YYÜ TAR BİL DERG
(YYU J AGR SCI)
2015, 25(3): 221-230

Geliş Tarihi (Received): 10.07.2015
Kabul Tarihi (Accepted): 17.09.2015

Araştırma Makalesi/*Research Article (Orjinal Paper)*

Some Physiological and Bichemical Traits of Two Wheat Cultivars Subjected to Salinity Stress

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Abstract: Wheat is one of the main crops occupying a large area in the world and also in Iran. Salinity is one of the factors that decrease wheat growth and productivity. Therefore, to study the effects of different salinity levels, changes in lipid peroxidation, antioxidant enzyme activities [catalase (CAT), ascorbate peroxidase (APX) and guaiacol peroxidase (GPX)], hydrogen peroxide (H_2O_2), membrane stability index (MSI), relative water content (RWC), soluble sugars, sodium ($Na^{+)}$ and potassium (K^+) ions in two wheat varieties (Hirmand and Karkheh) were investigated. Seedlings were subjected to 100 and 200 mM NaCl and hoagland's solution as control for 20 days. Salinity stress increased GPX and CAT in both cultivars compared with control. At the same time, the APX enzyme increased in 'Hirmand', but had no significant change in 'Karkheh' compared with control. The amount of H_2O_2 in 'Hirmand' did not significantly change in the saline condition compared with control, but showed a significant increase in 'Karkheh'. The amount of malondialdehyde (MDA) in both cultivars under saline conditions increased in comparison with control. However, the highest increase was related to 'Karkheh'. The results also showed a decrease in MSI in both of the cultivars. RWC decreased significantly in 'Karkheh' while in 'Hirmand' there was no significant change. Soluble sugars in both cultivars increased. The results indicated an increase in Na^+ in both cultivars. But K+ reduced in 'Karkheh' and did not change significantly in 'Hirmand'. The K^+/Na^+ ratio also decreased in both cultivars. These findings possibly suggest that by inducing the activity of antioxidant enzymes, no decrease in RWC, and K^+, low increase of Na^+ and increasing the soluble sugars, 'Hirmand' has a better protection mechanism against salt-induced oxidative damage than 'Karkheh'.

Key words: Antioxidant enzymes, Salinity, Osmotic regulation, Oxidative stress, Wheat

Tuz Stresine Maruz Bırakılan İki Buğday Çeşidinin Bazı Fizyolojik ve Biyokimyasal Özellikleri

Özet: Buğday dünyada ve aynı zamanda İran'da geniş bir alanı kaplayan ana ürünlerden biridir. Tuzluluk buğday büyüme ve üretkinliği azaltan faktörlerden biridir. Bu nedenle, iki buğday çeşidinde (Hirmand ve Karkheh) farklı tuzluluk seviyelerinde, lipid peroksidasyonu, antioksidan enzim faaliyetleri [katalaz (CAT), askorbat peroksidaz (APX) ve guaiakol peroksidaz (GPX)], hidrojen peroksit (H_2O_2)] membran stabilite indeksi (MSI) bağıl su içeriği (RWC), çözünür şekerler ile sodyum (Na^+) ve potasyum (K^+) iyonları incelenmiştir. Fideler, 20 gün süreyle 100 ve 200 mM NaCl ve kontrol olarak Hoagland çözeltisi ile muameleye tabi tutulmuştur. Tuzluluk stresi kontrol ile karşılaştırıldığında, her iki çeşitte GPX ve CAT seviyelerini artırmıştır. Aynı zamanda, APX enzimi Hirmand çeşidinde artmış, ancak kontrol ile karşılaştırıldığında Karkheh çeşidinde önemli bir değişiklik olmamıştır. Hirmand çeşidinde H_2O_2 miktarı tuzlu koşullarda kontrol ile karşılaştırıldığında önemli ölçüde değişmezken, Karkheh çeşidinde önemli bir artış gözlenmiştir. Tuzlu koşullar altında, her iki çeşitte malondialdehid miktarı (MDA), kontrol ile karşılaştırıldığında artmıştır. Ancak, en yüksek artış Karkheh çeşidinde gözlenmiştir. Sonuçlar ayrıca, çeşitlerin MSI seviyelerinde bir azalma olduğunu göstermiştir. RWC, Karkheh çeşidinde önemli ölçüde azalırken, Hirmand çeşidinde önemli bir değişikliğe rastlanmamıştır. Her iki çeşitte çözünür şekerler artmıştır. Sonuçlar, her iki çeşitte de Na^+ seviyesinde bir artış göstermiştir. Ancak, Karkheh çeşidinde K^+ azalırken, Hirmand çeşidinde önemli ölçüde değişiklik gözlenmemiştir. K^+/Na^+ oranı, her iki çeşitte de azalmıştır. Bu bulgular, muhtemelen çözünür şekerleri artan, antioksidan enzimlerin aktivitesini uyaran, K^+ ve RWC seviyesinde azalma olmayan ve Na^+ seviyesinde düşük artış olan Hirmand çeşidinin,

