J. Plant Biol., Vol. 34 (3), December, 2007, pp. 185–192.

ARTICLE

Differential Responses of Proline, Ion Accumulation and Antioxidative Enzyme Activities in Pearl millet [*Pennisetum glaucum* (L.) R. Br.] lines Differing in Salt Sensitivity

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MS received 24 January 2008; accepted 10 March 2008

Ten-day-old seedlings of pearl millet [*Pennisetum glaucum* (L.) R. Br.] mapping populations differing in their salinity tolerance levels were exposed to 0 and 150 mM NaCl concentrations for short durations of time (0 to 144 h) to assess the pattern of accumulation of proline, glutathione, Na^+, K^+, Ca^{2+} and Cl^- contents and their antioxidative enzyme activities. Salt-tolerant lines accumulated more proline and K^+ than the susceptible ones pointing their accumulation as a possible mechanism of salt tolerance. Specific activities of CAT, SOD and GR were higher in tolerant compared to the sensitive lines under salt stress conditions. High GST activity was noticed in the moderately tolerant line while the increase was transient (till 48 h) in the tolerant line. Lipid peroxidation as measured by MDA levels remained more or less same in the salt-sensitive line ICMB 90111, while it increased considerably in the tolerant line under salt stress till 96 h. The above comparative studies suggest that salt-tolerant and salt-sensitive lines of pearl millet possess differential oxidative components of both enzymatic and non-enzymatic machinery for scavenging ROS generated during salt stress.

Keywords: Antioxidant response, lipid peroxidation, ion accumulation, pearl millet, salinity stress.

Introduction

Irrigated lands are becoming increasingly saline year after year (Kavi Kishor *et al.*, 2005). Ion toxicity and decreased uptake of vital nutrients such as K^+ and Ca^{2+} under salt stress can also decrease growth and productivity of plants (Sangam, 1995). Organic compounds that accumulate in the cytoplasm may function as osmotica and thereby protect the conformation of macromolecules in the changing ionic environment (Ashraf and Foolad, 2007). The degree of oxidative cellular damage in plants exposed to salt stress is in general controlled by the response of the antioxidative enzymes such as SOD, CAT, APX, GR, and GST. Therefore, accumulation of osmoprotectants, ion homeostasis, exclusion or sequestration of the toxic ions such as Na^+ and Cl^- and effective enzymatic or non-enzymatic antioxidant responses are important components for the plants to survive and to reproduce under salt stress. Thus, understanding the molecular basis and mechanisms of gene regulation, signal transduction, ion transport and mineral nutrition will be helpful in developing selection strategies for improving salinity tolerance in crop plants. The mechanism of Na^+ exclusion, its transport and sequestering into vacuoles is not yet known for pearl millet. Estimations of proline, ion concentrations and measurements of reactive oxygen species in salt-tolerant and salt-sensitive lines of pearl millet treated with different concentrations of NaCl would give a clue for this type of information. This information is vital especially in mapping populations for later use in breeding programmes. The main objective of this study is to find out the short-term responses of proline, glutathione, ion accumulation, lipid peroxidation as measured by malondialdehyde levels and the antioxidative enzymatic activities in salt-tolerant and salt-sensitive lines of pearl millet.

