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Response of maize genotypes to salinity stress in relation to osmolytes and metal-ions contents, oxidative stress and antioxidant enzymes activity

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Abstract

Effect of long term soil salinity (control - S_0 and three levels S_1 to S_3) was studied in two maize (*Zea mays* L.) genotypes, PEHM 3 (comparatively tolerant) and Navjot (susceptible) at vegetative and anthesis stages during summer-rainy season. Salinity stress decreased relative water content (RWC), chlorophyll (Chl) and carotenoid (Car) contents, membrane stability index (MSI), potassium and calcium contents, and increased the contents of superoxide radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), thiobarbituric acid reactive substances (TBARS), proline, glycinebetaine, total soluble sugars, and sodium, and Na^+/K^+ and Na^+/Ca^{2+} ratios in both the genotypes. Contents of zinc, copper, manganese and iron increased up to S_2. Though under S_0 PEHM 3 had higher content of all the metals, Navjot recorded higher content of Zn at all salinity levels and contents of all metal ions at S_2 and S_3. Activities of superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT) and glutathione reductase (GR) increased upto S_2 in both the genotypes, and upto S_3 in PEHM 3 at the two stages. Salinity induced decrease in RWC, Chl, Car, MSI, K^+ and Ca^{2+} was significantly greater in Navjot, which also recorded higher Na^+ content and Na^+/K^+ and Na^+/Ca^{2+} ratios than PEHM-3. PEHM-3 recorded higher contents of proline, glycine-betaine, total soluble sugars, K^+, Ca^{2+}, activity of SOD, APX, CAT, GR, and comparatively lower $O_2^{\bullet-}$, H_2O_2 and TBARS contents compared to Navjot.

Additional key words: ascorbate peroxidase, calcium, catalase, glutathione reductase, glycinebetaine, heavy metals, osmolytes, potassium, proline, sodium, soluble sugars, superoxide, superoxide dismutase.

Introduction

Soil salinity is one of the main factors, which reduces crop production in semiarid and arid areas all around the world. Salt stress is accompanied by a decrease in uptake of K^+, Ca^{2+}, Mg^{2+} (Poonia *et al.* 1972), inhibition of growth and photosynthesis, readjustment of metabolic processes (Olmos and Hellin 1996) and compensation for osmotic and ionic changes (Lutts *et al.* 1996). Plants have developed different strategies to withstand salt stress. These include mechanisms, which facilitate ion exclusion/sequestration (Yeo and Flowers 1983), accumulation of compatible solutes allowing pressure potential maintenance such as proline or glycinebetaine (Bohnert and Jensen 1996, Serrano and Gaxiola 1994), metabolic switches to more efficient C_4 type of photosynthesis (Cushman et al. 1989) and detoxification of free radicals (Bohnert and Jensen 1996). Accumulation of proline has been widely advocated for use as parameter of selection for salt stress tolerance (Storey and Wyn-Jones 1975), whereas salt stress induced decrease in proline content has also been reported (Siddiqui and Krishnamoorthy 1987). Su and Bai (2008) suggested that salinity induced proline accumulation could be due to putrescine oxidation.

Very little information is available on the salinity induced changes in heavy metal concentration in crop plants. Hirpara *et al.* (2005) reported salinity induced

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Abbreviations: APX - ascorbate peroxidase; CAT - catalase; Chl - chlorophyll; Car - carotenoids; DAA - days after anthesis; DAS - days after sowing; DTNB - 5,5-dithiobis-2-nitrobenzoic acid; GB - glycine betaine; GR - glutathione reductase; GSSG - glutathione disulfide (oxidized glutathione); H_2O_2 - hydrogen peroxide; NBT - nitroblue tetrazolium chloride; ROS - reactive oxygen species; SOD - superoxide dismutase; TBARS - thiobarbituric acid reactive substances.

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increase in Zn, Cu and Mn in all parts of *Butea monosperma* with a concurrent decline in Fe content. In salt-stressed strawberry, contents of Fe and Mn increased in shoots, while contents of Zn and Cu increased in roots (Turhan and Eris 2005).