Karkheh çeşidine göre tuz kaynaklı oksidatif hasara karşı daha iyi bir koruma mekanizmasına sahip olduğunu düşündürmektedir.

Anahtar kelimeler: Antioksidan enzimler, Tuzluluk, Ozmotik düzenleme, Oksidatif stres, Buğday

Introduction

A high amount of sodium chloride in most saline soils is the main cause of salinity (Tejera et al. 2006). High levels of this compound in soil causes ionic and osmotic stresses on plants (Munnes and Tester 2008), so the assertion can be made that ion stress in plants is caused by the accumulation of sodium ion (Na^+) and chloride (Cl^-) (Gorbani Javid et al. 2011). High levels of Na^+ reduce a plant's intake of essential elements such as potassium (K^+), calcium (Ca^{2+}) and magnesium (Mg^{2+}) ions (Mansour et al. 2005; Murillo-Amador et al. 2006). In addition, high levels of Cl^- lead to nitrate (NO^{3-}) absorption loss (Hamed et al. 2007).

High levels of salt in the soil reduce the amount of absorbable water for plant roots and decrease in their gas exchange decreases (Munnes and Tester, 2008). The outcome of this will be osmotic stress in plant cells. Osmotic and ionic stresses resulting from salinity cause disruption in photosynthesis and respiration. Therefore the production of Reactive Oxygen Species (ROS) increases and results in an occurrence of oxidative stress in plant cells (Mittler el al. 2004; Jubany-Marí et al. 2010). Different types of ROS are produced by partial reduction of atmospheric oxygen in vital cell processes such as photosynthesis, respiration and photo-respiration (Mittler el al. 2004; Jubany-Marí et al. 2010). Among the most important types of ROS, the superoxide radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($HO^{\cdot}$) and singlet oxygen ($^1O_2$), are extremely dangerous and toxic to cells (Mitller 2002; Blokhina and Fagerstedt, 2010). Because these compound's high affinity, they could damage the cell's vital biomolecules such as lipids, proteins and nucleic acids (Murillo-Amador et al. 2006).

Aggregation of ROS damage and toxic compounds from the oxidization of biological materials results Programmed Cell Death (PCD) and lead to cell death (Marrs 1996). Notably, these toxic compounds can even be produced in optimum environmental conditions. Therefore it is necessary for plant cells to have special defense mechanisms to deal with these potentially damaging effects, both in favorable and stress conditions. These mechanisms, by the complete gathering of ROS and their reduction to water, prevent damage to the essential biomolecules. Cell defense mechanisms consist of antioxidants (such as ascorbate, glutathione, tocopherol and carotenoids) as well as antioxidant enzymes (such as superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (GPX), ascorbate peroxidase (APX) (Schafer et al. 2002; Edreva 2005). In the favorable environmental condition there is a balance between the production of ROS and the activity of defense mechanisms in plant cells which results in prevention of cells from oxidative stress (Mittler 2002). If the production of ROS conquest activity of the defense systems, oxidative stress will occur in plant cells (Edreva 2005; Jubany-Marí et al. 2010). With increasing severity of damage to the vital biomolecules and metabolic disturbances, the programmed cell death will run terminally and the cell will be destroyed (Esfandiari et al. 2007a).