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Materials and methods

Three inbred lines of pearl millet (ICMB 841 (=841B)-P3, 863B-P2 and ICMB 90111) obtained from the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, India were tested for salt stress tolerance over a range of salt concentrations (0–150 mM NaCl) and duration (0–144 h). Seedlings were allowed to grow for 10 days at 25°C under continuous fluorescent light ($30\ \mu Em^{-2}\ S^{-1}$) and then exposed to salt stress. Proline was determined by the method outlined by Bates *et al.* (1973). Proline content was expressed in terms of μg/mg dry weight of tissue. GSH was determined by Ellman's method (1959) and the glutathione content is expressed in moles of GSH/g fresh wt. For measuring Na^+, K^+, Ca^{2+} and Cl^- ions, 50 mg of dry weight of seedlings was used. Ions were extracted by boiling the dried seedlings in distilled water and incubated in a boiling water bath for an hour. Ion contents were estimated by using a Metrohm Ion Analyzer (Model No. AGCH-9101). Specific electrodes were used for estimations and standard solutions of Na^+, K^+, Ca^{2+} and Cl^- supplied by the company were used for calibration. Specific activities of antioxidant enzymes were determined as described previously by Jogeswar *et al.* (2006). Soluble protein content in the enzyme extract was measured according to the method of Bradford (1976). CAT activity was expressed as μl of H_2O_2 consumed/min/mg protein. Specific activity of SOD was expressed as units/mg protein. The unit is defined as the amount of the enzyme, which causes 50% inhibition of pyrogallol oxidation. Activity of GR was expressed in nmoles of NADPH oxidized/min/mg protein. GST activity is expressed in nmoles of CDNB conjugated/min/mg protein. Lipid peroxidation was determined following the method of Utley *et al.* (1967). The absorbance was read at 540 nm for the end product MDA, a TBARS, which is an index of lipid peroxidation. The content of MDA is expressed in nmoles/mg protein. Electrophoresis was carried out at 4°C according to a modified procedure of Gabriel (1971) with 10% polyacrylamide mini-slab gel in standard tris-glycine buffer (pH 8.3). After electrophoresis, a modified photochemical method of Beauchamp and Fridovich (1971) was used to locate SOD activities on gels. The data were analysed statistically and standard deviations were calculated using Sigma plot software. The level of significance was found out by performing standard *t*-test.

Results and discussion

The pearl millet inbred lines were categorized as sensitive (ICMB 90111), moderately tolerant (863B-P2) and highly tolerant (841B-P3) based on their differential abilities to maintain high germination levels and good seedling growth up to NaCl levels of 75 mM, 100 mM and 150 mM respectively (Mukhopadhyay *et al.*, 2005). Free proline levels were noticed to increase at 150 mM NaCl levels (Table 1) and were more pronounced in tolerant lines. Accumulation of ions (Na^+, K^+, Ca^{2+}, Cl^-) were measured and it was observed that increasing magnitude of salt exposure led to an increase in Na^+, K^+ and Cl^- ions (Table 2). The accumulation of Na^+ was slightly more in the ICMB 90111 (Table 2) compared to the other two lines. In contrast, accumulation of K^+ in the susceptible line was 3-folds, while it was 5 to 6-folds in the tolerant lines (Table 2). ICMB 90111 displayed no increase in the accumulation of Ca^{2+}, but it was much higher in the tolerant lines. The content of Cl^- did not vary much at 150 mM NaCl stress in the three lines.

Table 1. Proline content (μg/mg dry wt tissue) under short-term salt stress at 150 mM NaCl in pearl millet lines: sensitive (ICMB 90111); moderately tolerant (863B-P2) and highly tolerant (841B-P3).

	Proline content (μg/mg dry wt tissue)*	
	ICMB 90111	
Time	Control	150 mM NaCl
0	2.21 (± 0.18)	2.23 (± 0.28)
24	2.15 (± 0.33)	2.35 (± 0.19)
48	2.19 (± 0.41)	2.75 (± 0.29)
72	2.23 (± 0.25)	2.59 (± 0.56)
96	2.25 (± 0.21)	2.75 (± 0.36)
	863B-P2	
0	1.19 (± 0.12)	1.21(± 0.15)
24	1.25 (± 0.35)	1.56 (± 0.43)
48	1.16 (± 0.36)	2.89 (± 0.22)
72	1.21 (± 0.21)	3.58 (± 0.16)
96	1.25 (± 0.90)	3.65 (± 0.71)
	841B-P3	
0	0.55 (± 0.06)	0.61 (± 0.09)
24	0.52 (± 0.07)	0.90 (± 0.07)
48	0.63 (± 0.20)	1.95 (± 0.05)
72	0.65 (± 0.12)	2.79 (± 0.15)
96	0.59 (± 0.15)	4.05 (± 0.10)

*Data represent mean of ten replicates; Values in the parenthesis indicate standard errors, P value < 0.05.

Table 2. Ion content (mg/g dry wt.) in pearl millet under short-term salt stress at 150 mM NaCl: ICMB 90111; 863B-P2, and 841B-P3.

