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PHYSIOLOGICAL PERFORMANCE OF TWO KOREAN RICE CULTIVARS UNDER SALINE ENVIRONMENT AT SEEDLING STAGE

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ABSTRACT

Physiological performance of two Korean rice cultivars viz. Ilmi and Dong-Jin were examined in saline environment. Salinity caused a substantial decline in seed germination of two tested cultivars with considerable variations among them. Among the cultivars, Dong-Jin appeared to be more sensitive at germination stage compared to Ilmi. Similarly, in saline environment, seedling height, root and shoot biomass and root/ shoot biomass ratio was lower in Dong-Jin than Ilmi. While varietal differences in both the cultivars were evident at the highest NaCl (225mM) concentration for most of the considered growth parameters. Antioxidant enzymes activities were also observed and discussed in relation to physiological mechanism of stress tolerance. Dong-Jin exhibited a decrease in superoxide dismutase (SOD) activity and an increase in peroxidase (POX) activity under salinization. However, Ilmi showed only slight increase in SOD and decrease in POX activity in saline environment. Salt stress preferentially enhanced the content of H_2O_2 as well as the activities of the SOD and ascorbate peroxidase (APX), whereas it caused a reduction in catalase (CAT) activity.

Keywords: Salinity, Korean wild type rice (*Oryza sativa* L.), c.v. Ilmi, c.v. Dong-Jin, germination, seedling growth, superoxide dismutase, catalase, peroxidase, ascorbate peroxidase and hydrogen peroxidase.

INTRODUCTION

Plants in their natural environment are exposured to various types of biotic and abiotic stresses. Among abiotic stresses, salinization limits agricultural productivity world-wide (Bhatt *et al*., 2008; Bybordi, 2010; Anwar *et al*., 2011; Osakabe *et al*., 2011 and Jamil *et al*., 2010). Many investigators have reported reduced germination and growth under salt stress condition in some crop plants (Ali *et al*., 2013; Moud *et al*., 2008; Laüchli and Gratten, 2007).

Rice (*Oryza sativa* L.) is one of the highly valued staple food crop for Pakistan and Korea. Rice production is greatly affected by the salt stress that exerts drastic antagonistic effects on rice growth and its developmental processes in terms of morphological and physiological characteristics (Islam *et al*., 2008). Studying differential responses of genotypes with contrasting stress tolerances will help in revealing salt stress tolerance mechanisms (Jogeswar *et al*., 2006). Several investigations have been carried out in order to elucidate the effect of salt stress on growth in rice cultivars (Pattanagul and Thitisaksakul, 2008).

Salinity may cause an imbalance between antioxidant defense and reactive oxidant species production that ultimately results in the oxidative stress to plant (Foyer and Noctor, 2003). Injury caused by reactive oxidant species to biological macromolecules under salt stress is among the major deterrents to growth (Farooq *et al*., 2008, 2009; Razmjoo *et al*., 2008 and Jaleel *et al*., 2009). To counter act the adverse effects of salinity, plants develop different types of antioxidants that protect them against the harmful effects of reactive oxidant species. For our concern, due to inherent sensitivity of rice plant to salt (Francois and Maas, 1994), there has been a great interest in developing salt resistant varieties to ensure future in rice crop production by incorporating genes that encode different types of antioxidants for achieving salinity tolerance. It was hypothesized that rice genotypes Ilmi and Dongjin, of Korean origin, may exhibit traits in salinity tolerance. Comparison of these genotypes under salinity prone environments could be useful in identifying differences related to the relative ability of these cultivars to cope with salinity. Results from this study can greatly increase scientific knowledge on the possible involvement of activated oxygen species in the mechanism of damage by salt stress in rice plant at early seedling stage which could be useful in improving the salt tolerance of rice through genetic engineering.

MATERIALS AND METHODS

Seeds of two Korean wild type rice (*Oryza sativa* L.) cultivars Viz.: Ilmi and Dong-Jin were obtained from

National Center for GM Crop, National Academy of Agricultural Science, RDA, South Korea. The seeds were surface sterilized by dipping the seeds in 1 per cent mercuric chloride solution for 2 minutes and rinsed thoroughly with sterilized distilled water. There were four salinity treatments (0, 75, 150 and 225mM NaCl). Treatment having 0mM NaCl concentration served as control. These treatments were prepared by dissolving calculated amounts of NaCl in deionized water. All the experiments were conducted in 9cm Petri plate.