Abiotic stresses, including salinity contribute to formation of reactive oxygen species (ROS), superoxide radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\bullet-}$), the last one being the most cytotoxic. These ROS cause perturbation of basic metabolic pathways, and damage membranes and organic molecules, mainly proteins, DNA and pigments (Fridovich 1986, Davies 1987, Imlay and Linn 1988), and sulphur containing amino acids in proteins (Hernandez *et al.* 1993). Hydrogen peroxide produced by SOD is eliminated by ascorbate peroxidase (APX) at the expense of oxidizing ascorbate to monodehydroascorbate and/or dehydroascorbate (Asada 1994). Another important scavenger of H_2O_2 is catalase (CAT). There are reports of both increase and decrease in antioxidant enzymes activity under various abiotic stresses, including salinity. Melakeselam *et al.* (1999) reported increase in SOD activity under freezing stress and decrease under heat stress in rape seed mustard. Water stress induced decrease in APX and GR activity in wheat (Menconi *et al.* 1995), and salinity induced decrease in Mn-SOD and APX, and increase in Cu/Zn-SOD and GR in both tolerant and sensitive cultivars of citrus (Gueta-Dahan *et al.* 1997) have been reported. Benavides *et al.* (2000) reported increase in APX and GR at 100 mM NaCl and decrease at 150 mM NaCl.

Little information is available on the contribution of oxidative stress and antioxidant enzymes on susceptibility and tolerance of maize genotypes. Thus, the present study was conducted to elucidate the role of metal ions, osmolytes, oxidative stress and antioxidant enzymes in relation to salinity stress tolerance in maize genotypes, which can not only help in cloning of genes involved in salt stress tolerance, development of transgenics and better breeding programmes, but also help to chalk out accurate screening techniques ultimately aiding to crop improvement in saline soils.

Materials and methods

Present study was undertaken with two maize (*Zea mays* L.) genotypes, procured from the Directorate of Maize Research (Indian Council of Agricultural Research), IARI campus, New Delhi, comparatively tolerant PEHM 3 and susceptible Navjot during the summer-rainy season of 2004. Earthen pots of uniform size (30 × 30 cm) lined with two layers of polyethylene bags were filled with 10 kg of air-dried soil and farm yard manure in 3:1 ratio. Each pot was fertilized corresponding to 10, 6 and 6 g m^{-2} of N, P and K, respectively. Before sowing, pots were irrigated with 2.5 dm^3 of water (control, S_0) or saline solutions [S_1(50, 25, 25 mM), S_2(100, 50, 50 mM) and S_3(150, 75, 75 mM) NaCl, Na_2SO_4 and $CaCl_2$, respectively]. Actual salinity levels are expressed as electric conductivity, ECe determined at vegetative (30 d after sowing; 1.06, 6.82, 10.38, 18.81 dS m^{-1}) and anthesis (50 % tasseling; 1.06, 7.82, 11.38, 20.08 dS m^{-1}) stages. Plants were watered as and when required. Samples for various assays were collected between 10:00 - 10:30 from first fully expanded leaf (2^{nd} leaf from the top) at vegetative stage and flag leaf at anthesis stage from 4 plants. Observations for relative water content (RWC), chlorophyll (Chl) and carotenoid (Car) contents, membrane stability index (MSI), $O_2^{\bullet-}$, H_2O_2 and thiobarbituric acid reactive substances (TBARS) contents, SOD, APX, GR and CAT activities were recorded at vegetative (30 d after sowing) and anthesis stages, while osmolytes, metal ions and isozyme pattern of SOD, APX and CAT were studied at anthesis stage only.

Leaf relative water content (RWC) was estimated according to Weatherley (1950) and calculated as follows: RWC = [(fresh mass - dry mass)/ (saturated mass - dry mass)] × 100. Membrane stability index (MSI) was estimated as per Sairam *et al.* (1997) and calculated as follows: MSI = [1- (C_1/C_2)] × 100, where C_1 and C_2 are conductivity of electrolytes leached from leaf disks incubated at 40 and 100 °C, respectively. Chlorophyll and carotenoid contents were estimated by the method of Hiscox and Israelstam (1979). Absorbance of acetone extract was recorded by UV-visible spectrophotometer (*Specord 200*, *Analytik*, Jena, Germany) at 645, 665 and 470 nm, and Chl and Car contents were calculated as per Arnon (1949). For the estimation of alkali metals (Na^+, K^+ and Ca^{2+}) and heavy metals (Zn, Fe, Cu, Mn) samples were prepared by digesting 1 g dry plant material in 100-cm^3 *Pyrex* digestion tubes with 10 cm^3 of 2:1 (v/v) mixture of nitric acid:perchloric acid. Contents of Ca^{2+}, Zn, Fe, Cu and Mn were estimated with atomic absorption spectrometer (*AAS4141*, *Electronic Corporation of India*, Hyderabad, India). Na^+ and K^+ were estimated using digital flame photometer (*Century*, model *CFM-1*, Chandigarh, India) (Tandon 1995).