According to Jafarzadeh and Aliasgharzad (2007), more than 33 million hectares of Iranian agricultural land faces the problem of soil salinity constituting more than 55% of the country's arable land. Wheat is a staple food in developing countries such as Iran. Considering the vast distribution of salinity in agricultural land, wheat has a very significant role in human nutrition and the negative effects of salinity on yield and food security, this study was conducted on two wheat cultivars (Hirmand and Karkheh) to determine the plant's physiological and biochemical responses to salinity.

Materials and Methods

Plant material and induction of salt stres

In order to explore the effects of salinity on wheat growth and development, the seeds of two varieties of wheat (Hirmand and Karkheh) were selected and disinfected using a solution of 0.1% SDS for 20 minutes. The seeds were then washed several times with distilled water. Sterilized seeds were germinated at 25±1°C and 60%±2 relative humidity. The germinated seeds were transferred to a hydroponic medium and were fed with nutrient solution. The combination of nutrients during the growing period consisted of

macronutrients ($Ca(NO_3)_2$, KNO_3, $MgSO_4$, KH_2PO_4 in 2.5, 3, 1.5 and 0.17 mM values respectively) and micronutrients ($FeSO_4$, H_3BO_3, $MnSO_4$, $ZnSO_4$, $CuSO_4$, H_2MoO_4 in 50, 23, 5, 0.4, 0.2 and 0.1 µM values respectively). The wheat seedlings were fed with a 50% solution until the 2-3 leaf stage and then with a complete nutrient solution. In order to maintain balance between the nutrients, solutions were changed every 2 weeks. In addition, the pH level of the solution was kept within in the range of 5.2-5.5. During the growing period temperature, lighting period and light intensity were 25±2° C, 16 hours and 2500 lux respectively. At the 4-5 leaf stage, wheat seedlings were treated with different salinity levels (control, 100 and 200 mM). After 10 and 20 days of applying different levels of salinity the young and mature leaf samples were taken and immediately immersed in liquid nitrogen. Samples were kept in -20^0c pending taking measurements of the parameters.

Measuring the physiological and biochemical parameters

Enzyme extraction

For CAT and GPX extraction, 0.5 g of leaf sample homogenized in ice-cold 100 mM phosphate buffer (pH=7.5) containing 0.5 mM EDTA. Homogenized samples were then centrifuged in 15000g and 4^0C for 15 minutes. The resulting supernatant was used to measure hydrogen peroxide gathering enzyme activity (Esfandiari et al., 2007b).

For APX extraction, 0.5 g of leaf sample was homogenized in ice-cold 100 mM phosphate buffer (pH=7.5) containing 0.5 mM EDTA, 2 mM ascorbate (AsA) and 5% polyvinylpyrrolidin (PVP 6000) (w/v). The other stages were carried out with the same method to extract the other enzymes (Esfandiari et al., 2007b; Esfandiari and Pourmohammad 2013).

Enzyme activity assay

The activity of CAT enzyme was measured according to the Aebi method (1984). Reaction complex consisted of 100 mM potassium phosphate buffer (pH=7), 75 mM H_2O_2 and enzyme solution. Sample volumes were brought to 3 ml by the addition of distilled water. The reaction was started by adding H_2O_2, and changes in sample absorption at 240 nm wavelength were recorded in a minute period. Enzyme activity was calculated by using the 36.6 mM^{-1} cm^{-1}extinction coefficient.

APX activity was measured according to Yoshimura et al., (2000). Reaction complex consisted of 25 mM phosphate buffer solution (pH=7), 0.1 mM EDTA, 0.25 mM ascorbate (AsA), 1 mM H_2O_2, enzyme solution and distilled water. Changes in sample absorption were recorded at a 290 nm wavelength in one minute and enzyme activity was measured using the 2.8 $mM^{-1}cm^{-1}$ extinction coefficient.

