SALINITY STRESS-INDUCED ANTIOXIDANT ENZYME ACTIVITIES, LIPID PEROXIDATION AND ELECTROLYTIC LEAKAGE IN TWO RAGI VARIETIES

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Abstract: The effect of salinity on antioxidant enzymes, lipid peroxidation and electrolytic leakage were investigated in two ragi varieties (GPU-28 and Intaf-5). Salinity was given as a basal dose of different concentrations (0, 50, 100, 150mM) and sampling was done in leaves after 30 days of treatments. The activities of superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (POD), ascorbate peroxidase (APX) and glutathione reductase (GR) were assayed in the leaf extracts of control and salinity treated plants. Antioxidant enzyme activities were enhanced in two ragi varieties in response to salinity treatment. However, Intaf-5 showed significantly higher activities of all the five antioxidant enzymes in response to salinity compared to leaves of GPU-28. Lower rates of membrane lipid peroxidation and electrolytic leakage were noticed in the leaves of Intaf-5 than GPU-28. The present study suggested that the variety Intaf-5 has an efficient antioxidant mechanism, which could prevent the oxidative damage in the leaves caused by salinity stress.

Index Terms: Antioxidant enzymes, electrolytic leakage, lipid peroxidation, ragi, salinity.

I. Introduction

Plants experience a multiple of stress, of which salt stress is important one which affects tremendously the physiology of plants (El-Bastawisy, 2010, Negrao *et al*. 2019). As other major crops in India, ragi is also subjected to environmental stresses, particularly salinity. Finger millet (*Eleusine coracana* (L.) Gaertn) is an important minor cereal in India, rich in calcium, dietary fiber and known for its health benefits. The stresses most commonly associated with water deficits are drought, high salinity and low temperature (Bohnert *et al.* 1994). When CO_2 fixation is limited because of stomata closure caused by water deficit, the rate of active oxygen formation increases in chloroplasts because an excess of excitation energy that is not dissipated by the protective mechanisms, is used to form reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), superoxide ($.O_2^-$), hydroxyl radicals (.OH) and singlet oxygen ($^1O_2$) (Scandalios 1993, Mallik *et al.* 2011). Plants possess defense antioxidant mechanisms, which can overcome this oxygen toxicity and delay the deleterious effects of free radicals and these ROS attack lipids, proteins and nucleic acids, causing lipid peroxidation, protein denaturation and DNA mutation (Shao *et al.* 2008). Plants are endowed with a complex antioxidant system to cope with ROS (Noctor and Foyer 1998, Oliveira-Campos 2012, Gupta and Huang 2014), which includes three general classes: 1. lipid-soluble, membrane-associated antioxidants (e.g. $\propto$-tocopherol, β-carotene); 2. small, water-soluble antioxidant molecules (e.g. ascorbate, glutathione); and 3. enzymatic antioxidants. The enzymatic system in turn includes superoxide dismutase (SOD), which catalyze the reaction from superoxide ($.O_2^-$) to H_2O_2 and catalase (CAT), guaiacol-type peroxidases and enzymes of the ascorbate-glutathione cycle, e.g. ascorbate peroxidase (APX) and glutathione reductase (GR), which function to detoxify the H_2O_2 produced. Melondialdehyde (MDA) is decomposition product of polyunsaturated fatty acids of membranes under stress. The rate of lipid peroxidation level in terms of MDA can therefore be used as an indication to evaluate the tolerance of plants to oxidative stress as well as the sensitivity of plants to salt stress (Jain *et al.* 2001). Cell membrane stability is frequently related to salt tolerance in plants (Dionisio-Sese and Tobita 1998) and electrolytic leakage is usually used as an indicator of membrane injuries in salt treated plants (Mandhania *et al.* 2006). In this study, we investigated the relationship between salinity stress and the antioxidant responses in two ragi varieties. To find out the severity of salinity, also measured the membrane lipid peroxidation and electrolytic leakage.