Ten seeds were placed in sterilized Petri plate representing one treatment. This was replicated 4 times. Likewise, for other treatments, placed Ten seeds in Petri plate and replicated 4 times. The experiment was completely randomized. Each and every individual seedling was an observation. Petri plates were irrigated with 10 mL solution of respective treatment and incubated at 30 °C. The control and saline media were changed after 48 hours in order to avoid salt accumulation.

Germination Test

The emergence of radicle / plumule from seed was taken as an index of germination. Radicle promotion of 1.5mm is consdered to be the criterion of germination by Taylor (1942). The germination was recorded daily up to 9 days.

Germination Indices

Two germination indices were calculated: 50% germination (T50), and Coefficient of the velocity or rate of germination (CRG). The time to get 50% germination (T_{50}) was calculated according to the following formulae of Coolbear *et al*. (1984) modified by Farooq *et al*. (2005):

$$T_{50} = ti + \frac{\{(N/2) - ni\}\ (ti - tj)}{ni - nj}$$

Where N is the final number of germination and n_i , n_j cumulative number of seeds germinated by adjacent counts at times t_i and t_j when n, < N/2 < nj.

While coefficient of the of velocity or rate of germination (CRG) was calculated according to the following formulae of Bewley and Black (1985):

$$CRG = 100 \times ([N_1 + N_2 + N_3 + ... + N_n]/[(N_1 \times T_1) + ... + (N_n \times T_n)])$$

Where N_1 is number of germinated seeds at time T_1, N_2 is number of germinated seeds at time T_2 and N_n is number of germinated seeds at time T_n

Seedling growth

After 9 days, data on root length, shoot length and seedling height was recorded. Seedlings were harvested to determine root and shoot biomass. The height of the plants was measured using a meter rule. The fresh weight of root and shoot was then determined separately, placed the separated parts of the plant in marked paper envelope, and finally kept in an oven at 80 °C for 24 hours to obtain dry weight. Oven dried weights of roots and shoot were recorded. The means as well as standard errors were calculated from the data obtained.

Extraction

Randomly collected 500 mg leaf samples were crushed in liquid nitrogen at 4 °C and homogenized in 10 mL protein extraction buffer containing Tris-HCl pH 6.8, 50 mg PVP, 10 mL DDT, 0.1 mM EDTA. The contents were centrifuged at 10,000 RPM for 15 min. Total protein was estimated by the method of Bradford (1976).

Superoxide Dismutase (Enzyme number by NC IUBMB: EC 1.15.1.1)

The assay for superoxide dismutase **(**SOD) activity was performed by following the method of Beyer and Fridovich (1987). The assay mixture consisted of 27.0 mL of 0.05M potassium phosphate buffer (pH 7.8), 1.5 mL of L-methionine (300 mg per 2.7 mL), 1.0 mL of nitroblue tetrazolium salt (14.4 mg per 10 mL), and 0.75 mL of Triton X-100. Aliquots (1.0 mL) of this mixture were delivered into small glass tubes, followed by the addition of 20 mL enzyme extract and 10 mL of riboflavin (4.4 mg per 100 mL). The cocktail was mixed and then illuminated for 15 minutes in an aluminum foil-lined box, containing 25W fluorescent tubes. In a control tube the sample was replaced by 20 mL of buffer and the absorbance was measured at 560 nm. The reaction was stopped by switching off the light and placing the tubes in the dark. Increase in absorbance due to formation of formazan was measured at 560 nm. Under the described conditions, the increase in absorbance in the control was taken as 100% and the enzyme activity in the samples was calculated by determining the percentage inhibition per minute. One unit of SOD is the amount of enzyme that causes a 50% inhibition of the rate for reduction of nitroblue tetrazolium salt under the conditions of the assay.

Catalase (Enzyme number by NC IUBMB: EC 1.11.1.6)

Catalase (CAT) activity was estimated by the method of Patterson *et al*. (1984). The decomposition of H_2O_2 was measured at 240 nm taking De at 240 nm as 43.6 mM cm^{-1}. Reaction mixture (3.0 mL) consisted of 10.5 mM H_2O_2 in 0.05 M potassium phosphate buffer (pH 7.0) and the reaction was initiated after the addition of 0.1 mL enzyme extract at 25 °C. The decrease in absorbance at 240 nm was used to calculate the activity. One unit of CAT activity is defined as the amount of enzyme that catalyzes the conversion of 1 mM of H_2O_2 min^{-1} at 25 °C.