GPX activity was measured according to Panda et al. (2003). Reaction complex included 100 mM phosphate buffer (pH=7), 0.1 mM EDTA, 5 mM guaiacol, 15 mM H_2O_2 and extraction enzyme sample. Changes in sample absorption were recorded at 470 nm wavelength in one minute and enzyme activity was measured using the 26.6 $mM^{-1}cm^{-1}$ extinction coefficient.

MDA was measured using the colorimetric method. 0.5 g of leaf sample was homogenized in 5 ml of distilled water. An equal volume of 0.5% thiobarbituric acid (TBA) in 20% trichloroacetic acid (TCA) solution was added and the sample was incubated at 95°C for 30 min. The samples were then centrifuged at 10000×g for 30 min. Sample absorbance was recorded at 532 and 600 nm wavelengths for each sample. Using the difference between absorbed wavelengths and extinction coefficient the amount of MDA was calculated 155 $mM^{-1}cm^{-1}$ (Stewart and Bewley 1980).

Hydrogen peroxide content (H_2O_2) was determined according to Sergive et al., (1997). 0.5 g leaf sample was homogenized in 5 ml of 0.1% (w/v) TCA and centrifuged at 12000g for 15 minutes. Then the reaction complex was obtained by the combination of 0.5 ml supernatant, 0.5 ml of 10 mM potassium phosphate buffer (pH=7.0) and 1 ml of 1M potassium iodide (KI). Sample absorption was recorded in 390 nm wavelength and H_2O_2 was obtained using the standard curve.

The protein content of the samples was determined by the method of Bradford (1976). Bovine serum albumin was used as a standard.

MSI measurement was based on the electrical conductivity of ion leakage into the leaf cells in deionized water (Sairam et al. 2002). For this purpose 0.1 g of leaf sample (two sets) was immersed in test tubes containing 10 ml of deionized water. Then a series of samples was kept at 40^0 C for 30 minutes and another series was held at 100 ºC for 15 minutes. The electrical conductivity of the samples, after reaching room temperature, was measured and recorded using PH- EC meter (HANNA, HI9811, Hanna Instruments, Padova, Italy). The membrane stability index was calculated based on the following formula: Membrane stability index= [1 - (C1/C2)] × 100 C1 and C2 in the above equation represent the electrical conductivity of the samples at 40 and 100 °C, respectively.

Potassium and sodium contents were measured by the flame photometry method. Leaf samples were dried and pulverized. Samples of powdered leaf material (1 g) were kept at 560°C for 4 h for ash preparation. To these samples, 20 ml 1N HCl was added and the mixtures were heated at 90°C to drive off the hydrochloric acid. The digested ash was dissolved in 100 ml distilled water and then filtered. The filtrate was stored in a refrigerator until analysis. Concentrations of potassium and sodium ions were estimated by referring to 0, 5, 10, 20, and 30 ppm standard working solution. The test solution was diluted if its signal was above that of the highest standard. Contents of the elements were calculated by using the following equation (Bandehhag et al. 2004):

$$E = [(C \times V \times D)/ (M \times 106)] \times 100$$

Where E is the element (either potassium or sodium) content of the test sample, expressed in %, C is the element mass of the test solution, expressed in mg/l, read from the calibration graph. V is the volume, in ml, of the digested solution (V = 100). D is the dilution factor of the test solution carried out during the measurement step. M is the mass, in g, of the test sample used in the procedure.

Total soluble sugars were estimated by anthrone reagent (Yemm and Willis, 1954). D-glucose was used as the standard.

The plant end leaf was chosen to measure Relative Water Content (RWC). Then the leaf's fresh weight was measured immediately. To determine the inflammation weight, leaves were placed in low light and at room temperature for 4 hours in distilled water. After drying the water on the leaves, their inflammation weight was measured. Then the leaves were placed in an oven at 70 °C for 48 hours and were weighed after drying, using a 0.0001g precision scale. Relative leaf water content was calculated as cited by Ritchie et al. (1990):

$$RWC= [WF-WD/WT-WD] \times 100$$

In the above formula RWC¸ WF, WD, and WT show relative water content (RWC), fresh weight, dry weight and inflammation weight of the leaves, respectively.

