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SUPPLEMENTARY $CaCl_2$ AMELIORATES WHEAT TOLERANCE TO NaCl

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Summary: Nine-day-old seedlings of two wheat cultivars (Misr-1 and Sakha-93) were treated with NaCl applied at concentrations of 75, 150 and 225 mM with or without 10 mM $CaCl_2$. After 15 days, all concentrations of NaCl led to significant decreases in fresh and dry weights of only Sakha-93; however, Misr-1 seemed to be affected only by the highest NaCl concentration. Nonetheless, growth parameters were most likely similar in the control samples of both cultivars grown under normal conditions. On the other hand, lipid peroxides (malondialdehyde MDA) and H_2O_2 were strongly accumulated in both cultivars, particularly in Sakha-93; a significant increase was only detected in Misr-1 treated with 225 mM. Also, all concentrations of NaCl decreased glutathione (GSH) content in Sakha-93. Nevertheless, there was no great difference in GSH content among both cultivars grown under normal conditions. On the other hand, the activities of the enzymatic antioxidants, glutathione reductase (GR), glutathione-S-transferase (GST), catalase (CAT) and peroxidase (POD) were inhibited in Sakha-93 by all concentrations tested, but remained unaffected in Misr-1. The alternative oxidase (AOX) responded differently to NaCl, there was a decrease in Misr-1 at 75 and 225 mM and in Sakha-93 at 75 and 150 mM. These findings suggest that Misr-1 is a more tolerant cultivar to NaCl than Sakha-93. Nevertheless, $CaCl_2$ alleviated the negative impact of NaCl; there was a retraction in the magnitude of decrease in growth parameters to reach most likely those of the control in Misr-1. In addition, the accumulated MDA and H_2O_2 were greatly counterbalanced. On the contrary, the decreased GSH contents seemed unrecovered in Sakha-93 in spite of the alleviations in their magnitudes. Moreover, there were recoveries in the activities of GR and POD in Sakha-93, nevertheless, GST and CAT activities remained significantly inhibited. These findings revealed that ROS scavenging was efficient and became more inducible in the more tolerant cultivar Misr-1 than in the susceptible one. The response of AOX appeared to coincide with that of antioxidants so that the damage which was induced by NaCl was ameliorated by over-expression of antioxidants especially in the presence of $CaCl_2$.

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Keywords: Antioxidants,; alternative oxidase; $CaCl_2$; salinity; tolerance; wheat.

Abbreviations: ANOVA – analysis of variance; AOX – alternative oxidase; CAT – catalase; CDNB – 1-chloro-2,4-dinitrobenzene; GR – glutathione reductase; GSH – glutathione; GSSG – oxidized glutathione; GST – glutathione-S-transferase; LSD – least significant differences; MDA – malondialdehyde; $O_2^{\cdot-}$ – superoxide radical; POD – peroxidase; ROS – reactive oxygen species; TCA – trichloroacetic acid.

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INTRODUCTION

Salt stress is one of the major abiotic stresses that affect biochemical and physiological processes in plants, causing growth inhibition and yield loss (Yang et al., 2008). Salinity generates reactive oxygen species (ROS) in plants. ROS typically result from the excitation of O_2 to form $^1O_2$ or from the transfer of one, two or three electrons to O_2 to form a superoxide radical (O_2^{-}), hydrogen peroxide (H_2O_2) or a hydroxyl radical (HO^-), respectively (Mittler, 2002; Nemat Alla et al., 2008a, 2008b). ROS can seriously disrupt normal metabolism through oxidative damage of lipids, protein and nucleic acids. These detrimental effects of the overproduction of ROS could limit plant tolerance to stress (El-Shintinawy et al., 2004; Hassan and Nemat Alla, 2005). However, plants have developed a complex defense system to detoxify and eliminate the generated ROS (Mittler, 2002; Tuteja, 2007; Nemat Alla et al., 2008a, 2008b). This system includes non-enzymatic antioxidants as glutathione and enzymatic antioxidants as glutathione reductase (GR), glutathione-S-transferase (GST), catalase (CAT) and peroxidase (POD) (Mittler, 2002; Aravind and Prasad, 2005; Nemat Alla and Hassan, 2006; 2007) as well as alternative oxidase (AOX) (Yip and Vanlerberghe, 2001). AOX is proposed to play a role as a survival protein through its ability to maintain mitochondrial function; it plays also a central role in determining ROS equilibrium in cells and plants (Robson and Vanlerberghe, 2002; Amirsadeghi et al., 2007; Fu et al., 2012). Although it is expressed during normal growth and development, it is often dramatically induced at the transcript level by stresses. The increase in transcript abundance is typically accompanied by accumulation of AOX protein (Thirkettle-Watts et al., 2003; Escobar et al., 2006; Clifton et al., 2006). One possible approach to ameliorate plant responses to salinity is through the addition of Ca supplements (Abo-Kassem, 2007). Ca is extremely important in plants. It regulates the absorption of nutrients across plasma cell membranes and affects plant cell elongation and division, structure and permeability of cell membranes, nitrogen metabolism and carbohydrate translocation (Gobinathan et al., 2009; Barakat, 2011). Ca induces an increase in antioxidant enzyme activities and a decrease in MDA content in a plant's response to water stress environment (Shu and Fan, 2000). It can participate in the regulatory mechanism in the adjustment to adverse conditions such as salt stress (Cramer et al., 1985). Jiang and Huang (2001) stated that osmotic adjustment increased during stress, but was not affected by external Ca treatment under stress conditions. Moreover, it increased drought resistance in soybean (Yang et al., 1993) and increased salinity tolerance in bean roots (Cachorro et al., 1993). Ca functions as a regulator of plant cell metabolism (Pietrobon et al., 1990). Therefore, the aim of this work was to study the effect of $CaCl_2$ on two cultivars of wheat differing in their tolerance to NaCl and to clarify the role of $CaCl_2$ in ameliorating salinity tolerance.