Ascorbate Peroxidase (Enzyme number by NC IUBMB: EC 1.11.1.11)

Ascorbate Peroxidase **(**APX) activity was determined according to the method of Nakano and Asada (1981). The reaction mixture (2.0 mL) contained 0.05 M potassium phosphate buffer (pH 7.0), 0.2 mM EDTA, 0.5 mM ascorbic acid and 0.25 mM H_2O_2. The reaction was started after the addition of 0.1 mL enzyme extract at 25 °C. The decrease in absorbance at 290 nm for one minute was recorded and the amount of ascorbate oxidized was calculated from the extinction coefficient 2.8mMcm^{-1}. The unit of activity is expressed as micromole of ascorbic acid oxidized min^{-1} at 25 °C.

H_2O_2 Production

The content of hydrogen peroxide was measured according to the procedure of Velikova *et al*. (2000). Freshly harvested leaf sample (100mg) were homogenized with 3mL 0.1% (w/v) trichloro acetic acid (TCA) in ice bath and the homogenate was centrifuged at 12000g for 15min. Later, 0.5mL of 10mM phosphate buffer (pH 7.0) and 1mL of 1M Potassium iodide (KI) were added to 0.5mL of the supernatant. The absorbance of supernatant was read at 390nm. The amount of H_2O_2 was calculated using the extinction coefficient and expressed as mmole g^{-1} FW.

Statistical Analysis

Statistical analysis of the collected data was performed using two way ANOVA (Steel & Torrie, 1960) and Duncan's Multiple Range Test (Duncan, 1955) with the help of the personal computer software packages SPSS version 13.0

RESULTS

Salinity decreased the percentage of seed germination in both the tested cultivars (Fig. 1). Germination velocity of the two rice cultivars and also the salt concentration corresponding to 50% reduction in these germinating seedlings are reported (Table 1). Rate of germination also decreased with an increase in salinity in both the rice cultivars.

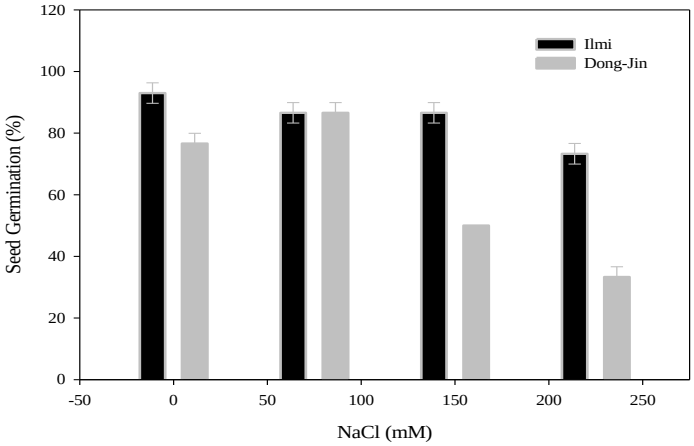

Fig. 1. Response at germination in salinity- prone environments. Vertical line on bar represent SE.

Seedling growth was recorded in terms of root length, shoot length, seedling length, root fresh weight, shoot fresh weight, shoot dry weight and root dry weight at different levels of NaCl treatment. There were marked differences between the control and different levels of salt treatments. Both the cultivars responded in same manner to salinity stress. However, the intensity of stress varied within the cultivars. The increase in NaCl concentrations decreased the shoot and root length of tested rice cultivars (Fig. 2). It was observed that salinity significantly

reduced seedling length of both the tested cultivars relative to the control (Fig. 2). Varietal differences exist between the two cultivars that are seedling length was comparatively more affected in Dong-Jin relative to Ilmi. The reduction of seedling length as compared to control, in response to salinity revealed that both the cultivars are salt-sensitive but Dong-Jin is comparatively more sensitive than Ilmi to some extent.

Table 1. Germination indices (i.e., 50% germination, T50 and coefficient of the velocity or rate of germination, CRG) of Korean wild type rice (*Oryza sativa* L.) cultivars at different salt concentraions.