Total $O_2^{\bullet-}$ generation was assayed by estimating the blue coloured formazan formed by tetrazolium dye with $O_2^{\bullet-}$ in the presence of SOD-inhibitor diethyldithiocarbamate in the extraction buffer (Chaitanya and Naithani 1994). Absorbance was recorded at 560 nm. Hydrogen peroxide content was estimated by measuring the titanium-hydroperoxide complex at 415 nm (Rao *et al.* 1997). Lipid peroxidation was estimated as TBARS (Heath and Packer 1968). Free proline content in the leaves was determined following the method of Bates *et al.* (1973). Glycine betaine estimation was done in dried leaf powder by cold periodite method (Greive and Grattan 1983). Total soluble sugars were estimated using anthrone reagent (Yemm and Willis 1954).

Enzyme extract for SOD, APX, GR and CAT was prepared from leaf samples (1 g) frozen in liquid nitrogen to prevent proteolytic activity followed by grinding with 10 cm^3 of cold extraction buffer (0.1 M phosphate buffer, pH 7.5, containing 0.5 mM EDTA and 1 mM ascorbic acid). Brie was passed through 4 layers of cheesecloth and filtrate was centrifuged for 20 min at 15 000 *g* and 4 °C, and the supernatant was used as enzyme extract. Total SOD activity was estimated by the inhibition of the photochemical reduction of nitroblue tetrazolium (NBT) by the enzyme (Dhindsa *et al.* 1981). To distinguish SOD isoforms, the sensitivity of Cu/Zn-SOD to cyanide (3 mM), and Cu/Zn-SOD and Fe-SOD to hydrogen peroxide (5 mM) were used, whereas Mn-SOD was unaffected (Yu and Rengel 1999). The absorbance was recorded at 560 nm, and one unit of enzyme activity was taken as that amount of enzyme, which reduced the absorbance reading to 50 % in comparison with tubes lacking enzyme. APX was assayed by recording the decrease in absorbance due to a decrease in ascorbic acid content at 290 nm (Nakano and Asada 1981). CAT was assayed by monitoring the decrease in absorbance at 240 nm due to decrease in H_2O_2 content (Aebi 1984). GR was assayed as per the method of Smith *et al*. (1988). Isozyme separation was done on native-PAGE. Samples containing equal amount of protein (200 µg) were subjected to non-denaturating PAGE (without sodium dodecylsulfate) (Laemmli 1970). For SOD samples were run at 4 °C in a 7.5 % resolving gel and 6 % stacking gel for 100 min at 300 V. Activity staining following electrophoresis was done with NBT (Beauchamp and Fridovich 1977, Sandalio *et al.* 1987). APX electrophoresis was done at 4 °C in 7.5 % resolving gel containing 10 % glycerol and 6 % stacking gel. Samples were pre run at 300 V and 30 mA for 60 min, followed by at 300 V and 40 mA for 120 min. The anodic buffer contained 2 mM ascorbic acid (Mittler and Zilinskas 1993). Gel was pre-incubated in buffer and ascorbic acid, followed by incubation in buffer, ascorbic acid and hydrogen peroxide. Staining was done with NBT. For catalase isozyme separation, the gel was incubated for 2 min in a solution of 5 cm^3 10 % $FeCl_3$ + 5 cm^3 10 % $K_3Fe(CN)_6$ + 40 cm^3 distilled water, followed by washing with distilled water (Woodbury 1971). Soluble proteins were estimated according to the method of Bradford (1976).

Results were analyzed by *ANOVA* and LSD values were calculated for cultivars, treatments and their interactions. Standard error of mean was also calculated.

Results

Relative water content declined significantly with increase in salinity levels (Table 1) during both the stages. Under all the salinity treatments PEHM 3 recorded significantly higher RWC than Navjot, except control plants during anthesis stage, where differences were marginal.