MATERIALS AND METHODS

Plant materials and growth conditions

A preliminary experiment was conducted using several wheat cultivars (*Triticum aestivum*) and different concentrations of NaCl to select the

most sensitive and the least sensitive cultivar. These were Sakha-93 and Misr-1, respectively. Moreover, the experiment was extended to find out the lowest efficient concentration of $CaCl_2$ to alleviate the effect of NaCl which turned out to be 10 mM. The grains of both cultivars were surface sterilized by immersing in 3% sodium hypochlorite solution for 10 min and thoroughly washed. The grains were soaked for 8 h, germinated in berlite in 7 cm-diameter pots and watered with tap water. On the 7th day, seedlings were thinned to only one per pot. The pots were placed in 40×60 cm trays containing 100% long Ashton nutrient solution for 2 days and then divided into 4 sets for NaCl treatments (0, 75, 150 and 225 mM). Each set was represented by 10 replications. A half of the replications of each treatment was subjected to treatment with 10 mM $CaCl_2$. The seedlings were distributed randomly into trays and kept for the following 15 days under controlled conditions (25/10 °C day/night temperature, 60% relative humidity, 10 h photoperiod and 300 µmol m^{-2} s^{-1} photosynthetic photon flux density). The nutrient solution was consistently made up to the appropriate volume daily and renewed every 4 days. At harvest, the plants were carefully removed from the perlite, washed thoroughly with the relevant nutrient solution, dipped into deionized water and plotted dry. Shoot system was separated and used for determination of fresh and dry weights. Other samples were frozen in liquid N_2 for subsequent analyses.

Determination of lipid peroxides, H_2O_2 and glutathione (GSH) contents

Lipid peroxides were extracted in 150 mM KCl. After centrifugation at 7000×*g* for 15 min, 1 ml of the supernatant was incubated at 37°C for 2 h with 1 ml of 0.6 M trichloroacetic acid (TCA). One ml of supernatant was taken with 1 ml of thiobarbituric acid and placed in a boiling water bath for 10 min, cooled and diluted with 1 ml distilled water. The absorbance was read at 535 nm (Buege and Aust, 1972). H_2O_2 was extracted in 200 mM perchloric acid and centrifuged at 5000×*g* for 10 min. The assay mixture contained 0.4 ml 12.5 mM 3-dimethylaminobenzoic acid in 375 mM phosphate buffer, pH 6.5, 0.08 ml 1.3 mM 3-methyl-2-benzothiazolinone hydrazone and 0.02 ml (0.25 units) horseradish peroxidase. The increase in absorbance at 590 nm was monitored for 3 min (Okuda et al., 1991). GSH was extracted in TCA (5%, w/v) containing 10 mM EDTA and centrifuged at 12,000×*g* for 15 min (Anderson and Gronwalds, 1991). GSH was assayed in 100 mM phosphate buffer, pH 6.8, containing 10 mM EDTA and 1 mM 1-chloro-2,4-dinitrobenzene (CDNB). The reaction was started by adding 1.0 U equine glutathione-S-transferase (GST) and incubated at 35 °C for 30 min. The absorbance was recorded at 340 nm.

Measurement of antioxidant enzyme activities

The enzymes were extracted in 50 mM sodium phosphate buffer (pH 6.9) containing 2 mM EDTA and 5 mM β mercaptoethanol and centrifuged at 12,000×*g* for 10 min at 4°C. Total soluble protein was determined according to Bradford (1976). Glutathione reductase (GR) was assayed in a reaction mixture containing 100 mM phosphate, pH 7.5, 0.5 mM EDTA, 0.75 mM 5,5-dithiobis-(2-nitrobenzoic acid), 0.1 mM NADPH

and 1 mM oxidized glutathione (GSSG) (Smith et al., 1988). The reaction mixture was incubated at 35°C and the absorbance at 412 nm was measured up to 5 min. Glutathione-S-transferase (GST) was extracted in 100 mM Tris-HCL (pH 7.5) containing 2 mM EDTA, 14 mM β-mercaptoethanol and 7.5% (w/v) polyvinylpolypyrollidone, then centrifuged at 15,000×*g* for 15 min and ammonium sulfate was added to 80% saturation (Dixon et al., 1995). GST was assayed in 100 mM phosphate buffer (pH 6.5) containing 5 mM GSH and 1 mM CDNB. After incubation for 1 h at 35 °C, 3 ml of 0.33 N HCl were added to stop the reaction and absorbance was measured at 340 nm. GST activity was assayed using the extinction coefficient E = 9.6 mM^{-1} cm^{-1}. Catalase (CAT) activity was measured by following the consumption of H_2O_2 at 240 nm in 50 mM sodium phosphate buffer (pH 7.0) containing 10 mM H_2O_2 (Aebi, 1984). The absorbance was read at zero time and after 1 min. Peroxidase (POD) activity was measured in 50 mM sodium phosphate buffer (pH 6.9) containing 3.2 mM guaiacol and 0.4 mM H_2O_2 (Chance and Maehly, 1955). The absorbance was measured at 470 nm.