II. Materials and Methods

The certified Finger millet seeds (varieties GPU-28 and Intaf-5) were procured from PASIC, Pondicherry. Seeds with uniform size were selected and the plants were raised in pots containing red and clay soil. After 20 days, seedings were thinned and plants of uniform vigor were maintained in each pot. The maximum irradiance (PAR, 400-700nm) available during growth was 1800-2000 µmol m^{-2} s^{-1} on clear day. Daily maximum and minimum temperatures were 29-33ºC and 20-22ºC, respectively. After germination, plants were watered for the first 20 days.

The seedlings were divided into four groups. One group of seedlings was maintained under non-salinized condition which served as control. The watering solution for control plants consists of tap water and one-fourth strength of Hoagland nutrients. Other three group were salinized by irrigation daily to soil capacity (500 ml d^{-1}) with the nutrient medium containing 50mM, 100mM and 150mM NaCl. All the plants used in this study were of comparable size. Young and fully matured leaves were taken at 30 days after salinity treatments for all the experiments described below.

Enzymes are extracted from leaf tissues using an ice-cold mortar and pestle, 60 mg polyvinylpolypyrrolidone and 1ml of following optimized extraction media: SOD (100mM K-phosphate buffer, pH 7.8, 0.1mM EDTA, and 0.1% Triton X-100); CAT, GR (100mM K-phosphate buffer, pH 7.0 and 0.1mM EDTA); APX (50mM K-phosphate buffer, pH 7.0 and 1mM ascorbate) and Peroxidase (POD)

(50mM K-phosphate buffer, pH 7.0). The resulting slurry was centrifuged at 15000Xg for 15min at 4^{o}C. The supernatants were collected and used for the assays of protein content by the method of Bradford (1976) and enzyme activities. The activity of SOD (EC 1.15.1.1) was assayed by measuring its ability to inhibit the photochemical reduction of nitro-blue tetrazolium (NBT) adopting the method of Beauchamp and Fridovich (1991). The 3ml reaction mixture contained: 50mM k-phosphate buffer (pH 7.8), 10mM methionine, 1.17mM rifoflavin and 56mM NBT and suitable enzyme extract. Rifoflavin was added in last and switching on the light started the reaction. The reaction was allowed to take place for 30 min. and stopped by switching off the light. The absorbance of solution was measured at 560 nm and from which the absorbance of the unirradiated reaction mixture that served as respective blank was deducted. A_{560} was plotted as a function of fresh matter equivalent of enzyme extract used in the reaction mixture. From the resultant graph fresh matter equivalents of enzyme extract corresponding to 50% inhibition of the reaction was read and considered as one enzyme unit.

The activity of CAT (EC 1.11.1.6) was estimated by measuring the rate of decomposition of H_2O_2 by the method of Havir and McHale (1987). Each 3 ml reaction medium contained 50mM K-phosphate buffer, pH 7.0, 12.5mM H_2O_2 and 20μl enzyme extract. The decrease in H_2O_2 followed the decline in optical density at 240nm and activity was calculated with the extinction coefficient (40mM^{-1}cm^{-1}at 240nm) for H_2O_2. Enzyme activity is expressed as mmol H_2O_2 reduced (decomposed) mg^{-1}protein min^{-1}. GR (EC 1.6.4.2) activity was measured by oxidized GSH – dependent oxidation of NADPH using the method of Foyer and Halliwell (1976). The reaction mixture contained 25mM Tris-$MgCl_2$ (pH 7.6), 5mM NADPH, 50mM GSSG and 1ml enzymes extract. The change in absorption at 340nm (E=6.2 mM^{-1}cm^{-1}) was recorded over 2.5 min. Enzyme activity is expressed as μmol NADPH oxidized mg^{-1}protein min^{-1}.

