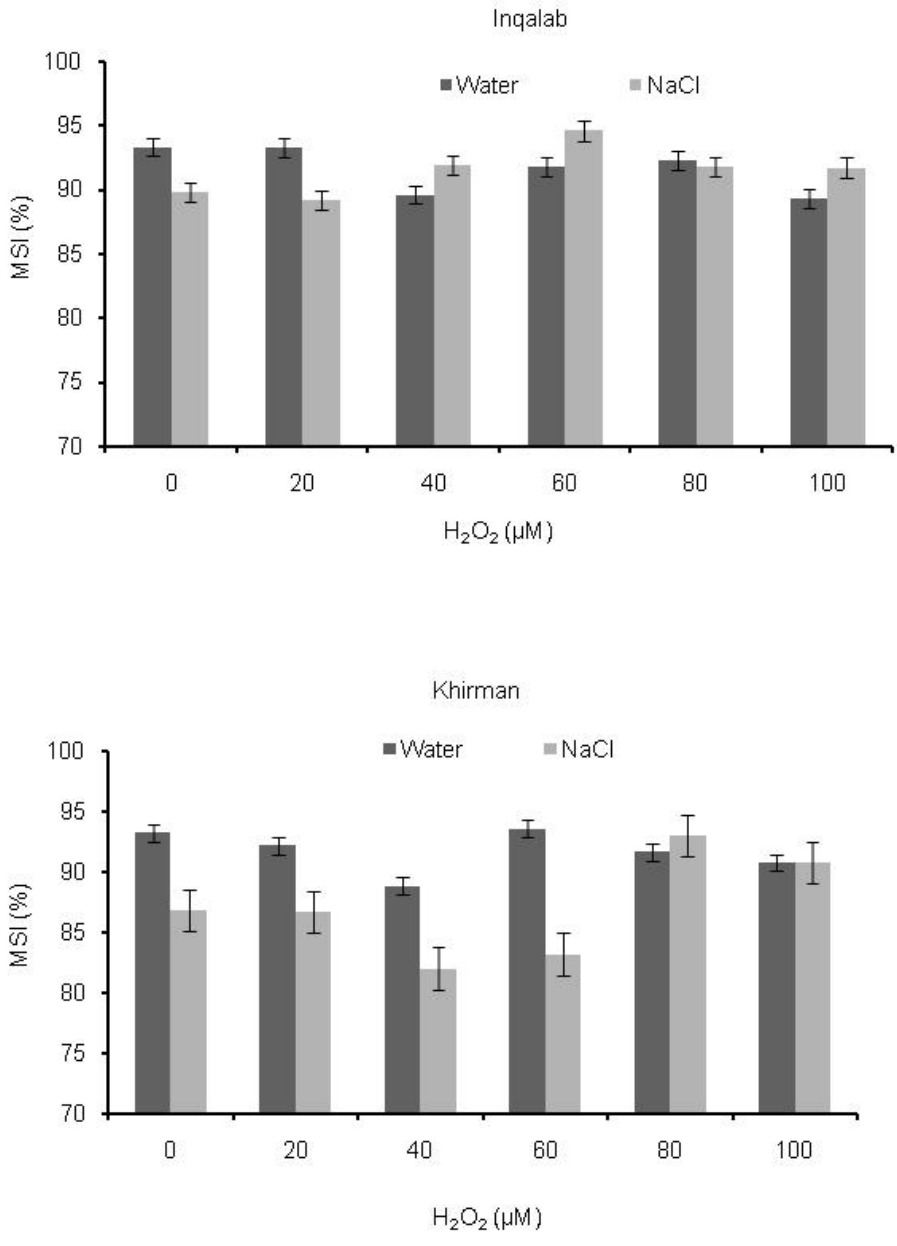

Figure 1. Effect of H_2O_2 **(*µM*)** and salinity levels on membrane stability index (%) of Inqlab and Khirman wheat genotypes

