Effect of Salt and Drought Stresses on Some Physiological Traits of Three Rice Genotypes Differing in Salt Tolerance

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Abstract

Salt and drought stresses are among major constraints to rice (*Oryza sativa* L.) production. In order to find some keys for better understanding of the mechanism of salt tolerance in rice, we compared the impact of salinity and drought on seedlings of three rice cultivars including IR29, FL478 and Gharib, which are salt sensitive, tolerant and moderately tolerant respectively. The two days seedlings were transferred to MS media complemented with iso-osmotic concentrations of NaCl (0, 50, 100 and 150 mM) or mannitol (0, 100, 180 and 275 mM) for 10 days. Both salt and drought stresses resulted in reduction of shoot biomass, alteration of protein content and antioxidant activities in all studied cultivars. However, some isoforms of antioxidant enzymes were induced by drought and suppressed by salinity. Moreover, proline content was considerably increased only under salt treatments. Also, there were some variations regarding response to each stress in different cultivars such as induction of some protein and peroxidase bands by salt stress as well as proline accumulation by drought which were exclusively observed in FL478. These responses together with higher superoxide dismutase activity of FL478 under salinity probably play some roles in higher osmotic tolerance of this recombinant cultivar. Comparative analysis of the studied rice genotypes confirmed the importance of the osmotic component of salt stress in rice, since the salt tolerant cultivar FL478 displayed less injuries caused by drought compared to Gharib and IR29 respectively.

Keywords: Oryza sativa, NaCl, mannitol, proline, protein, antioxidant enzymes

Abbreviations: DMRT—Duncan's multiple range tests; GR—gluthatione reductase; L-DOPA—L-3,4-dihydroxyphenylalanine; Mr—molecular mass marker; MS medium—Murashige and Skoog medium; NBT—nitroblue tetrazolium; POX—peroxidase; PPO—polyphenol oxidase; PVPP—poly vinyl poly pyrrolidone; ROS—reactive oxygen species; SOD—superoxide dismutase.

Introduction

Salinity and drought stresses are among the most serious challenges to crop production in the world today, particularly in developing countries (Zhou *et al*. 2007). It has been estimated that more than half of the yield potential of major crops are usually lost due to unfavorable growing environments such as drought or high salinity (Cortina & Culiáñez-Macià 2005). A great deal of plants injuries caused by these osmotic stresses is associated with oxidative damage at cellular level (Bowler *et al.* 1992). Oxidative stress caused by accumulation of reactive oxygen species (ROS) results in perturbation of the overall cellular metabolism (Mittler 2002). In order to protect from deleterious effects of ROS, plants have evolved an antioxidant defense system that includes non-enzymatic compounds such as ascorbate and glutathione and enzymes such as superoxide dismutase (SOD), peroxidase (POX), gluthatione reductase (GR) and polyphenol oxidase (PPO) (Agarwal & Pandey 2004). Also several small organic molecules, known as osmoprotectants or compatible solutes, such as proline are thought to act as effective scavengers of ROS (Smirnoff and Cumbes 1989). Accumulation of proline has been observed in many plants exposed to salt or drought stresses (Garcia *et al.* 1997, Ibarra-Caballero *et al*. 1988, Hoai *et al*. 2003), however its actual roles in osmotic stress tolerance of plants remain controversial (Gao *et al*. 2008).

Rice is the staple food for more than half of the world's population (Khush 2005). It is one of the most sensitive cultivated species to salt and drought stresses (Lefèvre *et al.* 2001), so both water/soil salinity and low water supply are growing obstacles to rice production worldwide (Flowers & Yeo 1995,

Munns 2002). The response of rice to osmotic stress and salinity varies with the developmental stage. Moreover, the post germination stage is one of the most sensitive stages to salinity in rice (Grattan *et al.* 2002; Flowers & Yeo 1981; Lutts *et al.* 1995; Suriya-arunroj *et al.* 2004).