APX (EC 1.11.1.11) activity was estimated by monitoring the decline in absorbance at 240nm following Nakano and Asada (1981). Each 3ml reaction medium contained: 0.25M ascorbate, 50mM K-phosphate buffer, pH 7.0, 0.1mM H_2O_2 and 20 μl enzyme extract. The amount of ascorbate oxidized was calculated using extinction coefficient of 2.8 mM^{-1}cm^{-1} and the activity has been expressed as mmol ascorbate oxidized mg^{-1}protein min^{-1}. POD (EC 1.11.1.7) activities were determined with guaiacol at 470nm (extinction coefficient 25.2mM cm^{-1}) following the method of Polle *et al.* (1994). The reaction mixture contained: 100mM K-phosphate buffer (pH 6.5), 16mM guaiacol, 10% H_2O_2 and enzyme extract in a 3ml volume. Enzyme activity is expressed as μmol substrate (guaiacol) oxidized mg^{-1} $^{-}$protein min$^{-1.}$

Lipid peroxidation rates were determined by measuring the malondialdehyde (MDA) equivalents according to Hodges *et al.* (1999). Total inorganic ions leaked out in the leaves during salinity stress were measured as described by Sullivan and Ross (1979).

For statistical analysis, five samples were taken for each treatment from five individual plants. Student's t-test and analysis of variance (ANOVA) were applied for analyzing significant differences between the control and treated plants ($P<0.05$).

III. Results and Discussion

Antioxidant levels and the activities of AOS scavenging enzymes have been correlated with tolerance to several different environmental stresses (Massacci *et al.* 1995). Superoxide dismutase, since discovered by McCord and Fridovich (1969) attracted the attention of many researchers because they are essential component in an organism's defense mechanism (Attia *et al* 2011). The SOD is the first enzyme involved in the antioxidative process (Rubio *et al.* 2002). This enzyme converts superoxide radical to hydrogen peroxide (H_2O_2) and molecular oxygen (O_2) (Zushi *et al.* 2009, Naji and Devaraj 2011). In this study, both the ragi varieties treated with salinity showed increased SOD activity compared to control plants (Fig. 1). For instance, SOD activity was enhanced to the tune of 47% (225.16 Units/mgprotein/min.) in Intaf-5 and 30% (165.11 Units/mgprotein/min.) in GPU-28 as compared to respective control plants (120.12 Units/mgprotein/min., 117.28 Units/mgprotein/min., respectively) under higher (150mM) salinity treatments. However, SOD activity in Intaf-5 in response to salinity was relatively high compared to GPU-28 at all the treatments. Although activity of SOD in the two ragi varieties in response to high salinity treatment may suffice to withstand the amount of oxidative stress, our results clearly show that Intaf-5 is more tolerant than GPU-28.

Catalase (CAT) and peroxidase (POD and APX) appear to play an essential protective role in the scavenging processes when coordinated with SOD activity (Massacci *et al.* 1995). They are chloroplastic or cytosolic enzymes which scavenge H_2O_2 generated primarily through SOD action. An increase in the activity of CAT was observed in both varieties of salinity treatment (Fig. 2). The activities of guaiacol peroxidase (POD) and ascorbate peroxidase (APX) increased almost coordinately with SOD activity in both ragi varieties (Table 1 and 2). As observed in case of SOD, higher activities of CAT, POD and APX were observed in Intaf-5. Another factor that would contribute to counter the oxidative stress by maintaining reduced glutathione content at the cellular level is the activity of the enzyme glutathione reductase (GR). An increase in the GR activity was also observed in both ragi varieties (Fig. 3) and we ascribe this due to *de nove* synthesis (Kaymakanova *et al.* 2010). In this study, 49% of GR activity was observed in Intaf-5, while 33% in GPU-28 under high salinity treatments. The elevated levels of GR might be able to increase the ratio of $NADP^+$/ NADPH, thereby ensuing the availability of $NADP^+$ to accept electrons from the photosynthetic electron transport chain (Baisak *et al.* 1994).