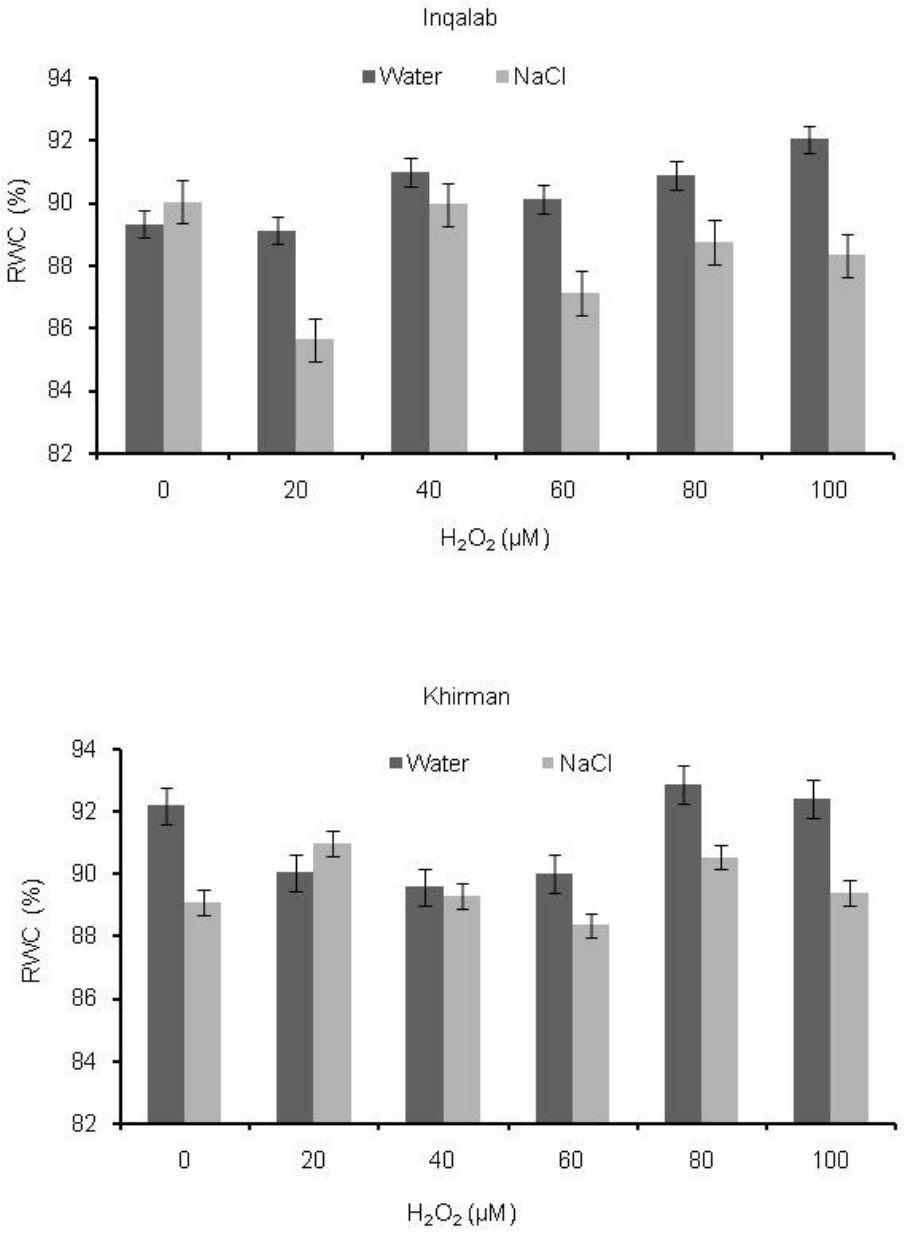

Figure 2. Effect of H_2O_2 (*µM*) and salinity levels on RWC (%) of Inqlab and Khirman wheat genotypes