Carotenoid content at vegetative stage showed gradual decrease in both the genotypes with salinity, while at anthesis tolerant genotype PEHM 3 showed slight increase at S_1 followed by decrease at higher salinity levels (Table 1). Under all salinity levels PEHM 3 maintained higher Car content than Navjot.

Total chlorophyll content (Table 1) decreased under salinity stress in both the genotypes. Chl contents were higher in PEHM 3 under all salinity levels during both the stages compared to Navjot. Chl *a*/*b* ratio (Table 1) increased significantly under salinity in both the genotypes at vegetative stage, higher being in PEHM 3 compared to Navjot. However, at anthesis only slight increase in Chl *a*/*b* ratio in PEHM 3 was observed at S_1, but decrease at higher salinity levels in both the genotypes.

Superoxide radical contents increased with increase in salinity levels in both the genotypes at both the stages (Table 2). However, the increase was greater at anthesis. Similar trend was observed for hydrogen peroxide

Table 1. Effect of salinity on relative water content [%], carotenoid and total chlorophyll contents [mg g^{-1}(d.m.)], and chlorophyll *a*/*b* ratio in tolerant (PHEM 3) and susceptible (Navjot) maize genotypes at vegetative and anthesis stages. Means ± SE (n = 8). Electrical conductivity at salinities S_0, S_1, S_2 and S_3 was 1.06, 6.82, 10.38 and 18.81 dS m^{-1}, respectively.

Parameters	Genotypes	Vegetative				Anthesis			
		S_0	S_1	S_2	S_3	S_0	S_1	S_2	S_3
RWC	Navjot	90.25±4.26	75.77±1.58	62.66±1.72	57.67±1.59	92.26±3.45	78.00±0.55	67.48±1.54	60.28±1.52
	PHEM 3	91.43±1.20	88.79±0.85	82.15±0.39	73.08±2.41	93.48±0.73	93.69±0.99	74.26±1.96	71.17±0.55
Car	Navjot	1.60±0.04	1.07±0.01	0.99±0.03	0.98±0.08	1.36±0.13	1.16±0.00	1.09±0.03	0.94±0.01
	PHEM 3	1.62±0.09	1.47±0.01	1.37±0.11	1.33±0.07	1.74±0.11	1.85±0.04	1.59±0.07	1.57±0.05
Chl *a*+*b*	Navjot	8.75±0.05	6.48±0.12	5.84±0.14	4.67±0.27	12.69±0.01	10.11±0.10	7.43±0.07	6.47±0.03
	PHEM 3	11.68±0.11	10.41±0.13	9.55±0.22	8.48±0.17	15.56±0.22	14.01±0.23	12.49±0.24	9.88±0.28
Chl *a*/*b*	Navjot	1.42±0.06	1.48±0.05	1.69±0.17	2.71±0.59	1.60±0.04	1.52±0.08	1.44±0.05	1.34±0.03
	PHEM 3	1.81±0.06	2.35±0.09	2.59±0.08	3.92±0.26	1.66±0.02	1.91±0.08	1.85±0.11	1.86±0.22

Table 2. Effect of salinity on contents of superoxide radical [ΔA_{560} mg^{-1}(protein)], hydrogen peroxide [μmol mg^{-1}(protein)], TBARS [nmol g^{-1}(d.m.)], membrane stability index [%] in tolerant (PHEM 3) and susceptible (Navjot) maize genotypes at vegetative and anthesis stages. Means ± SE, n = 8.