It is conceivable that acquisition of the stress tolenrance in any plant is a multi –factorial function and amelioration of AOS–scavenging systems is an important index to assess the abilities of ragi varieties to tolerate the stressful conditions like salinity. We presume that the metabolism of the active oxygen species under stressful environment is dependent on different functionally interrelated antioxidant enzymes. The increased activities of AOS scavenging enzymes should have a greater significance as invaluable tools in the elucidation of plant metabolic regulation under stressful environment. SOD is an essential component of these defense mechanisms as it dismutase to produce hydrogen peroxide and oxygen (Allen 1995; Scandalios 1993). Hydrogen peroxide has been implicated as an essential elicitor of several different genes related to both abiotic and biotic stress tolerance (Levine *et al.* 1994, Prasad *et al.* 1994). The reduction of H_2O_2 by ascorbate–glutathione cycle is an extremely efficient reaction sequence that dissipates energy and aids in the adjustment of ATP/NADPH ratios at times, when the severity of the salinity is more. Certain POD isomers utilize the phenolic compounds and H_2O_2 to initiate the biosynthesis of several secondary metabolites required for the plant growth, development and differentiation (Gaspar *et al.* 1991). A significant increase in the POD activity, using guaiacal as an artificial substrate under the stress conditions like salinity, indicates the formation of large amounts of H_2O_2 in ragi leaves which indicates that ragi is capable of effectively scavenging the AOS for the production of certain secondary metabolites to withstand during salinity stress. GR activity is believed to be an important factor, limiting the degree of photo–damage experienced by the ragi under salinity stress conditions. This enzyme has been suggested to play a pivotal role in the glutathione cycle in the eukaryotic cells (Noctor and Foyer 1998). GR over producing plants had a greater capacity to regenerate ascorbate during oxidative stress (Foyer *et al.* 1995). From this study, it is clear that the damage which was inflicted by salinity can be ameliorated by over–expression of antioxidant enzymes as noticed in Intaf-5 and there are certain variations in the activity of these antioxidant enzymes between two varieties to counteract the stresses.

Lipid peroxidation is a destructive chain reaction and it can directly damage the structure of membrane (Koca *et al.* 2006). Salt stress affected the activity of plasmamembrane ATPase activity (Mansour *et al.* 2000) and peroxidation of memebrane lipids is an indication of membrane damage and leakage under salt stress conditions (Misra and Gupta 2006). Lipid peroxidation and electrolytic leakage were more in variety GPU-28 compared to Intaf-5 under salt stress (Fig. 4). At higher salinity treatment, Intaf-5 showed 38% of membrane lipid peroxidation, while in GPU-28 by 50% when compared to respective control plants. Variation in MDA contents were found in rice (Tijen and Ismail 2005), cotton (Diego *et al*. 2003) and *Alfalfa* (Xiao-Shan and Jian-guo 2009) cultivars differing in salt tolerance. Lower levels of lipid peroxidation are associated with higher APX activity in drought or salt tolerant tomato (Shalata and Tal 1998), sugar beet (Bor *et al.* 2003) and rice (Demiral and Turkan 2005) plants. Also, higher electrolytic leakage was observed in GPU-28 under salinity stressed conditions (Fig.5). An undamaged plasma membrane is crucial to the survival of the whole cell (Bor *et al*. 2003, Azevedo-Neto *et al.* 2006) and electrolytic leakage can indicate plasma membrane injury induced by salt stress (Koca *et al.* 2007). Shi and Yin (1993) reported that salt stress increased the electrolytic leakage of plant cells.

IV. Conclusion

The two ragi varieties used in the present study have a different relative tolerance to high salinity and this tolerance was larger in Intaf-5 compared to GPU-28. The present study clearly shows that Intaf-5 is superior with respect to its antioxidant defense systems and should be more tolerant than GPU-28 due to higher AOS–scavenging systems and as well as showed lower rates of lipid peroxidation and electrolytic leakage. Such studies can be used in ragi breeding programmes or transgenic ragi research to generable plants with elevated activities of antioxidant systems for improved tolerance to salinity.

V. Acknowledgement

The authors thankful to Dr. R. Panneerselvam, Prof. and Head, Department of Botany for providing necessary Lab facilities.