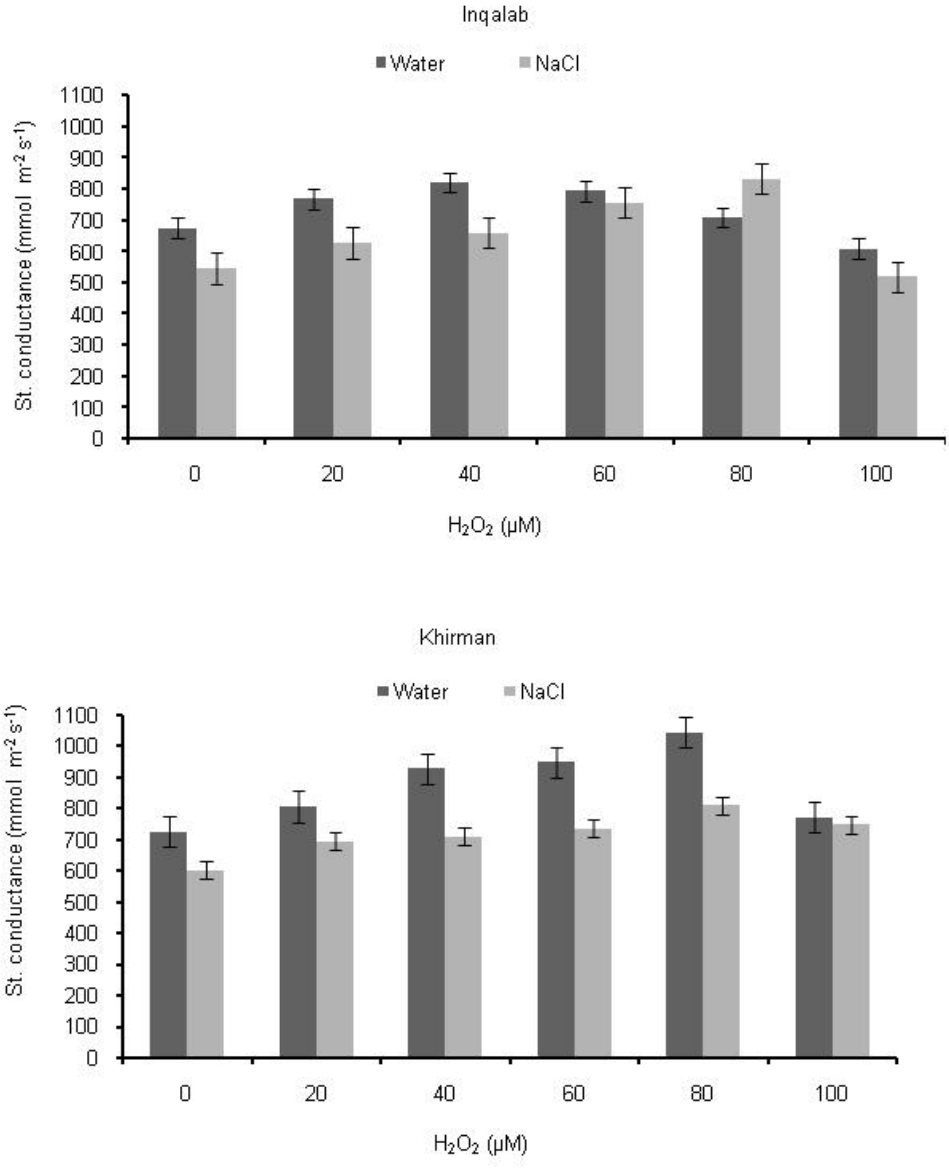

Figure 3. Effect of H_2O_2 (*µM*) and salinity levels on stomatal conductance (mmol $m^{-2}s^{-1}$) of Inqlab and Khirman wheat genotypes