In the present research, we studied salinity and drought induced changes in fresh weight, proline content, protein content and electrophoretic profile and activity of antioxidant enzymes in three rice cultivars including IR29, Gharib and FL478. IR29 is an improved indica cultivar currently used as a salt-sensitive standard (Bonilla *et al.* 2002) and Gharib is an Iranian indica cultivar which is moderately salt tolerant. FL478 is one of the lines identified from the recombinant inbred population developed from IR29 and Pokkali, showing salt tolerance higher than or comparable to the tolerant parent, Pokkali (Walia *et al.* 2005). Impact comparison of iso-osmotic concentrations of NaCl and mannitol on young rice seedlings would clarify the share of osmotic versus ionic damage at salinity stress and make it possible to compare the rice response mechanisms to salt and drought stresses in this sensitive developmental stage. Moreover, in spite of several studies about the role of osmoprotectants and antioxidant enzymes in abiotic stress tolerance of rice, data on the genotypes variations are limited (Morsy *et al.* 2005). Therefore, comparative analysis of the osmotic stresses effects on these cultivars will improve our understanding on the difference of osmotic stress response mechanisms in them and clarify the stress tolerance mechanisms in FL478.

Materials and methods

Plant Materials and Stress Treatments- Seeds of three rice (*Oryza sativa* L.) cultivars including IR29, FL478 and Gharib were dehulled and the surface was sterilized. Then they were germinated on petri dishes containing MS media (Murashige and Skoog, 1962) solidified with 7.5 g l^{-1} agar at 25 °C. For exposing to salt and drought stresses, two days seedlings were transferred to the glass containers having MS media complemented with iso-osmotic concentrations of NaCl (0, 50, 100 and 150 mM) or mannitol (0, 100, 180 and 275 mM) respectively. These various iso-osmotic concentrations corresponded to osmotic potentials of −0.5, −0.7 and −0.95 MPa determined by a vapor pressure osmometer (Wescore, 5520, United States). Control medium had an osmotic potential of −0.41 MPa. Seedlings were grown under 16 h photoperiod (white fluorescent lights; irradiance of 230-250 μmol m^{-2} s^{-1}) at 25 °C for 10 days. Fresh weight of shoot and root were recorded in 12 day-old seedlings with eight replicates. Whole shoot and root of each seedling were assayed in the experiments.

Physiological Assays

Proline- Free proline was determined by the method of Bates *et al*. (1973) using L-proline as the standard. About 0.5 g of plant fresh material was homogenized in 10 ml of 3 % aqueous sulphosalicylic acid completely. One ml of the extract was diluted with 1 ml of distilled water and reacted with 2 ml of glacial acetic acid for 1 h at 100 °C and then it was cooled rapidly to 0 ºC on ice. The reaction mixture was extracted with 4 ml toluene and the absorbance was measured by spectrophotometer (Shimadzu, UV-160, Japan) at 520 nm.

Protein and antioxidant enzymes- For determination of total protein content and enzyme activity, 1 g of fresh seedlings was homogenized in 3 ml of 25 mM sodium phosphate buffer (pH 6.8), 3% poly vinyl poly pyrrolidone (PVPP). The homogenate was centrifuged (Heraus centrifuge, D63, Germany) at 13000 *g* for 1 h and the supernatant was used for protein determination and enzyme assays. All the steps were carried out at 4 °C. Total protein content of the extracts was determined according to the spectrophotometric method of Bradford (1976), using BSA as the standard.

Superoxide dismutase- (SOD; EC 1.15.1.1) activity was determined by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) as described by Beauchamp and Fridovich (1971). The reaction mixture consisted of 0.05 ml enzyme extract and 1.5 ml contained 50 mM sodium phosphate buffer (pH 7.0), 13 mM L-methionine, 75 μM NBT, and 2 μM riboflavin. The reaction was started by exposing the mixture to white fluorescent light (irradiance of 50 μmol m^{-2} s^{-1}) for 15 min. The reduction in NBT was measured by reading absorbance at 560 nm. Blanks and controls were run in the same manner but without illumination and enzyme respectively. One unit of SOD was defined as the amount of enzyme required to cause 50 % inhibition of NBT reduction under the

assay conditions (Giannopolitis and Ries 1977).

Peroxidase- (POX; E.C. 1.11.1.7) activity was assayed following the method of Abeles and Biles (1991). The reaction mixture contained 2 ml of 0.2 M acetate buffer (pH 4.8), 0.2 ml H_2O_2 (3 %), 0.1 ml 20 mM benzidine in 50 % methanol and 0.05 ml enzyme extract. The rate of benzidine oxidation was measured by spectrophotometer at 530 nm.