Parameters	Genotypes	Vegetative				Anthesis			
		S_0	S_1	S_2	S_3	S_0	S_1	S_2	S_3
Superoxide	Navjot	7.610±0.168	10.38±0.133	14.74±0.192	16.15±0.197	18.38±0.172	21.66±0.148	27.62±0.193	35.88±0.327
	PHEM 3	6.320±0.161	7.27±0.189	10.14±0.447	11.33±0.259	11.24±0.162	12.12±0.131	13.74±0.081	16.20±0.120
H_2O_2	Navjot	0.359±0.040	0.888±0.025	1.378±0.045	2.163±0.038	0.566±0.026	1.317±0.023	1.765±0.030	2.239±0.024
	PHEM 3	0.389±0.013	0.588±0.055	0.945±0.037	1.426±0.123	0.494±0.021	0.664±0.038	1.132±0.042	1.459±0.047
TBARS	Navjot	178.5±9.20	201.5±15.30	250.8±16.59	374.0±12.84	334.5±18.18	528.8±35.11	566.2±15.85	687.6±6.87
	PHEM 3	137.5±1.33	143.6±3.52	159.8±3.52	179.0±1.33	316.2±15.92	351.0±7.33	434.4±36.23	820.6±38.73
MSI	Navjot	84.18±0.65	64.57±0.80	63.12±4.30	57.53±1.35	76.93±0.82	66.14±0.49	58.95±2.17	52.66±0.47
	PHEM 3	87.50±0.73	76.36±1.27	72.25±2.29	68.84±1.42	83.55±0.66	77.66±1.04	73.11±1.89	67.10±3.48

(Table 2) except for the fact that at the highest salinity level the H_2O_2 content was almost equal both at the vegetative and anthesis stages. Lipid peroxidation measured in terms of TBARS content also increased with salinity levels and the values were significantly higher at anthesis than at vegetative stage (Table 2). Susceptible genotype Navjot recorded significantly higher superoxide, H_2O_2 and TBARS contents than PEHM 3. Membrane stability was slightly lower at anthesis in both the genotypes compared to vegetative stage (Table 2). Membrane stability index was adversely affected by increasing salinity in both the genotypes during both the stages, however, PEHM 3 maintained greater MSI at all salinity levels than Navjot.

Contents of proline, glycine betaine and soluble sugars recorded at anthesis increased with salinity in both the genotypes, however, greater contents were observed in PEHM 3 than Navjot (Table 3). Sodium content, and Na/K and Na/Ca ratios increased with salinity levels, and were higher in Navjot than in PEHM 3 (Table 3). In contrast, contents of K and Ca decreased under salinity, however, PEHM 3 showed higher values as well as lower decline under stress than Navjot (Table 3). Contents of Zn, Fe, Cu and Mn estimated at anthesis stage increased up to S_2 and declined at S_3. At S_0 and S_1 the contents of Fe, Cu and Mn were greater in PEHM 3 than in Navjot, but Navjot recorded higher contents of Zn at all salinity levels and Fe, Cu and Mn at S_2 and S_3.

Total SOD activity increased under salt stress, and the pattern of increase in SOD activity and its isoforms under salinity was similar during both the stages (Table 4). Higher activity was observed in PEHM 3 than in Navjot at all salinity levels at both the stages, though the increase in activity was greater at anthesis in PEHM 3. Salinity induced increase in enzyme activity was more marked for Mn-SOD during both stages, and in Cu/Zn-SOD at anthesis only (Table 4). Among the SOD isoforms, the Cu/Zn-SOD activity was highest followed by Mn-SOD, very little Fe-SOD activity was observed, which actually showed slight decline at S_3.

Table 3. Effect of salinity on contents of proline [μmol g^{-1}(d.m.)], glycine betaine [μg g^{-1}(d.m.)], soluble sugars [μmol g^{-1}(d.m.)], potasium, sodium and calcium [mg g^{-1}(d.m.)], zinc, copper, manganese and iron [μg g^{-1}(d.m.)] in maize genotypes Navjot and PHEM 3 at anthesis stage. Mean ± SE, n = 8).