	Ion content (mg/g dry wt)*							
	ICMB 90111							
	Na^+		K^+		Ca^{2+}		Cl^-	
Time	0 mM NaCl	150 mM NaCl	0 mM NaCl	150 mM NaCl	0 mM NaCl	150 mM NaCl	0 mM NaCl	150 mM NaCl
0	0.019 (± 0.0)	0.021 (± 0.0003)	0.018 (± 0.0003)	0.022 (± 0.0009)	0.021 (± 0.0005)	0.020 (± 0.0006)	0.098 (± 0.0001)	0.100 (± 0.0005)
24	0.021 (± 0.0003)	0.029 (± 0.001)	0.020 (± 0.0005)	0.021 (± 0.0003)	0.019 (± 0.0006)	0.018 (± 0.0003)	0.095 (± 0.0001)	0.121 (± 0.0)
48	0.020 (± 0.0005)	0.048 (± 0.0005)	0.022 (± 0.0009)	0.023 (± 0.0002)	0.020 (± 0.0007)	0.020 (± 0.0004)	0.105 (± 0.0002)	0.156 (± 0.0001)
72	0.021 (± 0.0005)	0.062 (± 0.0004)	0.023 (± 0.0004)	0.041 (± 0.0005)	0.022 (± 0.0003)	0.017 (± 0.0009)	0.106 (± 0.0006)	0.126 (± 0.0002)
96	0.022 (± 0.0006)	0.072 (± 0.0003)	0.025 (± 0.0005)	0.054 (± 0.0005)	0.019 (± 0.0009)	0.016 (± 0.0002)	0.106 (± 0.0003)	0.128 (± 0.0003)
120	0.024 (± 0.0002)	0.078 (± 0.0001)	0.026 (± 0.0006)	0.059 (± 0.0009)	0.018 (± 0.0009)	0.019 (± 0.0001)	0.108 (± 0.0008)	0.134 (± 0.0009)
144	0.025 (± 0.0005)	0.082 (± 0.0004)	0.024 (± 0.0002)	0.069 (± 0.0008)	0.020 (± 0.0007)	0.021 (± 0.0005)	0.110 (± 0.0005)	0.142 (± 0.0002)
					863B-P2			
0	0.029 (± 0.0002)	0.031 (± 0.001)	0.021 (± 0.001)	0.023 (± 0.001)	0.020 (± 0.0005)	0.022 (± 0.0001)	0.085 (± 0.0004)	0.084 (± 0.0004)
24	0.029 (± 0.0002)	0.050 (± 0.0006)	0.023 (± 0.0005)	0.039 (± 0.0004)	0.021 (± 1.0002)	0.019 (± 0.0002)	0.082 (± 0.0006)	0.083 (± 0.0008)
48	0.031 (± 0.0006)	0.055 (± 0.0003)	0.022 (± 0.0003)	0.055 (± 0.0007)	0.019 (± 0.0009)	0.019 (± 0.0004)	0.085 (± 0.0006)	0.103 (± 0.0002)