Statistical analysis

All physiological and biochemical parameters were recorded with five replications. The data were analyzed with GenStat software. Mean comparisons were carried out with the LSD method.

Results and discussion

The results showed that the amount of GPX enzyme in 'Hirmand' increased as salinity levels increased at both harvest stages (Table 1E). However in 'Karkheh', the enzyme only increased in 200 mM level in the first harvest and 100 mM in the second harvest. Increase in GPX activity during salt stress has been reported (Jungklang et al. 2004). The GPX enzyme converts the toxic metabolite, H_2O_2, to H_2O. Thus, increased activity of this enzyme in stress conditions leads to collection of H_2O_2 (Asada 2006). CAT in both cultivars showed similar changes (Table 1F), in the first harvest the amount of CAT increased in both cultivars as salinity levels increased. CAT activity in the second harvest in both cultivars at 100 mM increased in comparison to the control, but at 200 mM there was no significant change. In other words,

CAT enzyme activity, at high salinity and long periods of stress in this research, did not increase. Esfandiari et al., 2007 and Costa et al., 2005 have reported an increase in CAT activity in salinity. CAT activity occurs in peroxisome, mitochondrion and glyoxysome (Seckin et al. 2010; Foyer and Nector 2000). CAT enzyme converts toxic metabolite, H_2O_2, into water and O_2 (Blokhina and Fagerstedt 2010; Seckin et al. 2010).

Change in APX activity was different in the two cultivars (Table 1D). In 'Hirmand', the APX activity, situations showed a significant increase in comparison to the control except for 200 mM salinity in the second harvest, But in 'Karkheh' there was no significant difference in the amount of APX with increasing salinity. Increase in APX activity has been reported by Mahmoud et al. 2009 and Seckin et al. 2010. APX enzyme exists in the chloroplast, cytosol, mitochondrion and peroxisome (Asada, 2006). This enzyme plays a role in glutathione-ascorbate and mehler cycles and converts H_2O_2 into H_2O. Thereby prevents the damage to the plant cells (Asada, 2000). The high activity of this enzyme in the above cycles, in addition to H_2O_2 gathering helps to moderate the $NADP^+$/NADPH, H^+ ratio in cells (Asada 2000). In fact the occurrence of these cycles is considered as an alternative route for consuming electron and hydrogen from the light reaction of photosynthesis, which prevents the closure of the electron transport chain and production of ROS.

The amount of H_2O_2 increased as the salinity levels increased in 'Karkheh'. However in 'Hirmand' except for 200 mM in the second harvest there was no significant difference in comparison to the control (Table 1C). H_2O_2 increase in 'Karkheh' variety also can be attributed to no increase in the amount of APX key enzyme. In most of the cell organelles, H_2O_2 produces during biological metabolic processes. This compound is highly toxic to cells. Accumulation of H_2O_2 damages key parts of the cell, and will therefore cause metabolic disorder.

The results showed that 'Karkheh' had the highest amount of MDA. In this variety, MDA increased at both harvest stages with increasing salinity levels. While in 'Hirmand', MDA increased only at 200 mM level in comparison to the control (Table 1A). In this study, changes of MDA were under the influence of H_2O_2 changes. Increase in MDA during salt stress has been reported (Seckin et al. 2010; Esfandiari et al. 2011b; Sairam et al. 2002). The results showed that under saline conditions, MSI decreased in both cultivars (Table 1B). However, 'Karkheh' showed the greatest decrease. High reduction of MSI in 'Karkheh' can be attributed to H_2O_2 and lipid oxidation increase in this variety. Sairam et al. (2002) reported a decrease of MSI at salinity stress in wheat that is consistent with the results of this study. Esfandiari et al., (2011a) reported a small decrease in MSI, a slight increase of MDA and no increase in H_2O_2 in resistant cultivars. The MSI and MDA were used as markers to illustrate damage caused by salinity stress (Esfandiari et al. 2007a; Esfandiari and Pourmohammad 2013).