Quantification and gene expression of alternative oxidase (AOX)

Total RNA was extracted using TRI-reagent (Sigma, UK) according to the manufacture's protocol. Poly A tail mRNA was then isolated by reacting 10 µl of RNA with 2 µl of oligo dT(18) and 3 µl RNase and DNase free H_2O for 5 min at 65°C and the reaction was terminated on ice for 2 min. The reverse transcription was conducted by using Revert Aid first strand cDNA synthesis kit (thermo scientific, Cat. No. K1621) according to the supplier's recommendations. Primers for AOX gene were designed to recognize conserved regions resulting from the alignment of the characterized AOX in *Triticum* species. The forward and reverse primers used for amplifying AOX were CGAGTGGAAGTGGTCTTG and GTTGAAGAAGACGCCCTG, respectively and for 18S rRNA were CCACCCATAGAATCAAGAAAGAG and GCAAATTACCCAATCCTGAC, respectively.

The PCR conditions were as follows: initial denaturation at 95°C for 3 min, followed by 27-40 cycles of denaturation at 95°C for 30 sec, annealing at 50°C for 30 sec for 18S rRNA or 55°C for AOX and extension at 72°C for 1 min. The number of PCR cycles was optimized to show the maximal differences among samples within the linear phase of amplification (35 & 40 for AOX and 27& 35 for 18S rRNA). PCR products were resolved by electrophoresis on 1% agarose gels, stained with ethidium bromide in 1X Tris–acetic acid-EDTA using Bio- Rad equipment and visualized and documented using Trans illuminator UViTec. The band volumes were measured by using Lab Image V 2.7.2 software. The measurements were normalized for equal 18S rRNA bands.

Statistical analysis

The experimental design was a complete block randomized design and repeated twice in at least triplicates so that the mean values ± SD were at least n=6. The design consisted of 320 pots [2 cultivars x 4 sets (4 NaCl treatments) x 2 $CaCl_2$ sets (2 $CaCl_2$ applications) 10 pots per set (10 replications per treatment, each pot containing one plant) x 2 experimental

repetitions]. The design exhibited that salinity without or with the application of $CaCl_2$ was in main plots, and wheat cultivars in sub plots with three random replications. The experiment was repeated twice. For each parameter, the three replications from each experiment (six determinations from two independent experiments) were used to calculate the mean value ± SD. The data obtained from both experiments were most likely consistent and were first subjected to analysis of variance (ANOVA) followed thereafter by least significant differences (LSD) at 5% level.

RESULTS

The results in Fig. 1 showed that both wheat cultivars (Misr-1 and Sakha-93) seemed to have likely similar values of fresh or dry weights. The differences in these parameters between both cultivars did not exceed 3%. Nonetheless,

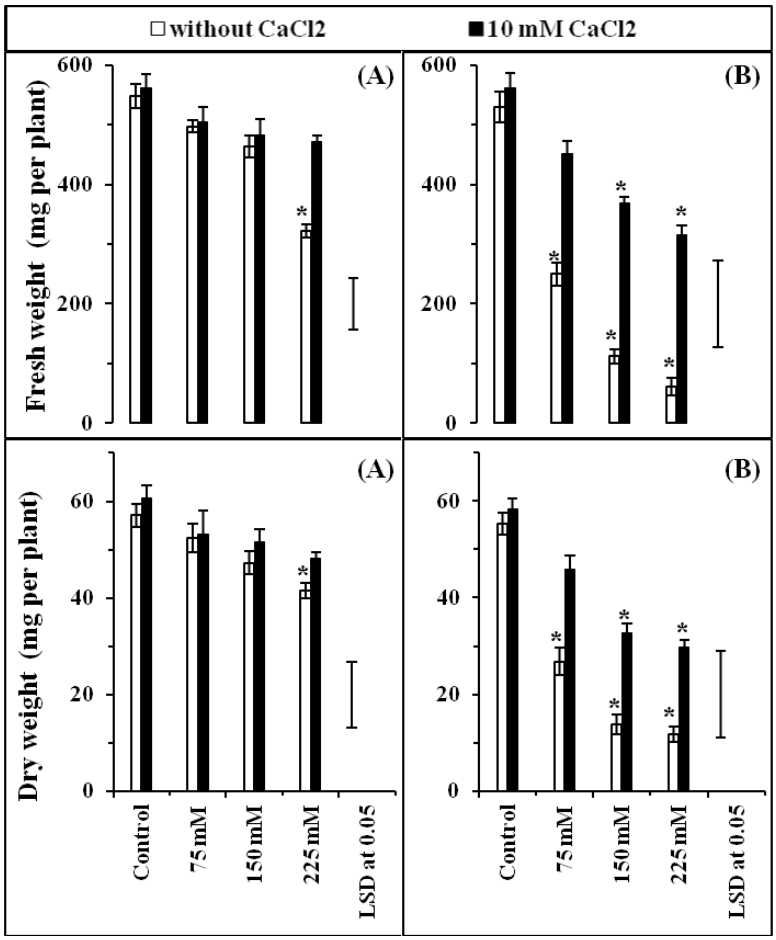

Figure 1. Changes in shoot fresh and dry weight of two wheat cultivars (A) Misr-1 and (B) Sakha-93 following treatment with 75, 150 and 225 mM NaCl for 15 days with or without 10 mM $CaCl_2$. Data are means (±SD) of at least six replications from two independent experiments. Vertical bars represent LSD at 5% level. The (*) represents significant change from the respective control.