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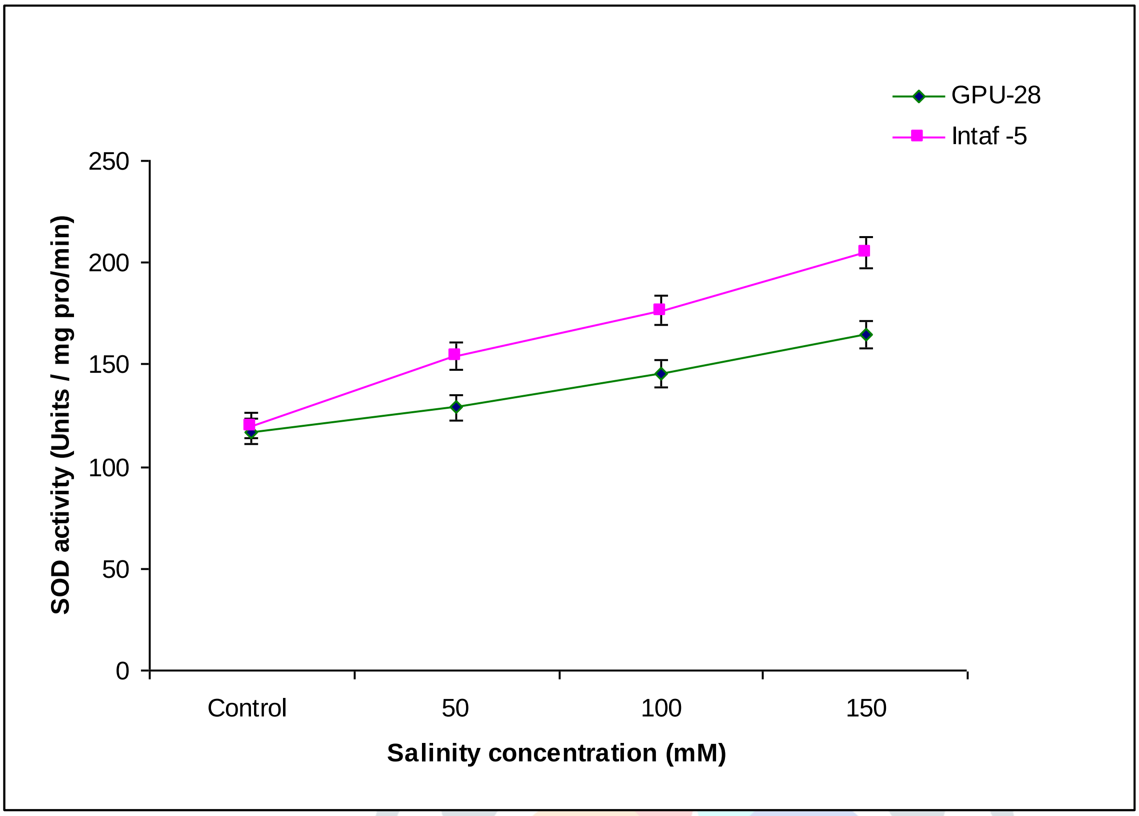
Fig. 1. The effect of salinity on the activity of superoxide dismutase in the leaf extracts of two finger millet varieties. Each value represents mean ± S.E. of five independent determinations



ig. 2. The effect of salinity on the activity of catalase in the leaf extracts two finger millet varieties. Each value represents mean ± S.E. of five independent determinations