In this study, changes in ion concentrations were observed with increasing salinity levels in the varieties under observation. The results showed that the amount of K^+ decreased significantly by increasing the salinity levels in 'Karkheh' (Table 2A). But in 'Hirmand', there was no significant change in other conditions except at 100 mM in the second harvest. Furthermore, the results showed an increase in Na^+ ion of both cultivars. However, the highest amount of Na^+ belonged to 'Karkheh' (Table 2B). More Na^+ uptake in plants in addition to cell damage can cause metabolic disorders (Hamed et al. 2007). But Na^+ ion accumulation in vacuoles can help osmotic adjustment and will be useful for the maintenance of turgor pressure in plant cells (Leidi and Saiz 1997). An increase in Na^+ content during salt stress in wheat has been reported (Sairam et al. 2002; Azizpour et al. 2010). Cachorro et al. 1994 reports that in some cases, despite a high amount of Na^+ ions in salinity, plants preferably absorb K^+. In this study, the results also showed a decrease in K^+/ Na^+ ratio. However, 'Karkheh' had the greatest decrease because of a high amount of Na^+ and a high reduction of K^+ in salinity levels (Table 2C). A decrease in K^+/ Na^+ ratio has been reported (Rejili et al. 2007).

Total soluble sugars content in both 'Karkheh' and 'Hirmand' cultivars in salinity levels increased in comparison to the control, but the greatest increase was seen in 'Hirmand' (Table 2D). According to the role of soluble sugars in decreasing the water potential and osmotic adjustment, it can be asserted that high amounts of this metabolite in 'Hirmand' in comparison to 'Karkheh' can have an effective role in plant's ability to tolerate salinity.

The results revealed that leaf RWC in 'Karkheh' at 200 mM stress has declined significantly in both harvests in comparison to the control. In 'Hirmand', leaf RWC not only did not decrease but also increased at 100 mM in the second harvest (Table 2E). Changes in leaf RWC in 'Hirmand' can be attributed to the increase in soluble sugars and K^+ ion which has led to appropriate osmotic stress adjustment and the maintenance of turgor pressure in stress conditions. In general, the decrease of leaf RWC, leads to turgor pressure reduction in the stomatal guard cells and can cause stomatal closure (Zhang et al. 2006; Azizpour et al. 2010). Stomatal closure reduse the CO_2 entrance (Zhang et al. 2006). CO_2 reduction impairs rubisco enzyme activity (Foyer and Noctor 2009) and thereby reduces the $NADP^+$/NADPH, H^+ ratio. The final result of these changes will lead to an accumulation of electrons in the electron transport chain which transfer the electrons from PSI to O_2 causing the toxic $O_2^{\cdot-}$ (Seppanen 2000). This radical is converted to H_2O_2 by the SOD activity (Jubany-Marí et al. 2010). Therefore ROS can cause metabolic disorder because of stomatal closure. But at these conditions a high level of APX enzyme could lead to the effective implementation of the mehler cycle and prevent the occurrence of oxidative stress. So no decrease in RWC and APX increase in 'Hirmand' could represent its resistance to salinity stress. Conversely, an RWC decrease and no change in APX in 'Karkheh' can be attributed to the high H_2O_2 and increased oxidative stress.

Overall, according to the results of this study it can be stated that due to a high activity of antioxidant enzymes, a low increase in MDA and the partial loss of MSI, the resistance of 'Hirmand' to oxidative stress is better than that of 'Karkheh'. No reduction in K^+, RWC and soluble sugars increase shows that 'Hirmand' cultivar has good osmotic ability and good resistance to salinity stress.

Table 1: The effect of salinity on MDA content (A), MSI (B), H_2O_2 content (C), APX activity(D), GPX activity (E) and CAT activity (F) in two wheat cultivars. The same letters are not significantly different at $P< 0.05$ by LSD test. □Control ⊡100 mM Salinity ■200 mM Salinity.