NaCl treatment led to a decrease in growth parameters depending on the concentration tested and wheat cultivar. Only 225 mM NaCl significantly reduced fresh and dry weights of Misr-1, however, all concentrations led to a significant decrease in Sakha-93. Treatment with NaCl at 75, 150 and 225 mM resulted in a reduction in fresh weight by 9, 15 and 41%, respectively in Misr-1 and by 53, 79 and 89%, respectively in Sakha-93. Dry weight was also reduced by 75, 150 and 225 mM NaCl by about 8, 17 and 27% in Misr-1, respectively relative to 52, 75 and 79% in Sakha-93. Nevertheless, application of 10 mM $CaCl_2$ to the treated seedlings appeared to overcome the impacts of the low concentration of NaCl but alleviated the effects of the high concentrations. The reduction in fresh and dry weights of Sakha-93 by 75 mM NaCl was completely withdrawn upon application of $CaCl_2$, however the reduction in fresh weight by 150 and 225 mM was retracted to 30 and 40%. Similarly, $CaCl_2$ retracted the decreases in dry weight induced by150 and 225 mM to become 41 and 46%, respectively in Sakha-93. Nonetheless,

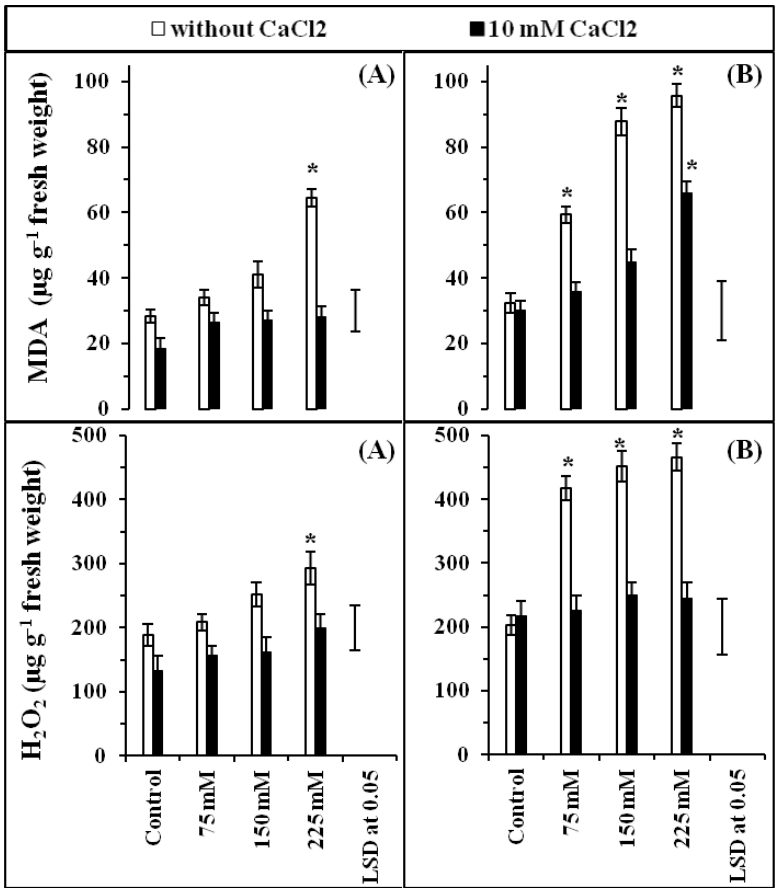

Figure 2. Changes in lipid peroxides as MDA and H_2O_2 of two wheat cultivars (A) Misr-1 and (B) Sakha-93 following treatment with 75, 150 and 225 mM NaCl for 15 days with or without 10 mM $CaCl_2$. Data are means (±SD) of at least six replications from two independent experiments. Vertical bars represent LSD at 5% level. The (*) represents significant change from the respective control.

the observed reduction in fresh and dry weights of Misr-1 by 225 mM NaCl was completely elevated.

Lipid peroxides content (represented as MDA) under normal growth conditions was higher (by about 14%) in Sakha-93 than in Misr-1, however, NaCl treatment resulted in a significant accumulation of MDA in both cultivars (Fig. 2). The magnitude of accumulation increased with increasing concentrations to become significantly higher than the respective control at all NaCl concentrations in Sakha-93 and at only 225 mM in Misr-1. Treatment with 75, 150 and 225 mM NaCl led to accumulation of 21, 45 and 128%, respectively in Misr-1 and to 84, 172 and 197%, respectively in Sakha-93. A similar trend of increase was also detected for H_2O_2; there was a higher level (by 7%) in Sakha-93 than in Misr-1 under normal growth conditions. Following treatments with 75, 150 and 225 mM NaCl, a significant accumulation of H_2O_2 was detected in Sakha-93 (by 107, 123 and 130%, respectively), however, in Misr-1 only 225 mM NaCl led to a significant accumulation of H_2O_2 (by 55%). Nonetheless, 10 mM $CaCl_2$ greatly counterbalanced the accumulation of MDA and H_2O_2 in both cultivars. In particular, the counterbalance by $CaCl_2$ was more obvious in Misr-1 than in Sakha-93, so that NaCl at 75 and 150 mM had an insignificant effect on MDA accumulation. On the other hand, the presence of 10 mM $CaCl_2$ greatly retracted the accumulated levels of MDA and H_2O_2 in NaCl-treated Misr-1 samples, however, the retraction in Sakha-93 was detected for only H_2O_2, but not for MDA.