72	0.030 (± 0.0007)	0.059 (± 0.0008)	0.023 (± 0.0003)	0.053 (± 0.0009)	0.017 (± 0.0007)	0.020 (± 0.0001)	0.089 (± 0.0008)	0.096 (± 0.0001)
96	0.031 (± 0.0001)	0.064 (± 0.0005)	0.025 (± 0.0002)	0.079 (± 0.0002)	0.018 (± 0.0008)	0.020 (± 0.0004)	0.088 (± 0.0003)	0.088 (± 0.0009)
120	0.032 (± 0.0009)	0.077 (± 0.0002)	0.025 (± 0.0005)	0.120 (± 0.0005)	0.020 (± 0.0008)	0.037 (± 0.0)	0.090 (± 0.0004)	0.091 (± 0.0002)
144	0.034 (± 0.0002)	0.086 (± 0.0003)	0.026 (± 0.0002)	0.159 (± 0.0003)	0.022 (± 0.0002)	0.032 (± 0.0002)	0.091 (± 0.0003)	0.106 (± 0.0004)
					841B-P3			
0	0.021 (± 0.0005)	0.023 (± 0.00023)	0.029 (± 0.0005)	0.031 (± 0.00065)	0.018 (± 0.001)	0.022 (± 0.00035)	0.045 (± 0.0007)	0.046 (± 0.0006)
24	0.020 (± 0.0003)	0.032 (± 0.00095)	0.029 (± 0.0012)	0.058 (± 0.0009)	0.020 (± 0.0009)	0.042 (± 0.00065)	0.048 (± 0.0001)	0.071 (± 0.00015)
48	0.023 (± 0.0003)	0.067 (± 0.00099)	0.032 (± 0.0009)	0.067 (± 0.00035)	0.020 (± 0.0012)	0.066 (± 0.00075)	0.052 (± 0.0009)	0.075 (± 0.0006)
72	0.025 (± 0.0004)	0.071 (± 0.00045)	0.033 (± 0.0007)	0.076 (± 0.00065)	0.023 (± 0.0007)	0.072 (± 0.00065)	0.051 (± 0.0002)	0.076 (± 0.0005)
96	0.026 (± 0.0009)	0.080 (± 0.00085)	0.035 (± 0.0008)	0.089 (± 0.00066)	0.026 (± 0.0008)	0.097 (± 0.00065)	0.056 (± 0.0008)	0.078 (± 0.00055)
120	0.028 (± 0.0002)	0.088 (± 0.00095)	0.039 (± 0.0008)	0.129 (± 0.00037)	0.032 (± 0.0006)	0.087 (± 0.00090)	0.059 (± 0.0003)	0.085 (± 0.00034)
144	0.031 (± 0.0008)	0.078 (± 0.00035)	0.042 (± 0.0002)	0.153 (± 0.00095)	0.037 (± 0.0005)	0.104 (± 0.00059)	0.064 (± 0.0004)	0.105 (± 0.001)