Polyphenol oxidase- (PPO; E.C. 1.10.3.1) activity was estimated according the modified method of Raymond *et al.* (1993) by measuring the increase in absorbance at 430 nm. The reaction mixture contained 0.8 ml 200 mM sodium phosphate buffer (pH 7.6), 0.02 ml pyrogallol 200 mM and 0.02 ml enzyme extract. The temperature of the reaction mixture was 28 °C.

SDS-PAGE- Discontinuous sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (1970) with 12 % acrylamide gels. Twenty five µg of protein from each extract sample was loaded. For detection of proteins, gels were stained with 0.03 % Coomassie Brilliant Blue G250.

PAGE- For isoforms assay, native gel electrophoresis (non-denaturing conditions) was carried out according to modified method of Davis (1964) with a 12 % (for SOD and PPO) and 10% (for POX) (w/v) polyacrylamide gel at 4 °C. A vertical electrophoresis apparatus (peQLab, Belgium) was used. The electrophoresis run was carried out with 120 mV (30 mA) per plate towards the cathode. To determine the pattern of SOD isoforms, gels were shaked for 40 min in the dark at room temperature, in 100 ml 0.05 M Tris-HCl buffer (pH 8) containing 4 mg riboflavin, 2 mg Na-EDTA and 20 mg NBT. Then, the bands became apparent in 5-10 min at light (Wendel and Weeden 1989). Electrophoresis patterns of POX and PPO were obtained as described by Van Loon (1971). For visualizing the isoforms of POX the gels were immersed in 80 ml 0.2 M acetate buffer (pH 4.8) containing 8 ml 3 % H_2O_2 and 4 ml 0.04 M benzidine in 50 % methanol shaking in the dark at room temperature till the brown color appeared. Isoforms of PPO were visualized by immersing the gel in 50 ml 0.2 M phosphate buffer (pH 6.8) containing 20 ml 0.5 % 3,4-dihydroxyphenylalanine (L-Dopa) and 0.7 ml 55.5 % $CaCl_2$ in the dark at room temperature on the shaker till the detection of enzymatic bands.

Statistical analysis- All the experiments were repeated at least three times and were done on a completely randomized design. The data were analyzed by one-way *ANOVA*, and the treatment mean values were compared by Duncan's multiple range tests (DMRT) at $P \leq 0.05$ using *MSTATC* software (version 1.42). Mean values and standard deviations for each treatment are shown in the table 1 and figures 1-3.

Results

Greater phenotypic modifications of rice seedlings were observed under drought compared to salinity, particularly in Gharib and IR29 cultivars. The color of leaf tips in IR29 seedlings turned to brown by 180 mM mannitol treatment. However, 275 mM mannitol caused brownish color of the shoots in both IR29 and Gharib cultivars.

Most of the stress treatments led to significant decline in fresh weight of seedlings shoots (Fig. 1a). However, drought stress had greater effect on IR29 and Gharib compared to FL478. Root fresh weight decreased by all salinity and drought treatments in IR29, except for 100 mM mannitol, but it was not affected by most of stress treatments in Gharib. Root fresh weight of FL478 even increased by drought stress, in spite of its decline under salt treatments (Fig. 1b).

All NaCl concentrations significantly increased proline content in all the cultivars, but it was not the case under drought stress. Only the proline content of FL478 increased by drought at highest concentration of mannitol (Fig. 1c).

Total protein content of the seedlings was raised under most of the stress treatments (Table 1).

According to the SDS-PAGE analysis, there were a few differences between the protein patterns of salt and drought treated seedlings (Fig. 2). A protein band with the molecular mass of higher than 116 kDa was detected at control and drought treatments while it was disappeared at salt treatments in all cultivars. The intensity of a protein band with the molecular mass of 45.0 to 66.2 kDa decreased under 100 and 150 mM NaCl in Gharib and IR29, while another protein band with the molecular mass of approximately 116 kDa was induced by salinity in Gharib. There were some other differences in the protein banding pattern of Garib with the molecular

mass ranging from 18.4 to 25.0 kDa under salt and drought treatments.

Table 1: Content of total proteins and activities of POX, SOD and PPO in seedlings of three rice cultivars under salt and drought stresses (N: mM NaCl, M: mM Mannitol). Values are means ± standard deviations (n=3). Values marked with different letters are significantly different within each cultivar according to DMRT at $P \leq 0.05$.