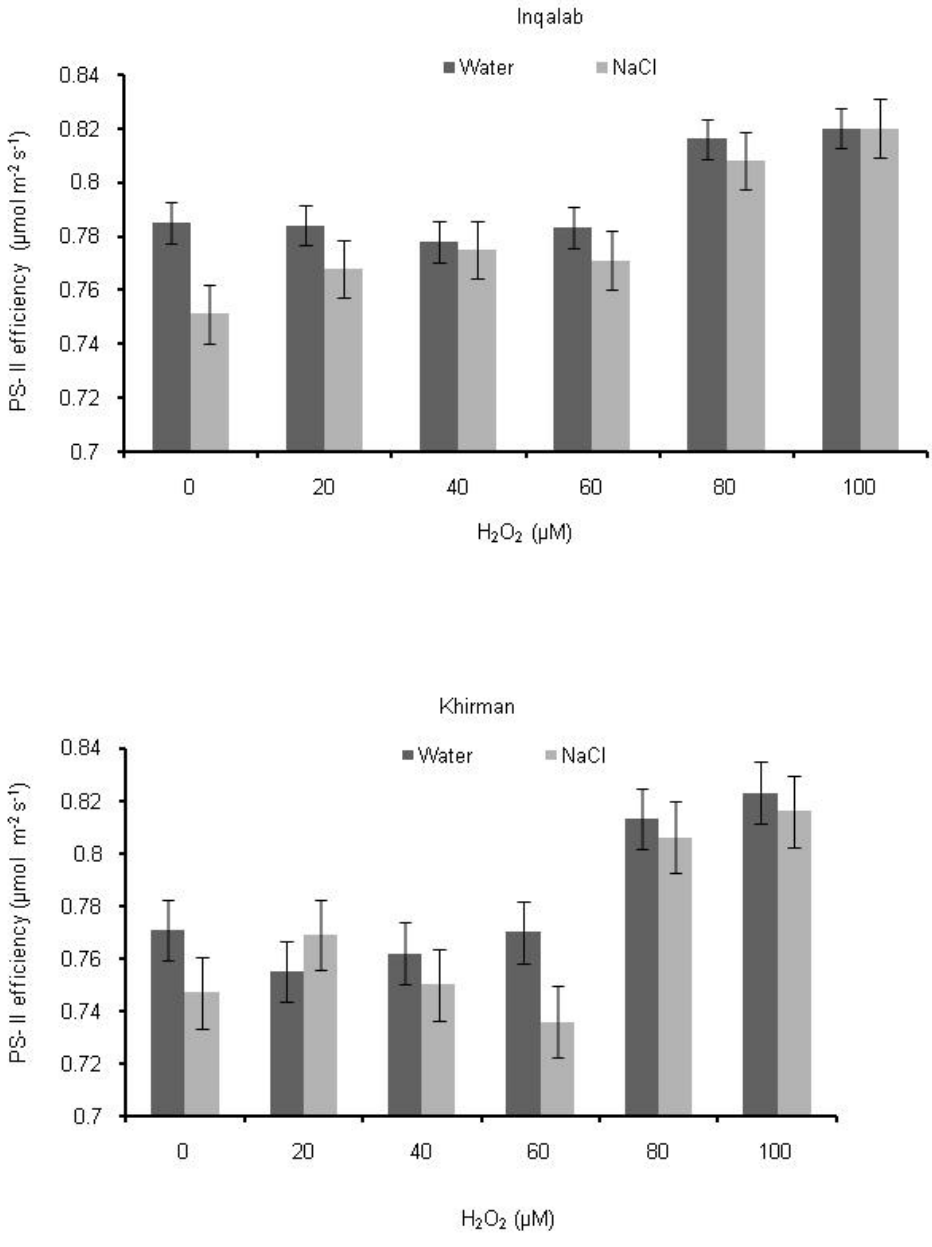

Figure 4. Effect of H_2O_2 (*µM*) and salinity on photosystem II (PS-II) efficiency Fv/Fm (µmole m^{-2} s^{-1}) of wheat Inqlab and Khirman genotypes