Species	**Treatments**	**CRG (%)**	**T_{50}**
Oryza sativa L. c.v. Ilmi	Control	16.172a ± 0.01	3.900d ± 0.002
	75mM NaCl	16.013b ± 0.00	3.888c ± 0.001
	150mM NaCl	15.537c ± 0.01	3.888a ± 0.002
	225mM NaCl	15.100d ± 0.02	3.857b ± 0.006
Oryza sativa L. c.v. Dong-Jin	Control	16.960a ± 0.01	3.888c ± 0.001
	75mM NaCl	15.923b ± 0.01	3.875b ± 0.001
	150mM NaCl	15.384c ± 0.00	3.800c ± 0.002
	225mM NaCl	15.510c ± 0.02	3.666a ± 0.003

Numbers followed by the same letter in the same treatment are not significantly different according to Duncan Multiple Range Test at $p<0.05$ level. ± represents Standard Error.

In Ilmi, root dry weight reduced significantly at 75, 150 and 225mM NaCl treatments while there are no significant differences in root fresh weight at 75 and 150mM NaCl concentrations but it significantly reduced at highest salinity treatment as compared to the control (Fig. 3). While shoot dry weight differs non-significantly at 75mM but reduced significantly at 150 and 225mM NaCl concentration relative to the control. Shoot fresh weight differs significantly at all salinity treatments (Fig. 3). Root biomass significantly reduced at 225mM NaCl as compared to 75 and 150mM NaCl treatment but non-significantly increased at 150mM NaCl as compared to 75mM NaCl treatment (Fig. 3). In Dong-Jin, root biomass decreased non-significantly at all NaCl treatment except for 75mM salinity treatment. Root biomass significantly reduced at 225mM NaCl with respect to 75 but reduced non-significantly relative to 150mM NaCl treatment. Shoot fresh weight differs significantly at 150 and 225mM but non significantly at 75mM salt treatment (Fig. 3).

Salt treatment has significantly altered the superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), ascorbate peroxidase (APX) activities (Fig. 4). SOD activities were higher in almost all saline environments than non-saline environment except for the 225mM NaCl concentration in both the tested rice cultivars. However, among both the cultivars SOD activity was quiet satisfactory in Ilmi comparative to Dong-Jin. SOD activities in 75mM salt concentration were increased remarktedly in Dong-Jin relative to Ilmi. CAT activities showed gradual increment with the increase in salt concentration in Ilmi and Dong-Jin but decreased substantially in 225mM NaCl treatment. CAT activity was comparatively more in Ilmi than Dong-Jin. Higher CAT activities were recorded in Ilmi treated with 150 mM salt concentration. POX activity was increased in 75mM salt treatment as compared to control. However, both the tested rice cultivars then showed a gradual significant decline in 150 and 225mM NaCl treatment. There was also a substantial increment in APX activities in all saline treatments as compared to control in both the rice cultivars. However, at the higher salt treatment APX activities were decreased with respect to 75 and 150mM salt concentration. Both the cultivars showed gradual enhancement in H_2O_2 contents almost in all saline treatments but higher H_2O_2 production were recorded in Ilmi with respect to Dong-Jin. On the whole the performance of antioxidant enzymes activities was greater in Ilmi relative to Dong-Jin. SOD, POX and CAT activities substantially increased with the increased H_2O_2 production then gradually caused a decline in both the rice genotypes (Fig. 5).