Cultivar	Treatment	Protein (mg/g fr w)	POX (unit/mg protein)	SOD (unit/mg protein)	PPO (unit/mg protein)
FL478	Control	3.58 ± 0.06 d	161.28 ± 10.29 a	6.29 ± 0.33 a	3.60 ± 0.27 b
	50N	2.74 ± 0.09 e	146.00 ± 20.49 a	6.35 ± 0.39 a	3.49 ± 0.03 b
	100N	3.69 ± 0.35 d	155.37 ± 7.80 a	6.50 ± 0.24 a	4.47 ± 0.42 a
	150N	5.18 ± 0.39 c	127.36 ± 4.53 b	4.17 ± 0.07 b	5.14 ± 0.73 a
	100M	5.15 ± 0.30 c	83.15 ± 1.86 c	4.18 ± 0.14 b	3.52 ± 0.51 b
	180M	7.12 ± 0.06 a	64.80 ± 11.54 d	3.17 ± 0.13 c	3.36 ± 0.17 b
	275M	6.53 ± 0.25 b	85.16 ± 4.37 c	3.84 ± 0.14 b	1.59 ± 0.07 c
IR29	Control	5.18 ± 0.29 d	132.00 ± 0.11 bc	2.24 ± 0.21 d	4.26 ± 0.34 b
	50N	5.82 ± 0.04 bc	112.99 ± 11.81 de	1.87 ± 0.05 e	4.98 ± 0.15 a
	100N	5.67 ± 0.06 bc	134.11 ± 12.98 bc	2.63 ± 0.29 c	4.07 ± 0.01 b
	150N	5.99 ± 0.37 b	140.82 ± 3.70 b	3.01 ± 0.10 b	3.87 ± 0.39 b
	100M	7.83 ± 0.08 a	97.92 ± 7.70 e	1.74 ± 0.03 e	4.20 ± 0.42 b
	180M	5.53 ± 0.22 cd	120.91 ± 11.02 cd	2.60 ± 0.14 c	3.12 ± 0.30 c
	275M	5.28 ± 0.05 d	159.02 ± 7.22 a	3.52 ± 0.25 a	2.22 ± 0.02 d
Gharib	Control	4.65 ± 0.61 bc	172.18 ± 2.72 ab	3.03 ± 0.30 a	3.09 ± 0.16 d
	50N	5.74 ± 0.38 a	101.95 ± 9.78 e	1.45 ± 0.18 d	4.88 ± 0.12 b
	100N	4.28 ± 0.05 bc	164.11 ± 9.60 b	2.27 ± 0.07 b	6.03 ± 0.58 a
	150N	4.95 ± 0.38 ab	121.50 ± 1.79 d	2.95 ± 0.12 a	2.43 ± 0.03 e
	100M	3.81 ± 0.68 c	186.67 ± 17.49 a	2.54 ± 0.20 b	3.77 ± 0.20 c
	180M	5.01 ± 0.16 ab	141.45 ± 11.80 c	2.29 ± 0.28 b	1.71 ± 0.07 f
	275M	5.67 ± 0.64 a	132.39 ± 6.30 cd	1.87 ± 0.10 c	1.65 ± 0.17 f

POX activity of IR29 was decreased under lower levels of salt and drought treatments while it was increased under highest exerted levels of them compared to control. POX activity was decreased by all stress treatments except 100 mM NaCl and mannitol in Gharib cultivar, while it was unchanged by 50 and 100 mM NaCl and decreased by other stress treatments in FL478 (Table 1). SOD activity of IR29 declined significantly under the least concentrations of NaCl or mannitol, but it was increased by higher levels of stress treatments. Moreover, iso-osmotic concentrations of NaCl and mannitol had nearly similar effects on the activity of total POX and SOD in IR29. In Gharib seedlings, SOD activity decreased by all stress treatments except 150 mM NaCl. Like POX, SOD activity of FL478 did not change significantly by 50 and 100 mM NaCl compared to control and decreased by other stress treatments. SOD activity was tremendously higher in FL478 compared to other cultivars under control and salt treatments (Table 1). The highest concentration of mannitol caused a decline in PPO activity of all studied cultivars, but other stress treatments affected the cultivars differently. Similar to the other studied enzymes, PPO activity of FL478 was not affected by mild salt stress (Table 1).