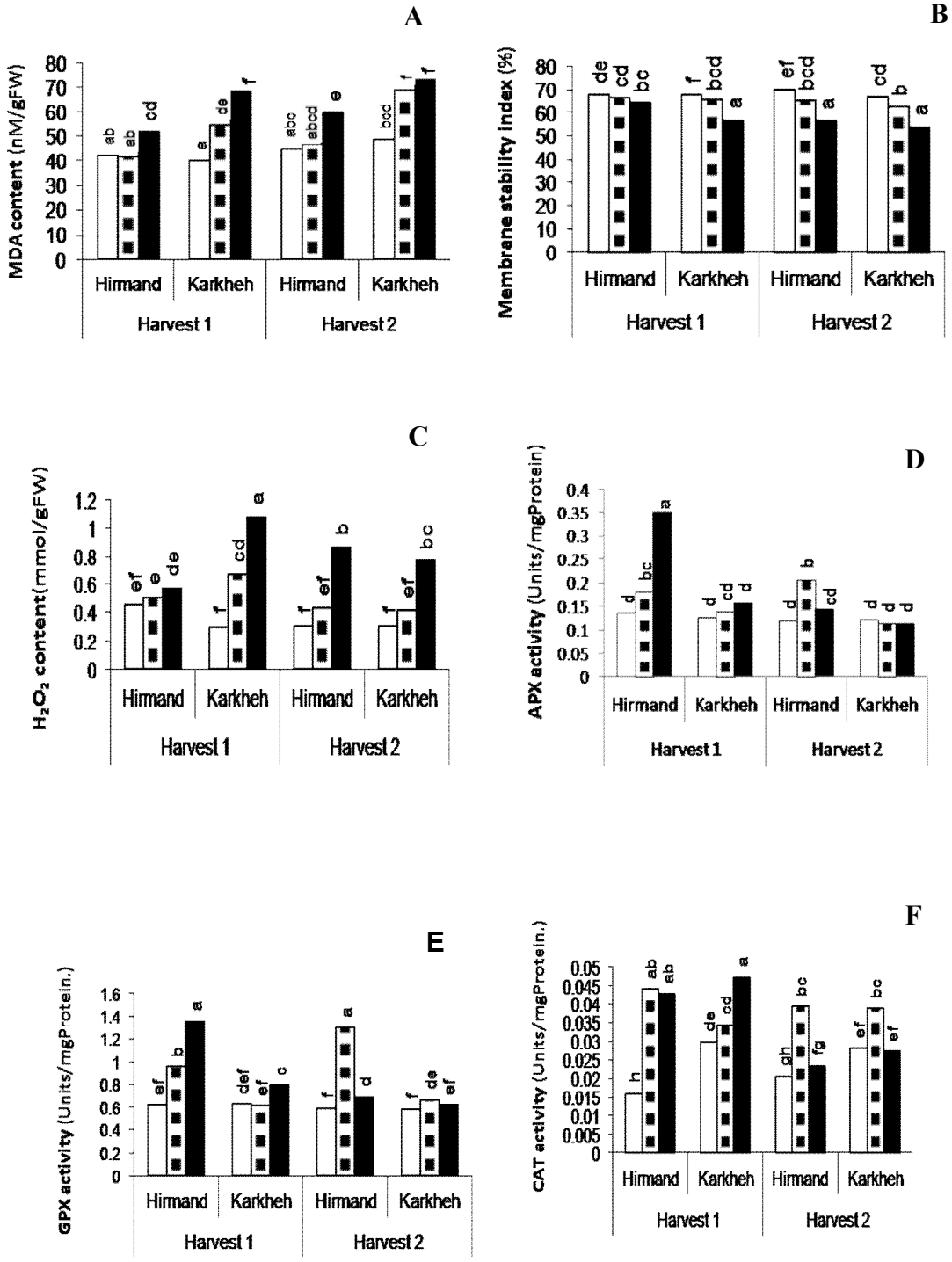
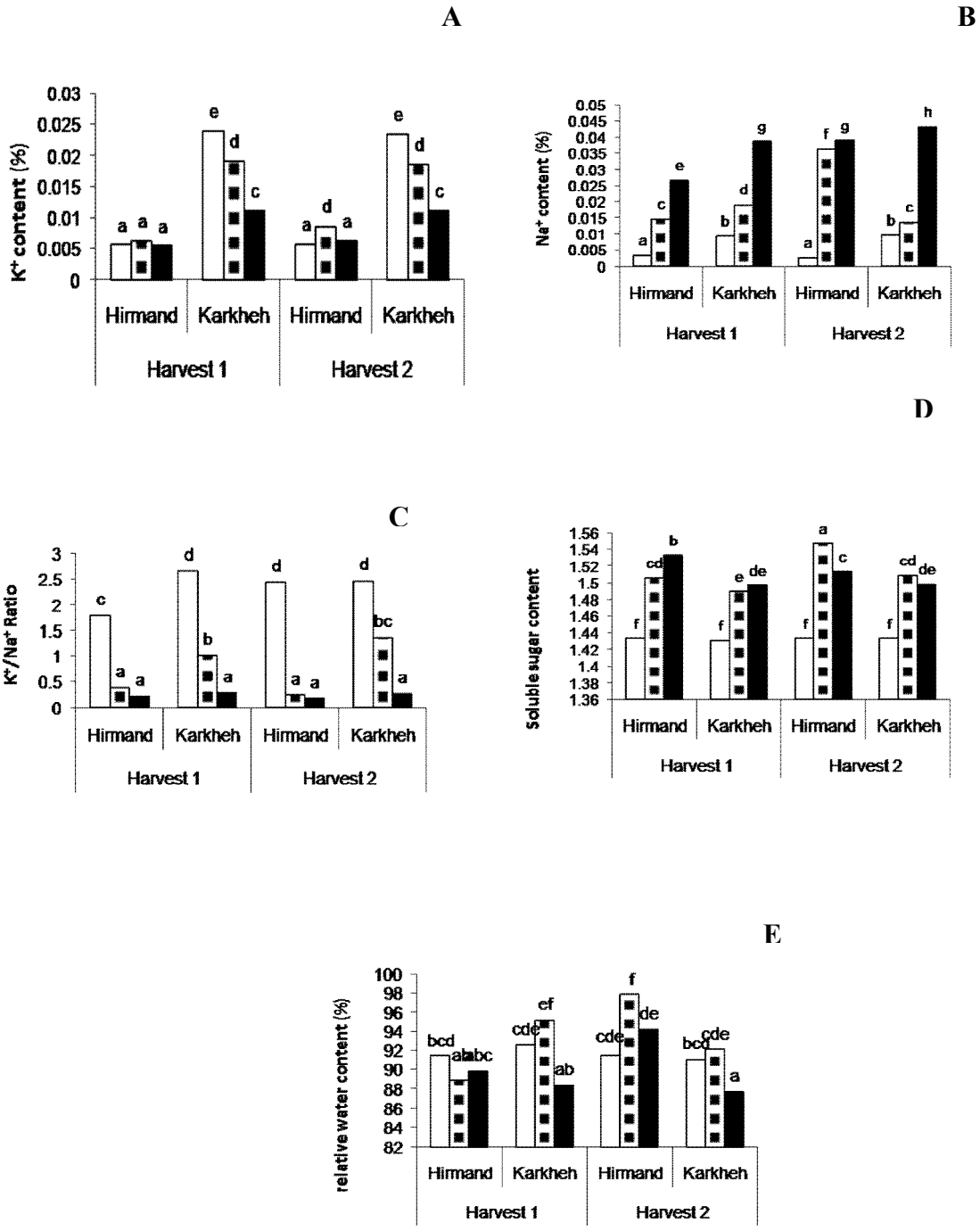


Table 2: The effect of salinity on K^+(A), Na^+(B), K^+/Na^+ (C), Sugar (D), and RWC(E) in two wheat cultivars. The same letters are not significantly different at P< 0.05 by LSD test. □Control ⊡100 mM Salinity ■200 mM Salinity.



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