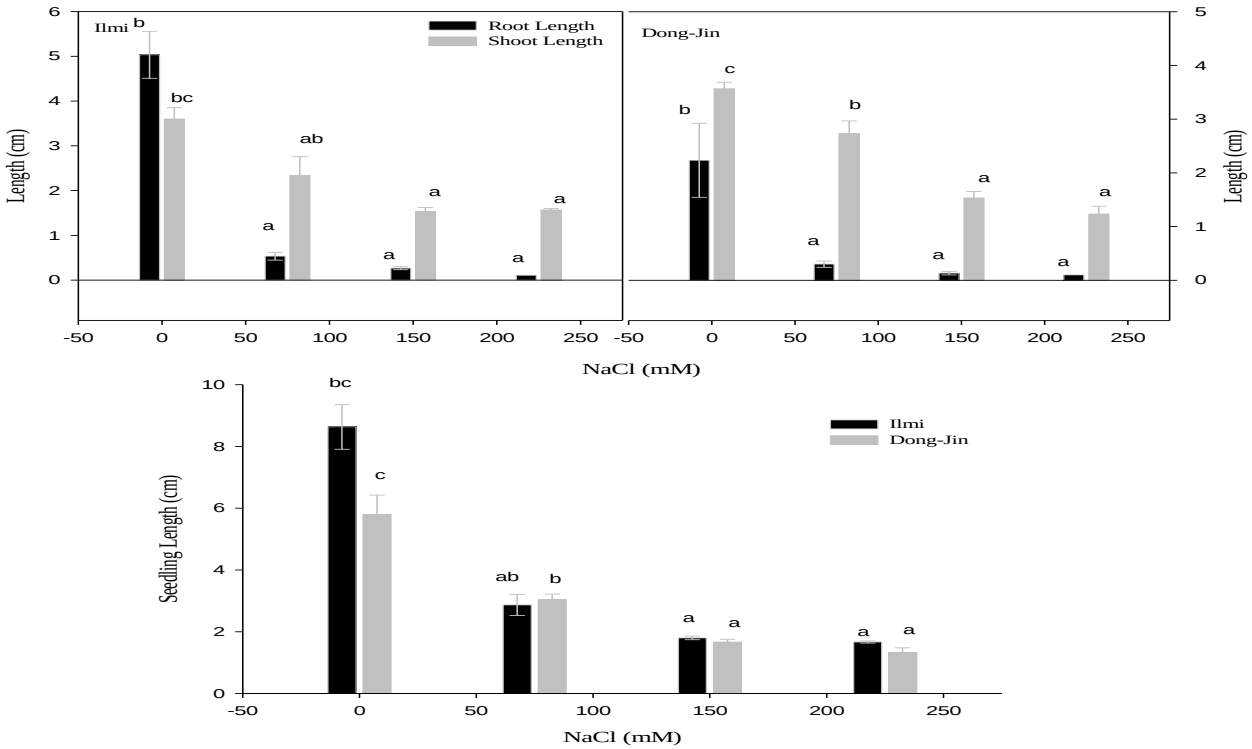

Fig. 2. Response of Korean wild type rice (*Oryza sativa* L.) cultivars at early seedling growth in salinity- prone environments. Bars followed by the same letter in the same treatment are not significantly different according to Duncan Multiple Range Test at p<0.05 level. Vertical line on bar represent SE.