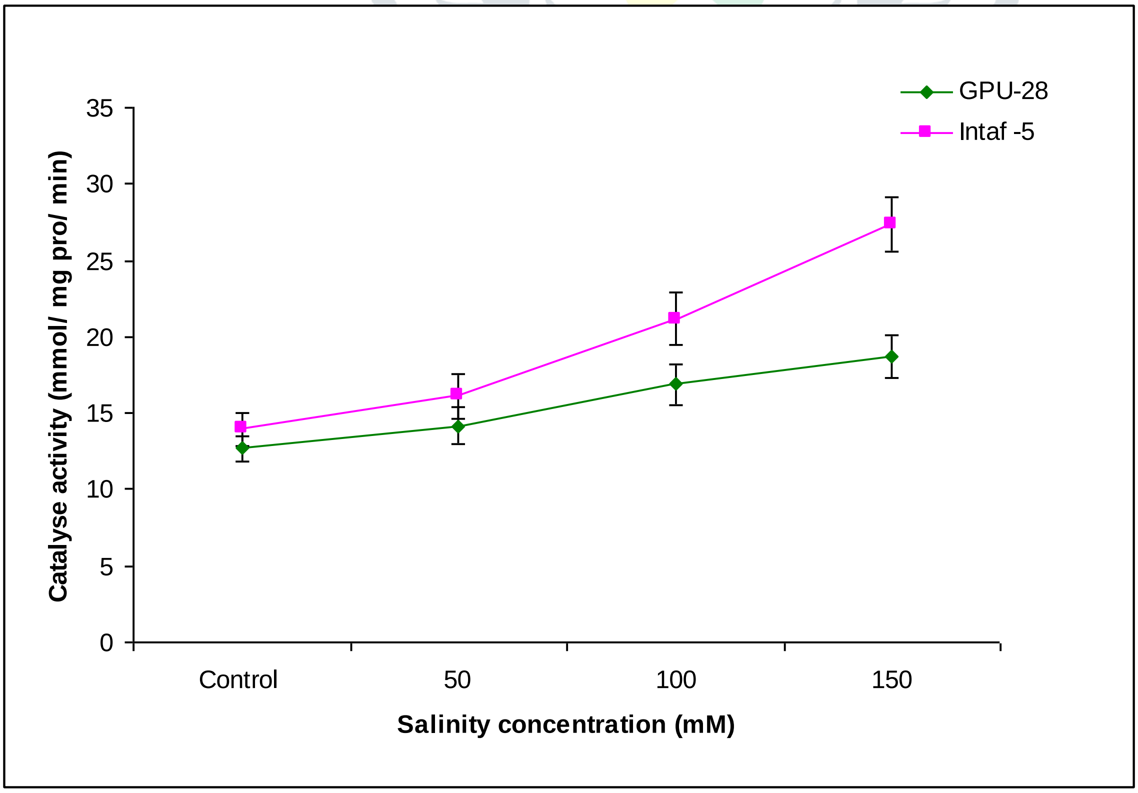


Fig. 3. The effect of salinity activity of on the glutathione reductase in the leaf extracts of two finger millet varieties. Each value represents mean ± S.E. of five independent determinations