*Data represent mean of ten replicates; Values in the parenthesis indicate standard errors; P value < 0.05.

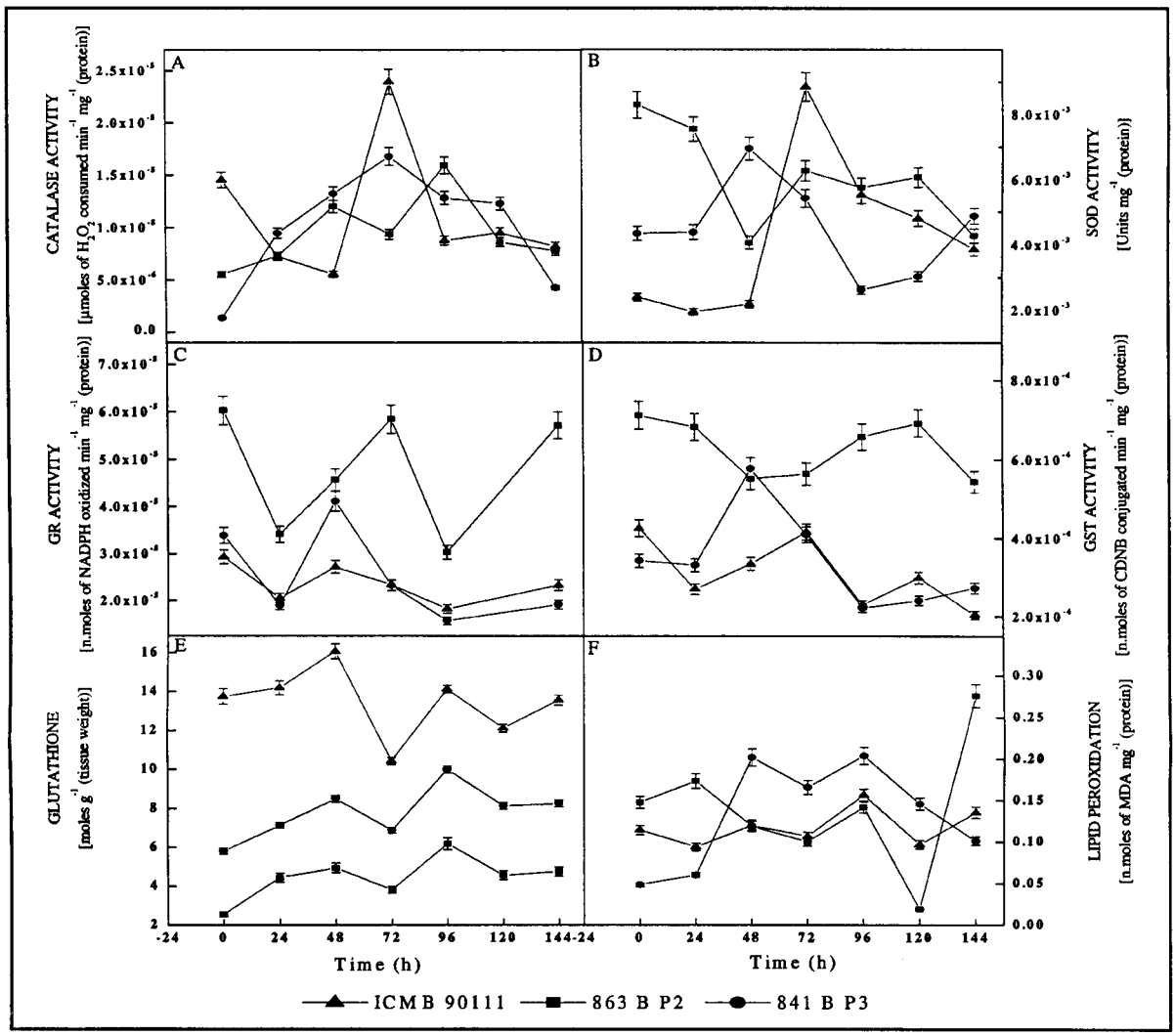

Figure 1. Changes in antioxidative enzyme activity during short-term salt stress at 150 mM NaCl in pearl millet lines. A, Catalase; B, Superoxide dismutase; C, Glutathione reductase; D, Glutathione-S-transferase; E, Reduced glutathione content; F, Lipid peroxidation.

The modulation of antioxidant components diverged significantly among three lines under salt stress conditions (Figure 1A–F). The constitutive activity of CAT was higher in the susceptible line compared to the tolerant ones. But the induced activity of CAT decreased in the sensitive line ICMB 90111 at 150 mM NaCl and showed the lowest activity throughout (except at 72 h), but increased activities were noticed in 863B-P2 and 841B-P3 (Figure 1A). Contrary to it, the native activity of SOD appeared lower in the sensitive line as compared to the other lines at 24 h and 48 h salt treatments (Figure 1B). A sudden spurt in SOD activity at 72 h was observed in the sensitive line ICMB 90111, which gradually decreased with time of exposure to 150 mM NaCl till 144 h. The native activities of GR and GST were lower in the susceptible line as compared to moderately tolerant and tolerant lines supporting the data on the content of glutathione (Figure 1C, D). The activity of GR in moderately tolerant line at 150 mM NaCl stress remained higher than ICMB 90111 all throughout. But the content of GSH in the line ICMB 90111 was higher compared to tolerant lines (Figure 1E). The sensitive line ICMB 90111 exhibited a decrease in GSH at 72 h and increased thereafter, supporting the GR activity data. The content of MDA increased by