GSH content seemed mostly alike in both cultivars under normal conditions; only about 6% higher level in Misr-1 than in Sakha-93 (Fig. 3). All concentrations of NaCl significantly decreased GSH content in Sakha-93, however, only 225 mM NaCl caused a significant decrease in Misr-1.

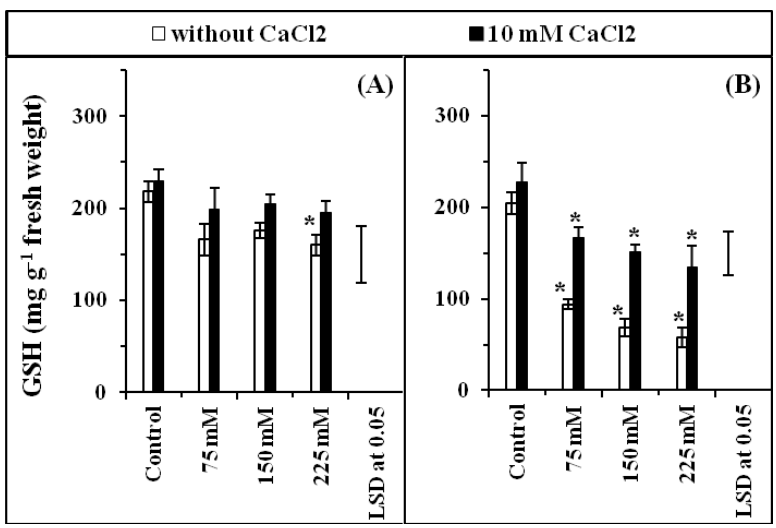

Figure 3. Changes in GSH content of two wheat cultivars (A) Misr-1 and (B) Sakha-93 following treatment with 75, 150 and 225 mM NaCl for 15 days with or without 10 mM $CaCl_2$. Data are means (±SD) of at least six replications from two independent experiments. Vertical bars represent LSD at 5% level. The (*) represents significant change from the respective control.