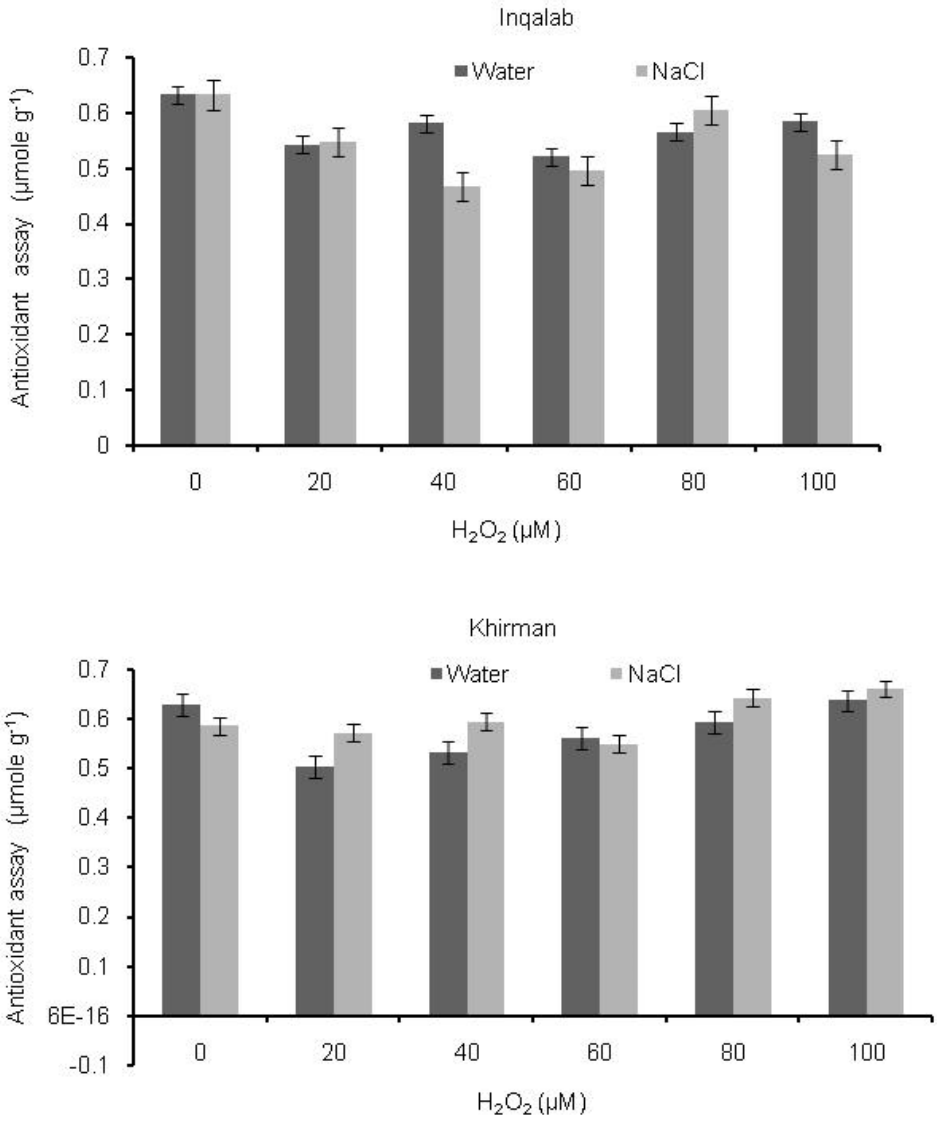

Figure 5. Effect of H_2O_2 (*µM*) and salinity on antioxidant assay (µmol/g^{-1}) of Inqlab and Khirman wheat genotypes

DISCUSSION

The membrane stability index of wheat genotypes under the effect of H_2O_2 seed soaking at various concentrations and salinity treatments (H_2O and NaCl) was significantly affected by genotypes and salinity ($P< 0.05$), while it was statistically non-significantly ($P> 0.05$) affect by H_2O_2 seed soaking concentrations. The MSI was significantly lowered by NaCl in Khirman, whereas in Inqalab, MSI was not altered significantly by salinity. The impact of H_2O_2 priming on physiology