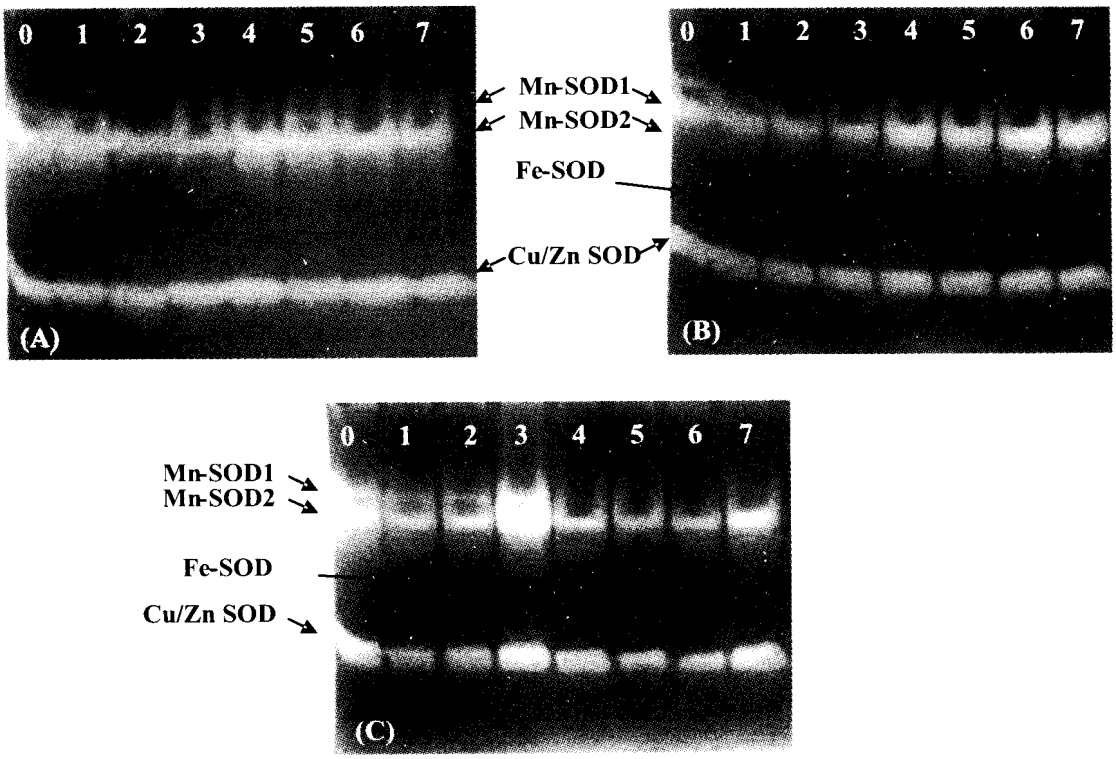

Figure 2. Isoenzyme patterns of superoxide dismutase in pearl millet lines: A, ICMB 90111; B, 863B-P2; C, 841B-P3 at 150 mM NaCl in short-term salinity treatments.

4–5-folds in the tolerant line 841B-P3 till 96 h which started declining there after. MDA levels rose sharply in the moderately tolerant and tolerant lines at 144 h (Figure 1F). Differences in the activities of Mn–SOD isoforms 1 and 2 and Cu/Zn–SOD were noticed with increased exposure to salt in all three lines (Figure 2A–C). A total of three isoforms of SOD were detected with *in situ* staining technique on the gel, of which two were Mn–SODs and one was a Cu–Zn SOD. The activity of Cu–Zn SOD increased in all the lines under NaCl stress. However, the Mn–SOD isoform 1 displayed differential expression in sensitive, moderately tolerant and highly tolerant lines. In the salt-sensitive line ICMB 90111, activity of Mn–SOD1 was low throughout the salt treatment, while in the moderately tolerant and tolerant lines, this isoform disappeared after 24 h and 72 h, respectively (Figure 2A–C). The second isoform Mn–SOD2 showed a gradual increase in activity in the sensitive, as well as in moderately tolerant and tolerant lines with increased duration of exposure to salinity. Low activity of Fe–SOD was observed in seedlings of moderately tolerant and tolerant lines after 24 h. However, no Fe–SOD isoform was observed in the sensitive seedlings. Fe–SOD bands were very faint in intensity in the tolerant lines.

The mechanisms examined to investigate salt tolerance in our studies include accumulation of proline and ions, and activities of antioxidative enzymes. Free proline levels were observed to increase with increasing salinity levels in all of the pearl millet lines, irrespective of their tolerance to salt. Proline accumulation to a higher degree under salinity stress is indicative of the fact that, proline acts as a cytoplasmic osmoticum and also protects the proteins against denaturation (Kavi Kishor *et al.*, 2005). Proline accumulation was also significant under salinity stress in salt-tolerant cultivars of green gram (Misra *et al.*, 2006), and is correlated with salt tolerance of many higher plants (Ghoulam *et al.*, 2002; Girija *et al.*, 2002). In the present studies, sensitive lines showed

more accumulation of Na^+ when compared to K^+. Generally, tolerant lines exhibit K^+/Na^+ ratio >1 indicating higher accumulation of K^+ than Na^+. Less accumulation of Na^+ and more accumulation of K^+ were observed in salt-tolerant varieties of rice (Sangam, 1995; Vaidyanathan *et al.*, 2003) and *Sorghum* (Jogeswar *et al.*, 2006). Lower uptake of Na^+ and comparatively higher accumulation of K^+ seems to be one of the mechanisms of their tolerance to salinity stress. Higher increase in Ca^{2+} in the tolerant lines may be due to its involvement as a second messenger in excluding Na^+ out of the cells via the salt overlay sensitive pathway as pointed out by Zhu (2003).