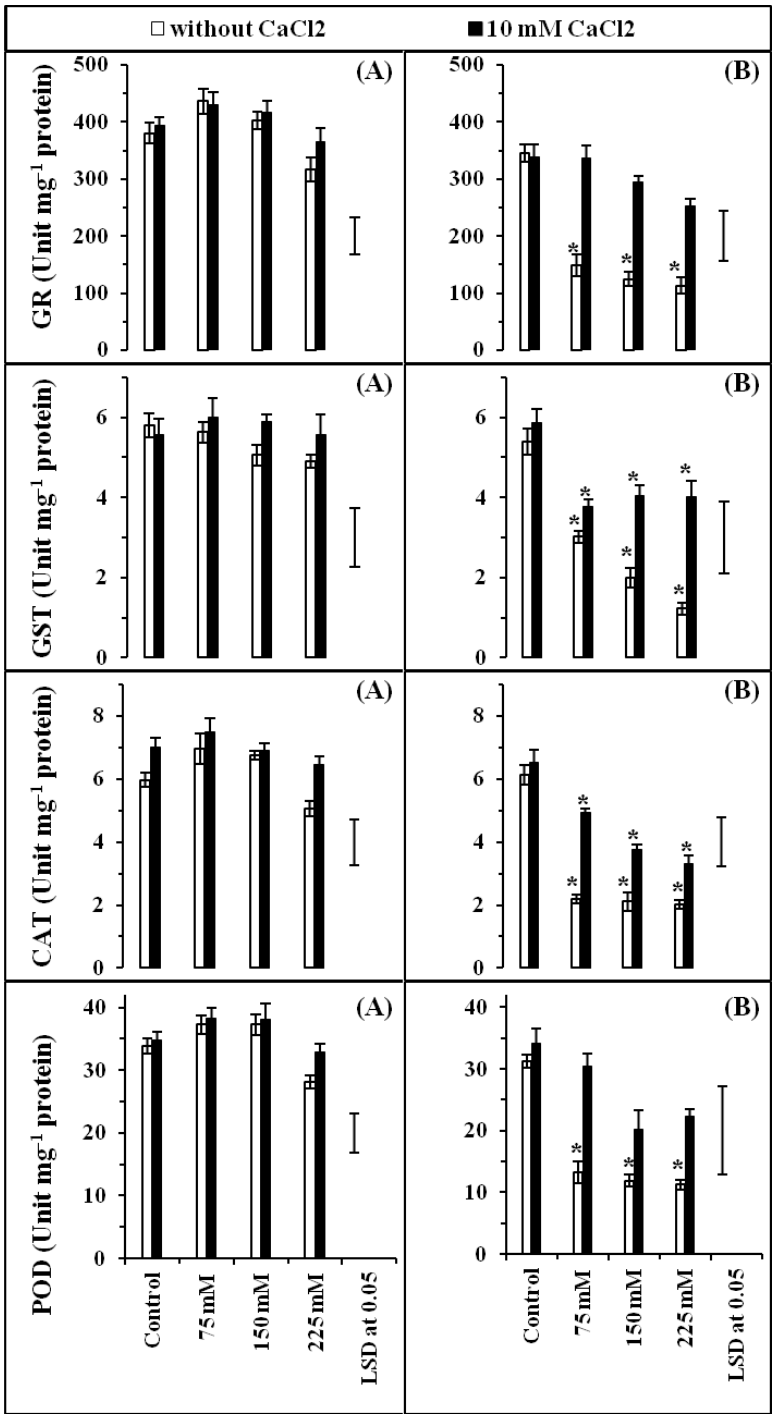

Figure 4. Changes in the activities of GR, GST, CAT and POD of two wheat cultivars (A) Misr-1 and (B) Sakha-93 following treatment with 75, 150 and 225 mM NaCl for 15 days with or without 10 mM $CaCl_2$. Data are means (±SD) of at least six replications from two independent experiments. Vertical bars represent LSD at 5% level. The (*) represents significant change from the respective control.

The magnitude of decrease was higher in Sakha-93 than in Misr-1 and augmented with increasing NaCl concentrations. NaCl at 75, 150 and 225 mM decreased GSH content in Sakha-93 by 54, 67 and 72%, respectively while only 225 mM led to a decrease (by 27%) in Misr-1. However, 10 mM $CaCl_2$ overcame the effects of NaCl only in Misr-1. In the presence of $CaCl_2$, the decreased GSH activity in Sakha-93 in the presence of 75, 150 and 225 mM NaCl was retracted to 18, 26 and 34%, respectively, in spite of being significantly lower than the control.

As shown in Fig. 4, the activities of the antioxidant enzymes GR, GST and POD were higher in Misr-1 than in Sakha-93 by 9, 7 and 8%, respectively. As a whole, all concentrations of NaCl seemed to have no significant effect on the enzyme activities in Misr-1. On the contrary, NaCl led to a significant inhibition of the enzyme activities in Sakha-93. Treatment with NaCl at concentrations of 75, 150 and 225 mM resulted in the inhibition of GR activity (by 57, 64 and 67%, respectively), GST activity (by 44, 63 and 77%, respectively), CAT activity (by 64, 66 and 67 %, respectively) and POD activity (by 58, 62 and 64%, respectively). However, 10 mM $CaCl_2$ greatly overcame the effects of NaCl on the activities of the above enzymes. $CaCl_2$ alleviated the impact of NaCl at all concentrations tested on the activities of GR and POD in Sakha-93. On the other hand, the effects of NaCl on GST and CAT activities still remained significant even in the presence of $CaCl_2$ in spite of the retraction induced.

The transcript levels of AOX in both cultivars responded differently to NaCl treatment. Our results showed a decrease by about 33 and 51% in Misr-1 in the presence of 75 mM and 225 mM NaCl while 150 mM NaCl led to an increase by 54% (Fig. 5). However, the enzyme was decreased in Sakha-93 upon treatment with 75 and 150 mM NaCl by about 6 and 29%, respectively while 225 mM NaCl induced an increase of 47%. Application of $CaCl_2$ seemed with no effect on the changes in the the AOX transcript level in Misr-1 at NaCl concentrations of 75 and 150 mM NaCl, however, an increase of 77% was detected at 225 mM NaCl. Nonetheless, $CaCl_2$ did not change the response of AOX transcript level in Sakha-93 plants treated with 75 and 150 mM NaCl, however, it resulted in further diminution in Sakha-93 treated with 225 mM NaCl to reach the minimal level.

DISCUSSION

The general response of plants to salinity is a reduction in growth (Ghoulam and Fares, 2001; Bandeoglu et al., 2004). In the present study, in spite of having similar values of growth parameters under normal conditions, the growth of the wheat cultivar Sakha-93 was inhibited more than that of Misr-1. These findings could indicate that Sakha-93 was a more sensitive cultivar to NaCl than Misr-1. The different sensitivity to NaCl could be related to deteriorations in various processes. Parida and Das (2005) have indicated that salt stress induces various biochemical and physiological responses in plants and affects almost all plant metabolic processes. Salinity causes alterations in the integrity of cell membranes (Bor et al., 2003) and inhibition of different enzymatic activities (Moradi and Ismail, 2007).