According to the PAGE analysis of POX, some POX isoforms induced by drought but suppressed by salinity in Gharib and IR29. In contrary, POX bands observed under control and drought treatments were also detected under high salinity in FL478 (Fig. 3). Drought induced similar SOD bands in FL478 and Gharib while these bands were not detected in IR29. Also, some PPO bands were induced by drought but were not observed under salinity in all cultivars (results not shown).

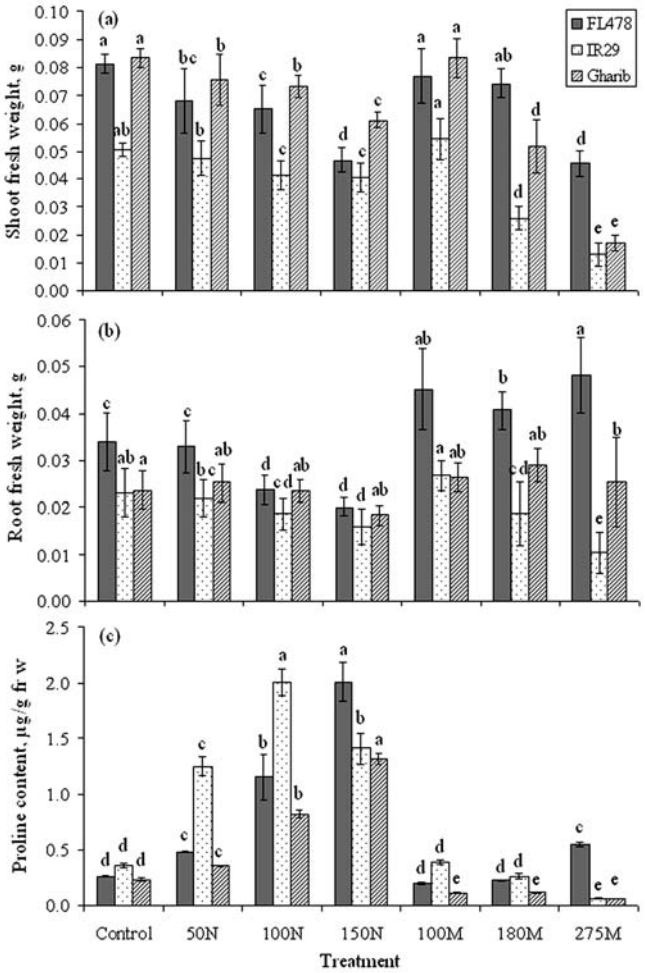

Fig. 1. Changes of shoot fresh weight (a), root fresh weight (b) and proline content (c) in seedlings of three rice cultivars under salt and drought stresses (N: mM NaCl, M: mM Mannitol). Values are means and standard deviations (n=8 for shoot and root fresh weight and n=3 for proline analysis); Values marked with different letters are significantly different within each cultivar according to DMRT at $P \leq 0.05$.

Discussion

Comparative analysis of the impact of salt and drought stresses on contrasting rice genotypes can improve our understanding about the stress tolerance mechanisms, clarifying similarities and distinctions of the systems involved in the responses to these two osmotic stresses in rice. In the present research, some physiological responses of three contrasting rice genotypes to iso-osmotic concentrations of NaCl and mannitol were studied comparatively at post germination stage. FL478 and Gharib are salt tolerant and moderately tolerant respectively, however, their mechanism of salt tolerance is not completely understood. It is just reported that FL478 maintains a lower Na-to-K ratio than both the parent lines (Suriya-arunroj *et al.* 2004) and also have good tillering capacity under salt stress (Walia *et al.* 2005).

According to the obtained results, mannitol treatments caused greater phenotypic changes such as color of the shoots in rice seedlings compared to NaCl. It can be due to NaCl absorption which declines the osmotic gradient between roots and medium under salt treatments compared to mannitol (Castillo *et al.* 2007).

The reduction of shoot fresh weight under most of the stress treatments is in agreement with some previous reports such as Castillo *et al.* (2007) who reported the reduction in aboveground biomass of another rice cultivar by salt and drought stresses.