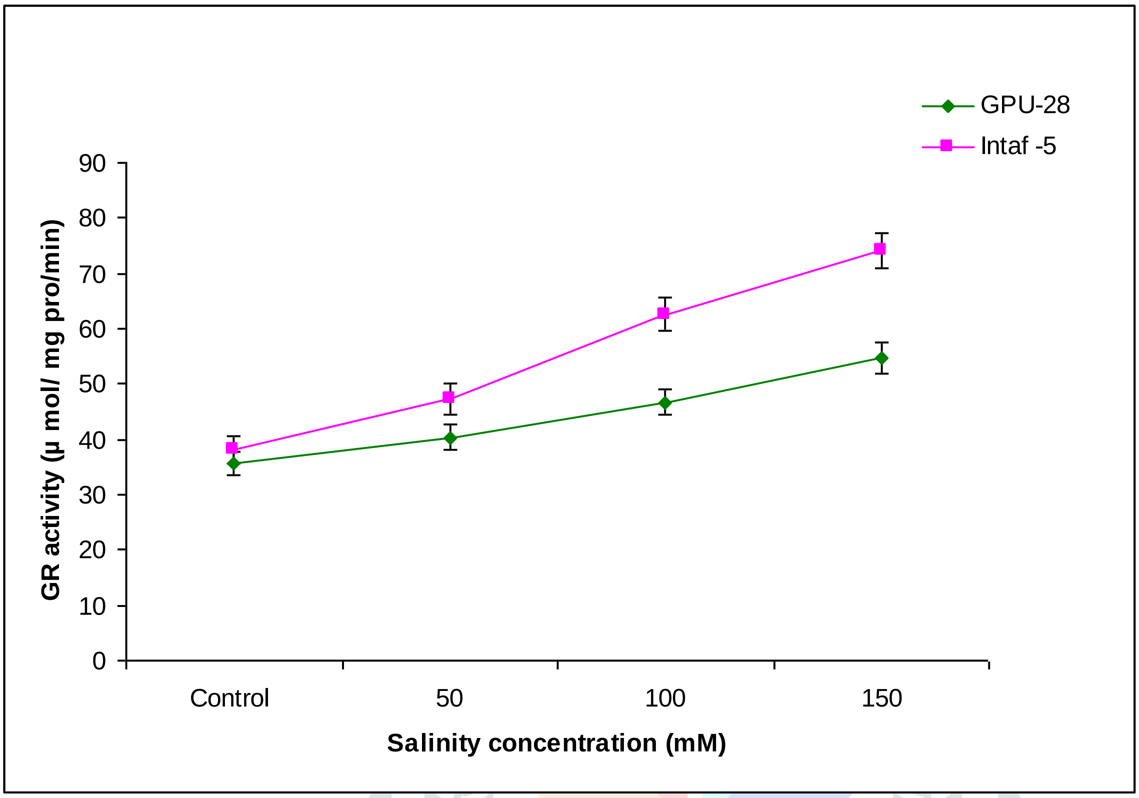


Fig.4. Salinity stress effects on lipid peroxidation in the leaves of two finger millet varieties. Each value represents mean ± S.E. of five independent determinations

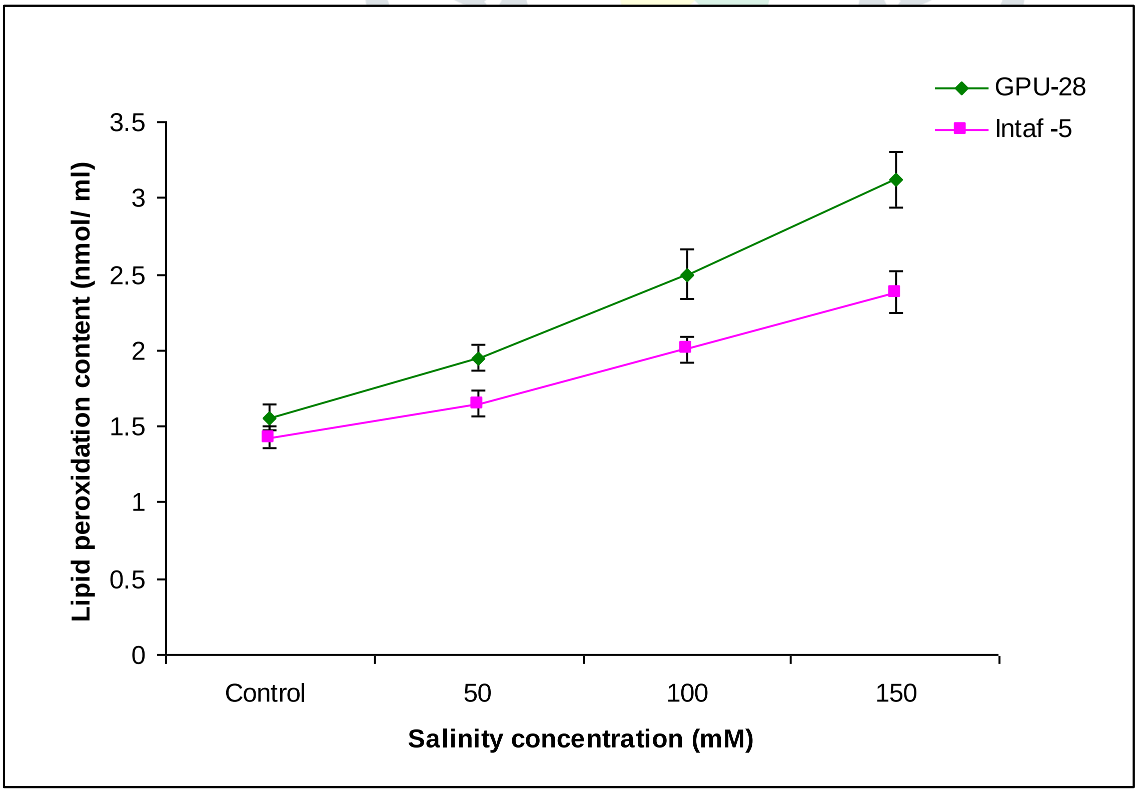


Fig. 5. Influence of salinity stress on electrolytic leakage in leaves of two finger millet varieties. Each value represents mean ± S.E. of five independent determinations