In the present study, application

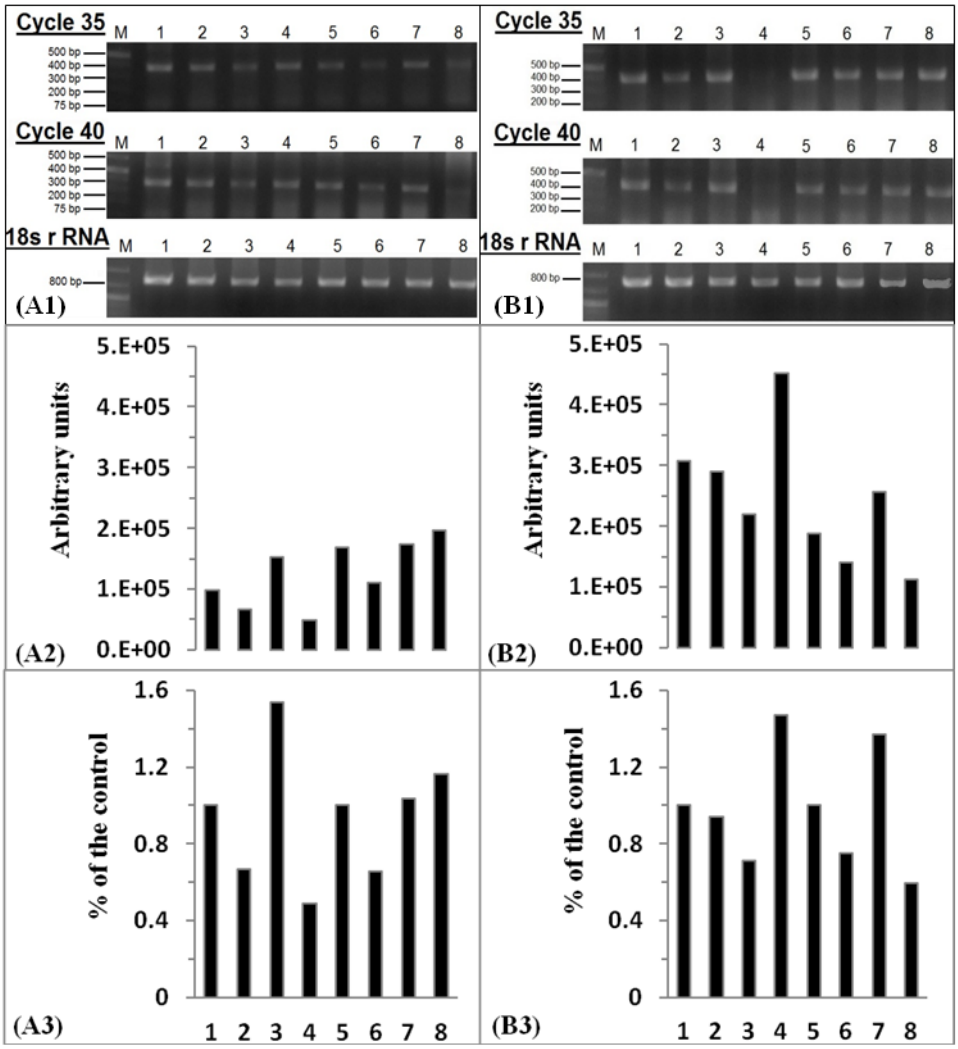

Figure 5. Changes in AOX of two wheat cultivars (A) Misr-1 and (B) Sakha-93 following treatment with 75, 150 and 225 mM NaCl for 15 days with or without 10 mM $CaCl_2$. 1) Stained gels after different cycles, (2) quantification of expression in terms of band volumes, and (3) the relative increase in the bands after normalization with the internal control 18S rRNA. Key to lane numbers: control (1), 75 mM NaCl (2), 150 mM NaCl (3), 225 mM NaCl (4), control +10 mM $CaCl_2$ (5), 75 mM NaCl +10 mM $CaCl_2$ (6), 150 mM NaCl +10 mM $CaCl_2$ (7), 225 mM NaCl +10 mM $CaCl_2$ (8).

of $CaCl_2$ seemed to mitigate the deterioration of salinity stress. It greatly counterbalanced the reduction in growth parameters particularly in Misr-1. These findings proved that the response of Misr-1 to $CaCl_2$ was stronger than that of Sakha-93. The response of a plant cultivar under NaCl stress to $CaCl_2$ amelioration might be related to tolerance to salinity. Ca^{2+} acts as a signaling molecule in salt stress signaling in addition to its effect on preventing Na^+ entry into cells (Knight et al., 1997; Sanders et al., 1999). In this regard, Zhu (2003) reported that cytosolic

Ca^{2+} oscillations during salt stress were regulated through the activities of mechanosensitive and ligand-gated Ca^{2+} channels on the plasma membrane, endoplasmic reticulum and vacuole.

Moreover, one of the most obvious effects of salinity stress is the accumulation of reactive oxygen species (ROS) that would react with lipids, proteins and pigments causing lipid peroxidation and membrane damage (Hernandez et al. 2001; Mittler, 2002; Nemat Alla et al., 2008b). ROS initiate a variety of autooxidative chain reactions on membrane unsaturated fatty acids, producing lipid hydroperoxides and thereby cascade of reactions ultimately leading to destruction of organelles and macromolecules (Aravind and Prasad, 2005). In the present study, the accumulation of lipid peroxides (as MDA) in both wheat cultivars could indicate oxidative stress status provoked by salinity treatment. However, MDA accumulation in Sakha-93 was higher than in Misr-1 showing that oxidative stress is related to the sensitivity to salinity. Therefore, the deleterious effects of NaCl might depend on the sensitivity of plants to salinity. Yasar et al. (2008) reported that NaCl treatments led to a gradual increase in the levels of MDA in green bean and its accumulation was higher in the sensitive cultivar than in the tolerant one. Also, NaCl caused greater accumulation of H_2O_2 in the more sensitive cultivar than in the less sensitive one. Nonetheless, the accumulated levels of MDA and H_2O_2 were greatly retracted following $CaCl_2$ application especially in the less sensitive cultivar. These findings could suggest that the response to $CaCl_2$ application also depended on plant sensitivity to salinity. So, the response of wheat plants to the improvement of salinity tolerance in the presence of $CaCl_2$ was better in the more tolerant cultivar than in the less tolerant one.