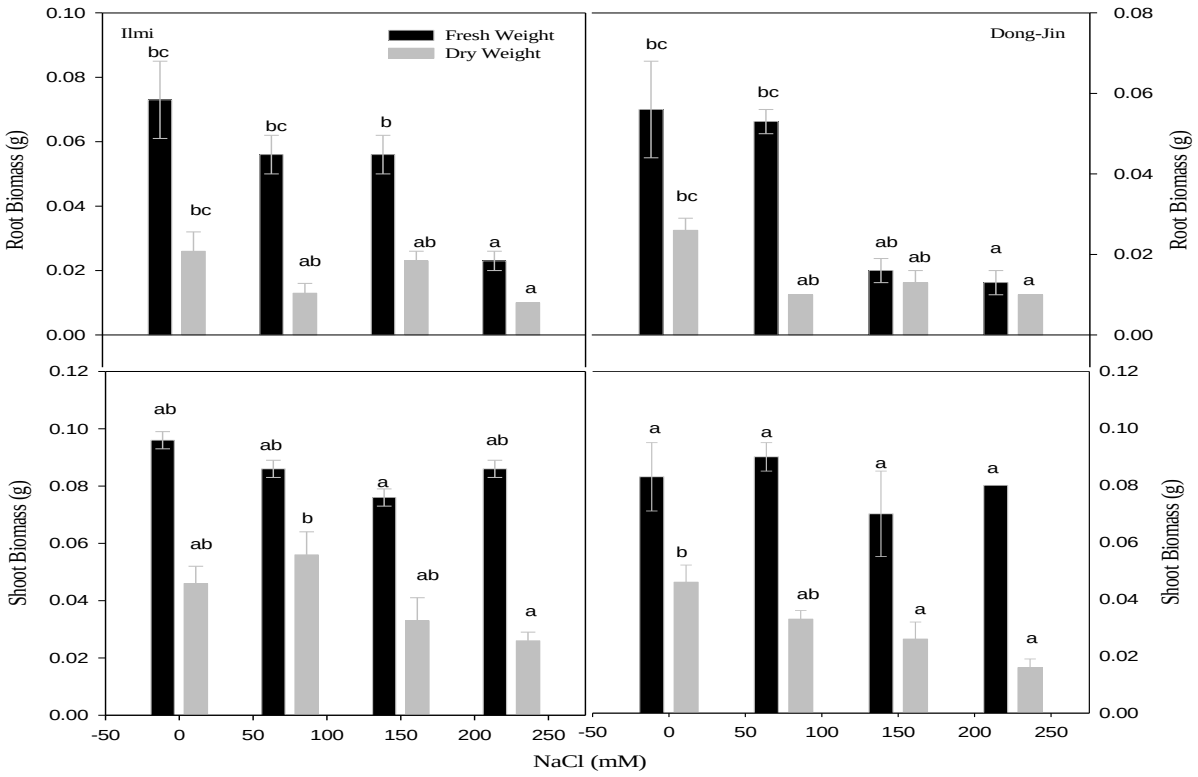

Fig. 3. Response of Korean wild type rice (*Oryza sativa* L.) cultivars at early seedling growth in salinity- prone environments. Bars followed by the same letter in the same treatment are not significantly different according to Duncan Multiple Range Test at p<0.05 level. Vertical line on bar represent SE.

DISCUSSION

Effect of different levels of salt treatments on seed germination and early seedling growth varied in both the tested rice physiotypes depending on the degree of salt concentration. Our results are lined with the findings of Mirza and Mahmood (1986) that germination was directly related to the amount of water absorbed and delay in germination to the salt concentration of the medium. Among the tested cultivars Dong-Jin appeared to be more sensitive at germination stage than Ilmi. Seedlings that were raised from 225mM NaCl showed decline in almost all the growth parameters. Salinity reduces the ability of plants to take up water and this quickly causes reductions in the seedling growth rate of plants (Munns, 2002 and Tezara *et al*., 2003). Chlorosis was quite obvious in both the seedlings exposed to 225mM NaCl concentration that may be due to chlorophyll destruction and cell injury. It is cleared from the results that there is inhibitory effect of salt stress on rice to seedling growth. Increase in salt concentration resulting in the damage of seedling growth.

It was observed by many workers that ROS toxification reduced the plant productivity by peroxidation of macromolecules (Becana *et al*., 2000; Majeed *et al*., 2010). In the present study, it was observed that H_2O_2 quantity rose with the increasing salt concentrations. The quantity of H_2O_2 was less in Dong-Jin as compare to Ilmi (Fig. 4) this attribute can be correlated with the higher antioxidant enzymes activity in Dong-Jin.