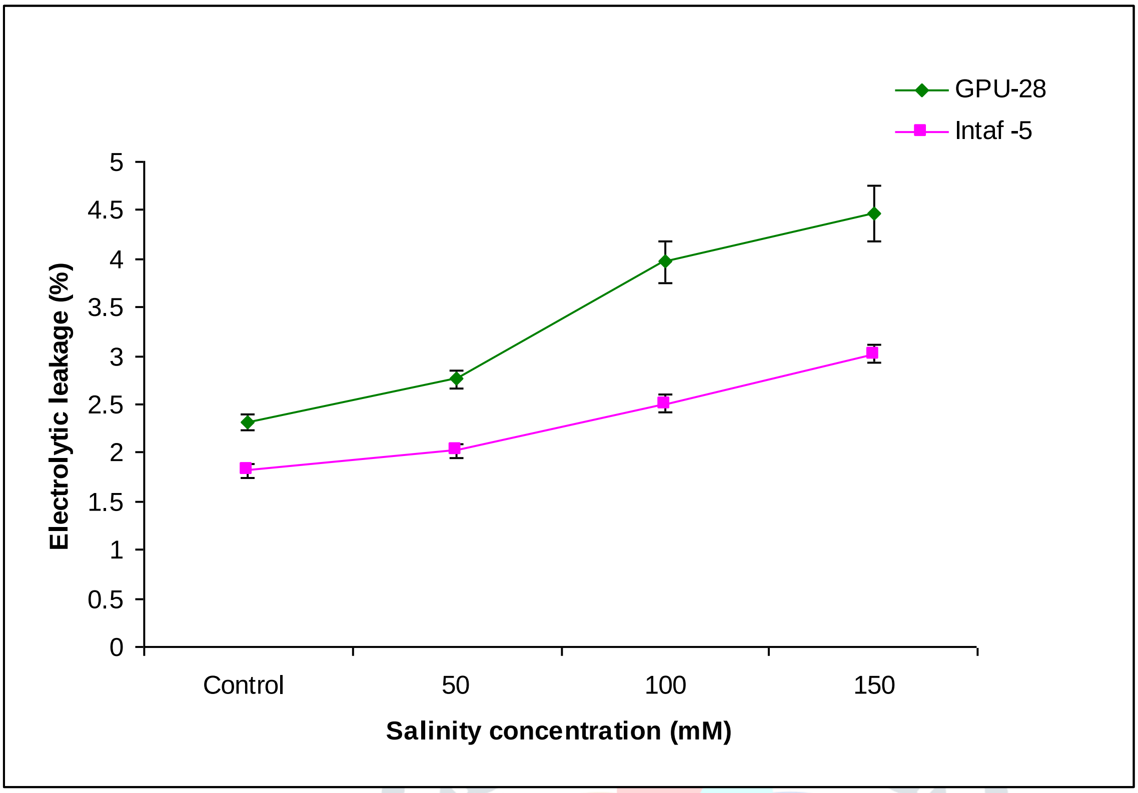


Table 1. Influence of salinity stress on ascorbate peroxidase (mmol/mgprotein/min) activity in two finger millet varieties.

Species	Salinity treatments (mM)			
	control	50	100	150
GPU-28	26.97 ±1.92	30.35 ±2.01	35.84 ±2.12	40.22 ±2.20
Intaf-5	28.72 ±1.95	35.02 ±2.09	45.18 ±2.22	54.47 ±2.31

The data are expressed as mean ±s.e. for five independent determinations (P<0.05).

Table 2. Peroxidase activity (μmol/mgprotein/min) in two finger millet varieties subjected to salinity stress.

Species	Salinity treatments (mM)			
	control	50	100	150
GPU-28	12.02 ±1.22	14.29 ±1.32	18.19 ±1.41	20.72 ±1.45
Intaf-5	13.11 ±1.29	16.98 ±1.38	23.12 ±1.47	26.31 ±1.54

The data are expressed as mean ±s.e. for five independent determinations (P<0.05)