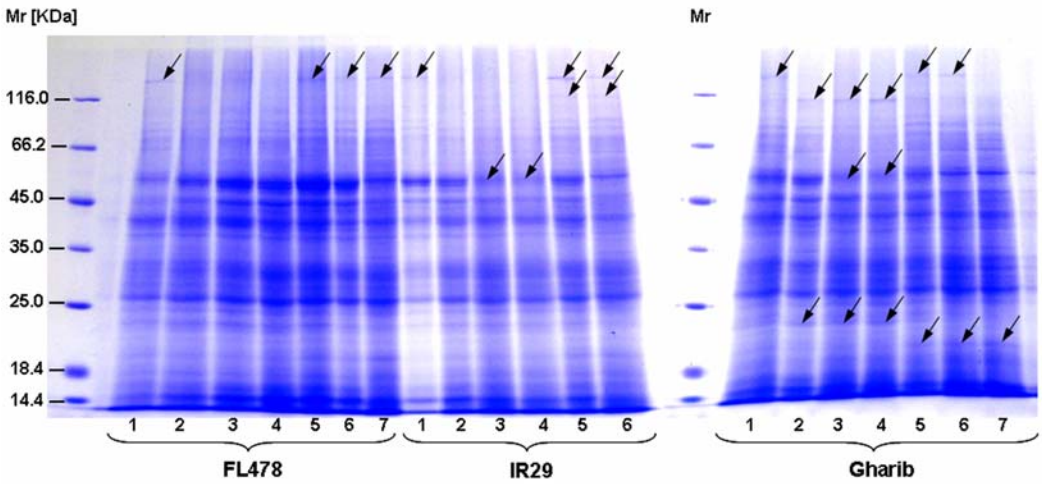

Fig. 2: SDS-PAGE pattern of proteins in seedlings of three rice cultivars under different levels of salt and drought stresses: Molecular mass marker (Mr), Control, 50, 100 and 150 mM NaCl (1 to 4), 100, 180 and 275 mM mannitol (5 to 7). *Arrows indicate the affected proteins.*

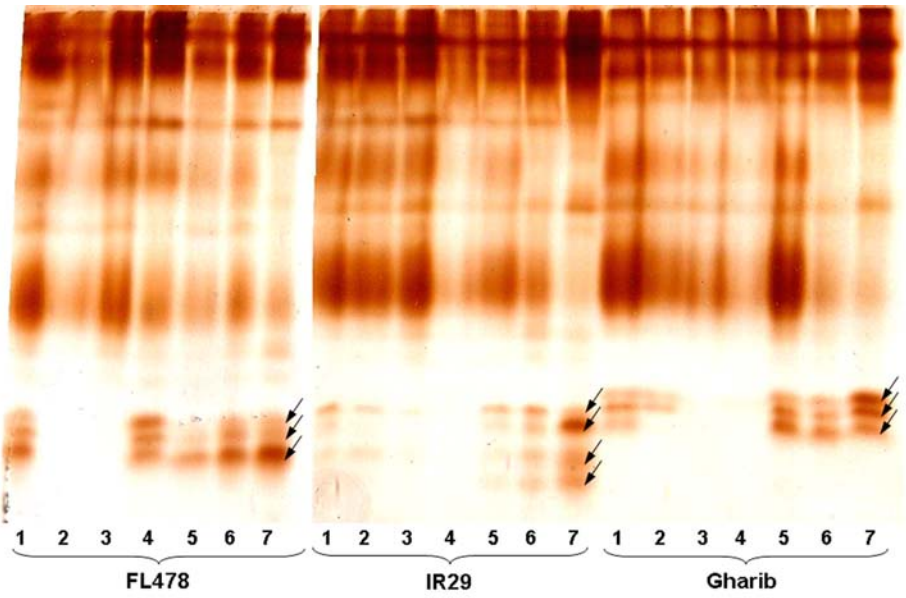

Fig. 3: Activity staining for POX in seedlings of three rice cultivars under different levels of salt and drought stresses: Control, 50, 100 and 150 mM NaCl (1 to 4), 100, 180 and 275 mM mannitol (5 to 7). ***Arrows indicate the affected isoforms.***