indicated the improvement in salt-tolerance in Inqalab. The activities of anti-oxidative enzymes in treated seeds are enhanced by priming (Hsu *et al.*, 2003). Moreover, the activities of glyoxysome enzymes in primed seeds are also increased by priming (Lin and Sung, 2001). Also, there was no significant effect of treatment, so changes in membrane stability were probably not one of the main mechanisms by which H_2O_2 protected growth and yield under salinity stress. Sairam *et al.* (2002) supported that salinity-stress affects MSI. Many genes are involved in salinity-tolerance but advancement has been made in studying the mechanism original a plant's response to salinity (Negrao, 2017). Wheat genotypes Inqalab and Khirman were evaluated for their leaf relative water content under H_2O_2 seed soaking at various concentrations and salinity treatments (H_2O and NaCl); and suggested non-significant (P>0.05) effect of H_2O_2 seed soaking concentrations on genotypes and salinity for RWC. It was expected that salinity alters water relations in plants. The RWC, carotenoids (CAR), MSI, biomass and grain yield increased H_2O_2, proline, glycine-betaine (GB), soluble sugars, superoxide dismutase (SOD), thiobarbituric acid reactive substances (TBARS), catalase (CAT) and glutathione reductase (GR) activity in Kharchia 65 (tolerant) and KRL- 19 (moderately tolerant) wheat genotypes and all the stages are decreased by salinity-stress (Sairam *et al.,* 2002). Rodriguez *et al.* (2005) showed that plant under saline and water stress conditions show reduction in RWC. The stomatal conductivity showed an interesting effect, and salinity tends to decrease conductivity.

It is concluded that the main protective effects of H_2O_2 treatment on salinity stress that had been identified were on stomatal conductivity. Treatment also had a significant effect, and H_2O_2 treatment increased conductivity that possibly allowed better gas exchange and more photosynthesis under salt stress. The priming of seeds in right concentrations and conditions of hydrogen peroxide is extremely beneficial for plants (William, 2003). The changes in plant growth, relative water content, stomatal conductance, lipid peroxidation and antioxidant system in relation to the tolerance to salt stress were investigated in salt-tolerant *Plantago maritime* and salt-sensitive *Plantago media* (Sekmen *et al.,* 2007). The H_2O_2 treatment can potentially improve photosynthesis via beneficial effects on photosynthesis II efficiency (Fv/Fm) which was significantly affected by H_2O_2 seed soaking concentrations (P<0.05), while non-significant effect on photosynthesis was observed due to genotypes and salinity (P>0.05). Related to this, although salinity did not significantly affect Fv/Fm (maximum efficiency of photosystem II), there was a significant effect of H_2O_2. Treatment tends to increase Fv/ Fm; so again, H_2O_2 is supposed to increase photosynthesis under all conditions, including salinity. Slesak *et al*. (2007) discussed the potential role of H_2O_2 in the photosynthetic mode of carbon assimilation, such as C4 metabolism and CAM (Crassulacean acid metabolism) and speculated that early in the evolution of oxygenic photosynthesis on earth, H_2O_2 could have been involved in the evolution of modern photosystem II efficiency. The data determining antioxidant assay was significantly influenced by H_2O_2 seed soaking concentrations and genotypes. Beneficial effects have been observed, when seeds were soaked in hydrogen peroxide pretreatment in plants (William, 2003). Under heat stress hydrogen peroxide plays a key role in regulating the activity of antioxidant enzymes (Wael and Rady, 2014).

CONCLUSION

The effects of H_2O_2 treatment on plant physiology might explain the improvement in salt-tolerance of Inqalab. The data conclude that H_2O_2 seed treatment seems to protect the salt-sensitive genotype Inqalab against yield reductions caused by salinity. The Khirman, salt-tolerant genotype was significantly affected. On the whole beneficial effects are seen around 60 µ*M* H_2O_2, with yield reduced was again observed in the plants with the highest levels of H_2O_2 treatments. Leaf relative water content was not significantly affected by H_2O_2 in both (H_2O and NaCl) applications. The most important observations are protective effects of H_2O_2 treatment in salinity-stress and are on stomatal conductivity. The presoaking of seed in H_2O_2 can potentially improve phosynthesis via beneficial effects on Fv/Fm. The H_2O_2 signals the activation of antioxidant in seeds, which persist in the seedlings to offset the ion-induced oxidative damage. These changes led to the expression of stress improved physiological attributes, which support growth under salinity.

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