ROS generated during salt stress need to be effectively scavenged which otherwise can damage lipid membranes of the cells (Polidoros and Scandalios, 1999). Plants possess not only enzymatic machinery for such activities but also non-enzymatic to scavenge them. While the native activity of CAT was higher, SOD appeared lower in the sensitive line when compared to the tolerant lines. In several salt-tolerant species, high activity of SOD was reported under salt stress conditions (Acar *et al.*, 2001). This suggests that SOD may function as an effective ROS scavenger, by converting O^{2-} to H_2O_2 as pointed by Alscher *et al.* (2002). Higher activities of CAT were reported in roots of lentil under NaCl stress (Bandeoglu *et al.*, 2004). An increase in the activity of CAT was recorded in the tolerant genotypes of *Beta maritime* (halophyte) and the non-halophyte *Beta vulgaris* (Bor *et al.*, 2003). Similar comparisons were made between genotypes of rice (Sudhakar *et al.*, 2001) and wheat (Sairam *et al.*, 2002) differing in salt tolerance. It appears therefore that some genotypes utilize catalase as an effective antioxidative enzyme to convert hydrogen peroxide. In some species, salt tolerance was associated with an increase in both APX and GR activities (Bor *et al.*, 2003; Harinasut *et al.*, 2003), the key enzymes of the ascorbate–glutathione cycle; however, only an increase in GR was observed in pearl millet. Earlier, similar increases in the activities of this enzyme were recorded in *Sorghum* (Jogeswar *et al.*, 2006). Tolerant lines of pearl millet may further be depending on antioxidants like reduced glutathione and ascorbate for the conversion of O^{2-} to H_2O_2. The constitutive activity of GST was high in the tolerant line, while it increased transiently in the tolerant line. Contrary to this, the specific activity did not change much in the susceptible line. The constitutive as well as salt-induced activity of GST was higher in the tolerant lines compared to the susceptible ones. This indicates that the salt–tolerant species possess effective mechanisms to conjugate with GSH and detoxify the electrophiles when exposed to salt stress conditions. Higher lipid peroxidation in sensitive cultivars of pea (Hernandez *et al.*, 1993) and rice (Dionisio-Sese and Tobita, 1998) were reported earlier; suggesting that the elevated levels of the antioxidative enzymes protect plants against the ROS during salt stress in the tolerant cultivars (Comba *et al.*, 1998; Shalata *et al.*, 2001). Surprisingly, MDA content was higher under salt stress conditions compared to the controls in the tolerant lines in spite of higher enzyme activities. The increase in exposure to salinity stress led to an increased intensity of the Mn–SOD2 and Cu/Zn–SOD isoforms in native gels. Lee *et al.* (2001) observed two isoforms of Mn–SOD and five isoforms of Cu/Zn–SOD in rice, while Fe–SOD isoform was not detected in the activity gels. The difficulty involved in identifying Fe–SOD in different species of higher plants is related to their low enzymatic activity and low expression (Almansa *et al.*, 1991; Gueta-Dahan *et al.*, 1997). Also, Fe–SOD is usually inactivated by H_2O_2 (Alscher *et al.*, 2002). Salt-stress increased the activities of leaf mitochondrial Mn–SOD and chloroplastic Cu/Zn–SOD in NaCl-tolerant pea cultivars (Hernandez *et al.*, 1995) and this has also been observed in shoot cultures of rice (Fadzilla *et al.*, 1997). Our results showed a strong correlation between salt tolerance and accumulation of osmolytes, ions and activity of antioxidative enzymes. This suggests that the balance between the activities of H_2O_2-producing and H_2O_2-scavenging enzymes plays an important role in providing a defense mechanism against salt-induced oxidative damage in plant cells. The results from this study may provide base-line information and a system necessary to conduct further studies related to the molecular and genetic basis of salinity tolerance in pearl millet to elucidate the importance of the relationship between antioxidant activity and development of salt-tolerant lines.

Acknowledgements

RM thanks the CSIR, New Delhi, for providing financial assistance to carry out this work. The work was

supported in part by a research grant to the ICRISAT from the Plant Sciences Research Programme of the UK's Department for International Development (DFID-PSP). The opinions expressed are those of the authors and are not necessarily those of DFID or ICRISAT.

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