Unlike other stress treatments 100 mM mannitol did not affect fresh weight of the shoots, but reduced the shoot length. It can be due to increased accumulation of soluble proteins (observed in IR29 and FL478), soluble carbohydrates or other compatible solutes. Increase of root fresh weight in FL478 by drought stress can implicate on the root elongation in order to achieve more water and to accumulate compatible solutes in this tolerant cultivar. The effect of NaCl on proline accumulation is consistent with previous studies such as Garcia *et al.* (1997) and Hoai *et al.* (2003) who reported an increased accumulation of proline during salt stress in rice. We suggest that proline accumulation under salinity is a consequence of ionic component of salt stress, because the iso-osmotic concentrations of mannitol did not have such an affect on proline content. There are contrasting reports on the role of proline in salt or drought tolerance. Some reports implicate on the positive correlation of proline accumulation to drought and salt tolerance in several species including rice (Ahmad *et al.* 2007; Su and Wu 2004; Ghoulam *et al.* 2002), while other reports suggest that proline accumulation might mainly be a consequence of salt stress rather than being involved in its alleviation (Garcia *et al.* 1997, Hoai *et al.* 2003). In this study, proline accumulation was highest in the salt sensitive cultivar under 50 and 100 mM NaCl, so it might be a consequence of injury rather than being involved in the stress tolerance. However, highest level of drought stress had increasing effect on proline content of FL478, the cultivar which displayed less damage under drought compared to IR29 and Gharib.

The raise of seedlings protein content can be due to induction of specific proteins involved in stress tolerance/response. Beside their specific functions, proteins which are accumulated in the plants by stress exposure may provide a storage form of nitrogen that is reutilized when stress is over and probably play a role in osmotic adjustment (Niknam *et al.* 2006; Ahmad *et al.* 2007). The disappearance of a protein band with the molecular mass of higher than 116 kDa under NaCl treatments seems to be due to ionic component of salt stress which is a conserved response among different rice cultivars in this stage of growth. The observed difference between the intensity of a protein band with the molecular mass range of 45.0 to 66.2 kDa in FL478 and other cultivars, suggests a probable role of this protein in salt tolerance. Also the induction of some protein bands under stress treatments which exclusively occurred in Gharib, may play a role in higher osmotic stress tolerance of Gharib compared to IR29.

There are much evidence indicating alteration of the amount and activities of ROS scavenging enzymes by salinity, drought and other abiotic stresses in plants (Niknam *et al.* 2006). Based on present quantitative analysis of antioxidant enzymes, the enzyme activities were significantly

affected by the least concentrations of salt in IR29 and Gharib, while they were not significantly affected by mild salinity in FL478. These results were expected, as based on previous studies, FL478 maintains a low Na^+ to K^+ ratio in aboveground tissues, so it may be relatively less stressed at the cellular level compared to other cultivars and thus have a more limited response to salt treatments (Walia *et al.* 2005). Although SOD activity in FL478 decreased under some stress treatments, it was higher under all treatments in comparison with other studied cultivars even under control condition. So we can suggest high SOD activity of FL478 as a probable reason for its salt tolerance. Similarly, Salekdeh *et al.* (2002) suggested that greater salt-tolerance of rice cv. Pokkali compared with IR29 is due to a higher constitutive level of antioxidant capacity.

PAGE analysis revealed some differences between the effect of salt and drought on the isoforms of antioxidant enzymes in all studied cultivars. However, high salinity induced similar isoforms of POX to that of drought induced one exclusively in FL478. These POX isoforms probably play a role in higher salt tolerance of FL478.

In conclusion, exposure of salt or drought stress resulted in reduction of shoot fresh weight, alteration of protein content and antioxidant activities in all studied cultivars. Unlike drought, salinity caused accumulation of proline in the seedlings. On the other hand, some isoforms of POX, SOD and PPO were induced by drought and suppressed by salinity, though the activity of total isoforms of the enzymes were not higher under drought treatments compared to salinity. There were some variations between responses to each stress in FL478 and the other cultivars, that probably play some roles in higher tolerance of FL478 to salinity and drought, e.g. proline accumulation under drought was exclusively observed in FL478. SOD activity of FL478 was so higher than other cultivars under salt treatments and some of protein and POX bands induced by salinity exclusively in FL478. Phenotypic comparison of the studied rice genotypes, suggests a positive correlation between salt and osmotic stress tolerance in rice, since IR29 (salt sensitive cultivar) displayed more injuries caused by drought than Gharib and FL478, respectively. However, further work is required to understand the divergence of osmotic stress response/tolerance mechanisms in contrasting rice genotypes.

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