To cope with oxidative stress, plants have developed an antioxidant defense system which plays an important role in response to stress. A correlation between the resistance to environmental stresses and the efficiency of the antioxidant defense system has been established (Yasar et al., 2008). Plants are endowed with an array of non-enzymatic antioxidants such as GSH and enzymatic antioxidants such as GR, GST, CAT and POD as well as AOX for removal of ROS (Mittler, 2002; Aravind and Prasad, 2005; Nemat Alla et al., 2008a). GSH is the most abundant low molecular weight non-enzymatic antioxidant in plant cells participating in ROS scavenging through the AsA-GSH cycle (Murgia et al., 2004; Nemat Alla et al., 2008a). During ROS scavenging, GSH is oxidized to GSSG (the oxidized form of glutathione) while GR maintains glutathione in its reduced form. GST activates the protection of plants from some xenobiotics and ROS (Mittler, 2002; Anderson and Davis, 2004). Therefore, the decrease in GSH content and in activities of GST and GR in Sakha-93 revealed that the scavenging of ROS was less efficient in the more sensitive cultivar, thus suggesting a more severe state of stress imposed by NaCl. Following $CaCl_2$ application, the ROS-scavenging system was highly ameliorated particularly in the more tolerant cultivar Misr-1.

The response of alternative oxidase (AOX) in both cultivars to NaCl appeared to coincide with that of antioxidants with a stronger response in the more sensitive cultivar, Sakha-93 than in the more tolerant

one. These findings suggest a possible role of this enzyme in tolerating salinity through ROS scavenging particularly in the more sensitive cultivar at high NaCl concentrations. AOX is involved in reducing the production of ROS (Yip and Vanlerberghe, 2001). It is proposed to play a central role in determining ROS equilibrium in cells and plants (Robson and Vanlerberghe, 2002; Amirsadeghi et al., 2007). On the other hand, some experimental observations suggest a role of ROS in the increase of AOX protein (Wagner and Krab, 1995; Wagner and Wagner, 1995; Vanlerberghe and McIntosh, 1996). So, the fluctuations in the expression of AOX of wheat cultivars at low and high NaCl concentrations could be regarded as reflections from the balance between AOX role in ROS scavenging and its induction by ROS.

In addition, AOX pathway of plant mitochondria uncouples respiration from mitochondrial ATP production and may ameliorate plant performance under stressful environmental conditions (Fiorani et al., 2005). These authors have stated that AOX not only functions to prevent excess ROS formation in whole tissues, but also affects metabolism through more pervasive effects, including some that are extramitochondrial. So, Juszczuk and Rychter (2003) reported the physiological role of AOX as a "survival" protein allowing plants to cope with stress. Indeed, Sieger et al. (2005) concluded that AOX played a role in nutrient availability. Nonetheless, the presence of $CaCl_2$ particularly with the high concentration of NaCl led to an increase in the enzyme activity only in the more tolerant cultivar Misr-1, but contrarily caused a decrease in the less tolerant cultivar Sakha-93, suggesting a role of AOX in salinity tolerance. These findings indicate that the response of AOX to Ca might depend on the cultivar tolerance to NaCl to exhibit its role in avoidance of damage by ROS via minimizing their production. In contrast, the less tolerant cultivar in which the expression of AOX was reduced contained more ROS than control. Future studies need to be performed to verify the involvement of inheritance in the tolerance mechanisms to salinity through investigation of some genes related to NaCl such as sodium overly sensitive (*SOS1*) and sodium hydrogen antiporter (*NHX1*).

In conclusion, both cultivars, Misr-1 and Sakha-93, responded differentially to NaCl in spite of having similar values of growth parameters. The results showed that Sakha-93 was more sensitive to NaCl treatment than Misr-1. Greater was the accumulation of lipid peroxides and H_2O_2 caused by NaCl in Sakha-93 than in Misr-1. On the contrary, both enzymatic and non-enzymatic antioxidants were inhibited by salinity more in Sakha-93 than in Misr-1. So, it could be concluded that Sakha-93 suffered from NaCl treatment more than Misr-1. These findings suggested that Sakha-93 was more sensitive to NaCl than the other cultivar, Misr-1 which can tolerate and combat salinity tolerance. The ability of the plant to combat environmental stress is determined by its efficiency to sense the stress and activate its defense machinery. Our results confirm the relationship between the potential antioxidants and NaCl stress tolerance in wheat. The application of $CaCl_2$ seemed to alleviate the effects of NaCl and enhance salinity tolerance of wheat, particularly in the more tolerant wheat cultivar, Misr-1.

However, supplemented $CaCl_2$ enhanced the defense mechanisms (antioxidants and AOX) so as to counteract certain effects of injury caused by NaCl treatment. Therefore, the damage induced by NaCl can be ameliorated by over-expression of antioxidant enzymes.

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