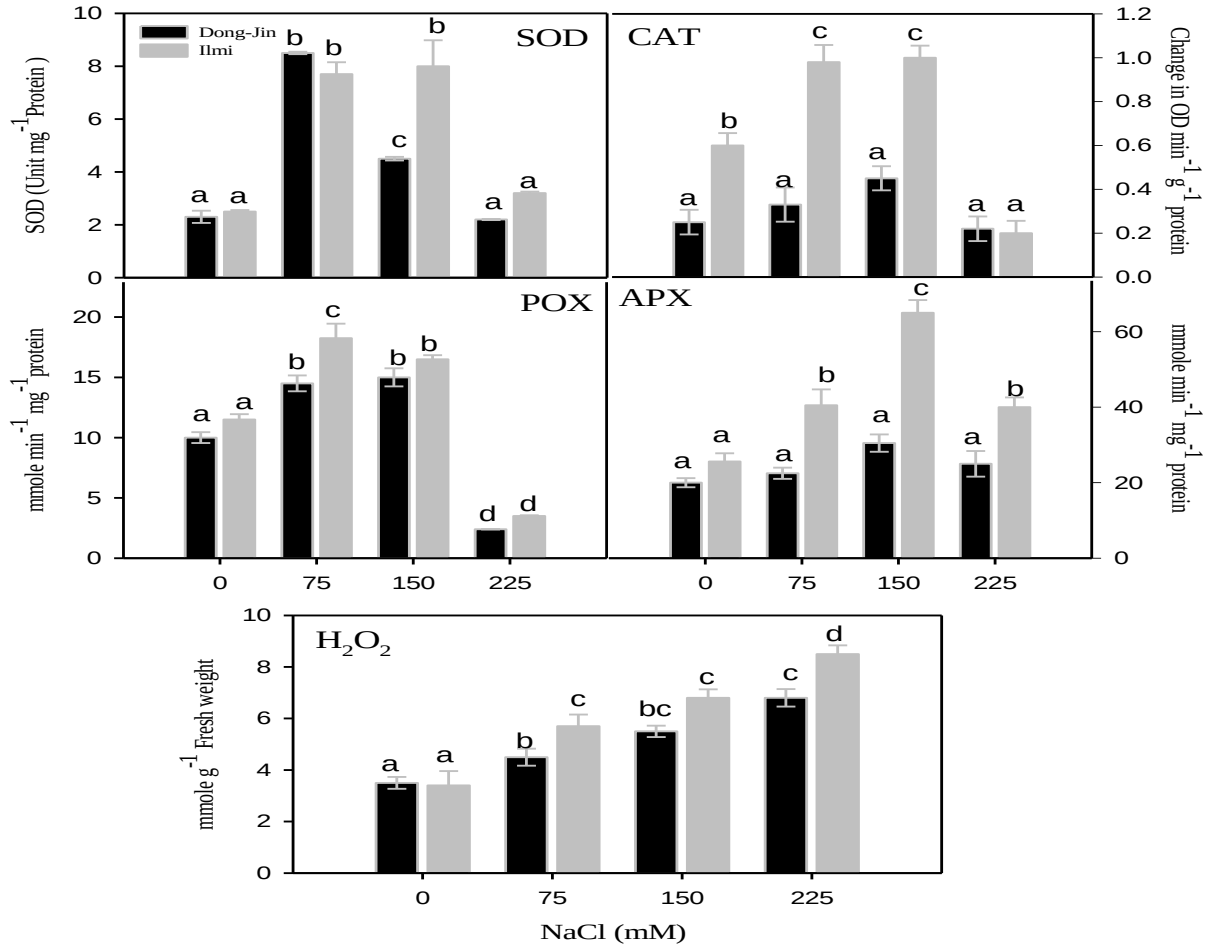

Fig. 4. Antioxidant enzymes activities of Korean wild type rice (*Oryza sativa* L.) cultivars in salinity prone environment. Abbreviations: SOD - superoxide dismutase, CAT- catalase, POX- peroxidase and APX- ascorbate peroxidase and H_2O_2- Hydrogen peroxide activities. Bars followed by the same letter in the same treatment are not significantly different according to Duncan Multiple Range Test at $p<0.05$ level. Vertical line on bar represent SE.

The protective role of SOD against salt stress is well established. Our results showed that salinity caused a significant decline of SOD activity in the Dong-Jin variety while in Ilmi, SOD activity slightly enhanced in response

to salt treatment (Fig. 4 and 5). Singha and Choudhuri (1990) also observed that NaCl decreased SOD activity in rice seedlings. However, the possible reason of the inhibition of SOD activity under salt stress may be a consequence of an altered synthesis and accumulation of less active enzymes in salt-treated plants that cannot be entirely ruled out. While the increase in SOD activity in response to stresses is possibly attributed to the *de-novo* synthesis of the enzymatic protein (Slooten *et al*., 1995 and Allen *et al*., 1997). APX is one of the important enzymes after SOD that has a major role in the detoxification of ROS (Luwe *et al*., 1993) and is required for the complete neutralization of H_2O_2 (Ushimaru, 1992). In this experiment, APX activity was at peak in 150 mM NaCl and the decline was noticed in 225mM NaCl treatment. Among the observed enzymes, APX activity relatively higher at 225mM as compared to other enzymes. The enhancement of APX activity observed in rice cultivars which may be due to the expression by oxidative stress as observed by Morita *et al*. (1999) in the germinating rice embryos. Our results are in accordance with the findings of Siddiqui *et al*. (2008) and Majeed *et al*. (2010) they described that in single stress it works more efficiently to get rid of H_2O_2 against abiotic stress.

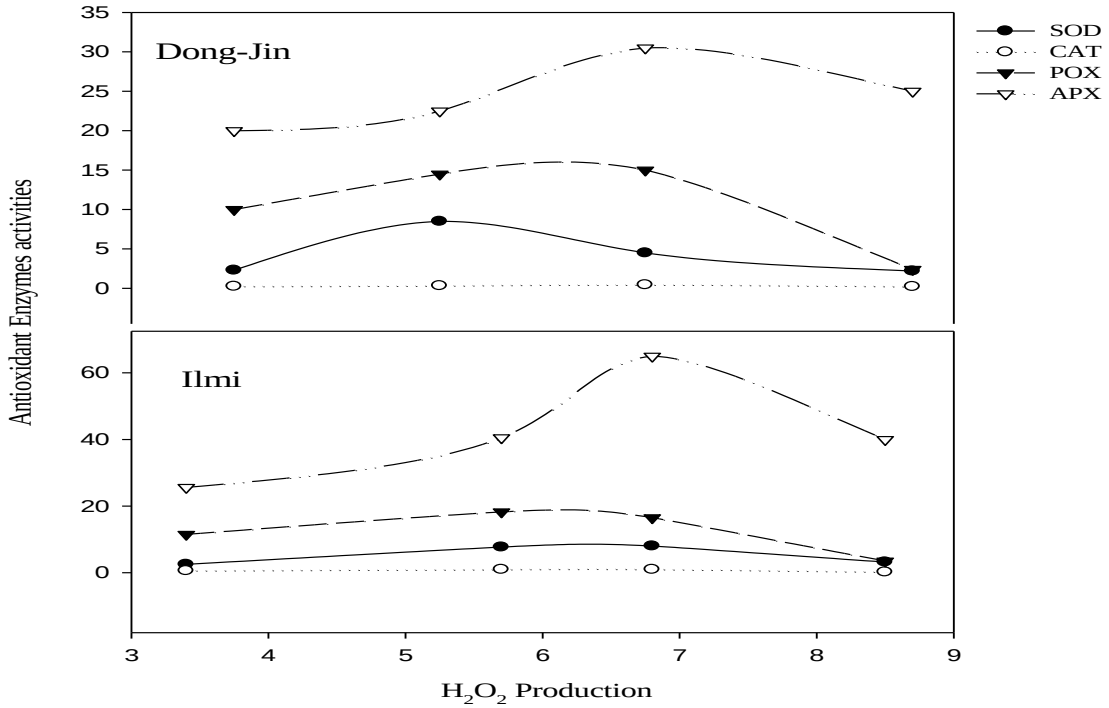

Fig. 5. Antioxidant enzymes' activities of Korean wild type rice (*Oryza sativa* L.) cultivars relative to hydrogen peroxide production under salinity prone environment. Abbreviations: SOD - superoxide dismutase, CAT- catalase, POX- peroxidase and APX- ascorbate peroxidase and H_2O_2- Hydrogen peroxide activities.

CAT degrades hydrogen peroxide to H_2O and O_2 by dismutation. It works together with SOD. There was considerable decline in the CAT activity under different salinity regime in both the tested cultivars. Comba *et al.* (1998) reported that salt induced stress reduces catalase activity. POX enzyme in plants is involved in the biosynthesis of cell wall (Negrel and Lherminier, 1987) including lignification and suberization (Polle *et al*., 1994 and Espelie *et al*., 1986). Our results showed that at higher level of salinity treatment, the activity is much affected as compared to other antioxidant enzymes whereas POX activity was observed to be much greater under 75 mM and 150 mM NaCl concentration. Various researchers dealing with rice (Mittal and Dubey, 1991) have also reported increase in POX activity in salt-sensitive cultivars under salt stress. Considerable evidence shows that high POX activity is correlated with the reduction of plant growth (MacAdam *et al*., 1992 and Zheng and Huystee, 1992). It is not clear whether the observed increase in POX activity under salt stress was due to increased activity of POX encoding genes or increased activation of already existing enzymes. Notwithstanding the other physiological mechanisms involved, the observed decrease in Dong-Jin growth at high salinity level might then be due to salt-induced increases in POX activity. The results of this study show that there were substantial differences between the growth and antioxidant responses of the both rice varieties, of Korean origin, to salinity treatment. During salt stress, Dong-Jin showed a decline in SOD activity, increase in POX activity and decrease in growth rate. Salinity however, had a minimal effect on antioxidant metabolism in the Ilmi, and its growth rate slightly enhanced at moderate

salinity level. Thus, from the present investigation it can be concluded that increased antioxidant activity could not alleviate salinity damage completely because dry matter production was not in accordance to high levels of antioxidants activity. The results of this study are in line with previous reports, which showed that plants when imposed to salinity stress produce ROS which scavenge by the antioxidant enzymes.

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