Parameters	Navjot				PHEM 3			
	S_0	S_1	S_2	S_3	S_0	S_1	S_2	S_3
Proline	12.64±2.19	23.10±1.18	30.60± 2.50	50.39±1.90	21.82±2.41	56.37±2.78	70.21±0.76	115.87±1.67
Glycine betaine	442.29±4.78	482.99±2.92	612.80±10.88	529.30±12.40	565.39±9.52	677.38±16.29	962.48±11.64	1036.2±26.50
Soluble sugars	374.50±13.43	447.28±8.26	791.71±11.79	542.49±10.80	535.43±3.89	760.49±7.35	864.44±10.64	871.79±7.26
Potassium	11.71±0.183	10.14±0.032	7.93±0.075	6.30±0.016	13.78±0.091	12.47±0.091	10.72±0.160	9.02±0.038
Sodium	1.45±0.015	2.45±0.077	4.09±0.051	5.64±0.118	1.32±0.048	2.52±0.037	3.57±0.086	4.66±0.054
Calcium	0.79±0.009	0.72±0.002	0.64±0.005	0.54±0.007	1.09±0.005	0.99±0.006	0.94±0.005	0.87±0.011
Na/K ratio	0.13±0.001	0.24±0.077	0.52±0.051	0.90±0.118	0.10±0.018	0.20±0.038	0.33±0.086	0.52±0.011
Na/Ca ratio	1.91±0.041	3.39±0.114	6.35±0.029	10.40±0.075	1.21±0.035	2.52±0.016	3.80±0.079	5.34±0.091
Zinc	43.02±3.42	65.38±0.54	75.60±0.91	56.88±1.87	52.38±2.63	55.16±0.45	64.02±2.51	50.22±1.56
Copper	18.00±0.51	24.36±0.17	30.48±0.34	25.14±0.14	21.62±0.31	28.20±1.30	28.76±0.34	23.22±0.14
Manganese	108.54±1.67	147.38±4.16	184.44±3.00	146.16±4.86	127.50±3.65	161.92±5.94	167.94±3.99	127.72±2.54
Iron	284.74±3.82	299.06±2.74	351.26±3.20	323.94±0.93	314.86±3.76	326.80±3.11	340.82±3.54	287.38±4.33

Table 4. Effect of salinity levels on the specific activities of total SOD, Cu/Zn-SOD, Mn-SOD, Fe-SOD [U mg^{-1}(protein) min^{-1}], APX [μmol(asc.) mg^{-1}(protein) min^{-1}], GR [ΔA_{412} mg^{-1}(protein) min^{-1}], and CAT [μmol(H_2O_2) mg^{-1}(protein) min^{-1}] in maize genotypes Navjot and PHEM vegetative and anthesis stages. Means ± SE, n = 8.

Parameters	Genotypes	Vegetative				Anthesis			
		S_0	S_1	S_2	S_3	S_0	S_1	S_2	S_3
Total SOD	Navjot	4.964±0.427	5.745±0.391	7.158±0.143	6.490±0.357	4.352±0.229	5.889±0.243	6.491±0.217	7.867±0.388
	PHEM 3	6.973±0.302	8.035±0.297	10.38±0.375	12.64±0.371	8.661±0.236	10.07±0.256	12.44±0.487	14.65±0.376
Cu/Zn-SOD	Navjot	2.935±0.575	3.272±0.685	3.580±0.490	3.041±0.554	2.314±0.301	2.554±0.055	2.832±0.005	3.274±0.242
	PHEM 3	4.497±0.326	4.291±0.436	5.107±0.938	6.824±0.303	4.429±0.443	4.538±0.329	5.958±0.867	7.082±0.242
Mn-SOD	Navjot	1.373±0.253	1.643±0.590	2.474±0.213	2.913±0.335	1.643±0.251	2.299±0.303	2.673±0.140	3.755±0.588
	PHEM 3	1.355±0.293	2.618±0.408	3.311±0.266	3.973±0.123	3.124±0.365	3.543±0.275	4.614±0.451	6.105±0.321
Fe-SOD	Navjot	0.657±0.080	0.828±0.112	1.104±0.138	0.536±0.081	0.395±0.109	1.037±0.041	0.986±0.035	0.839±0.051
	PHEM 3	1.121±0.133	1.125±0.106	1.962±0.770	1.844±0.197	1.109±0.250	1.990±0.248	1.866±0.536	1.467±0.359
APX	Navjot	17.97±0.16	19.64±1.00	22.96±1.65	19.47±0.75	13.74±0.34	16.29±0.71	20.18±0.67	15.55±1.44
	PHEM 3	23.15±3.15	28.41±1.30	31.61±2.33	34.46±1.72	26.43±0.75	27.38±3.79	35.29±2.30	43.29±1.35
GR	Navjot	15.27±0.83	19.46±0.59	21.18±0.37	20.69±3.80	9.60±0.32	11.58±0.37	14.29±0.20	13.87±0.49
	PHEM 3	25.71±0.78	29.38±0.50	33.20±0.85	36.21±0.34	16.77±0.41	20.15±0.63	22.72±0.41	25.96±0.41
CAT	Navjot	3.68±0.46	5.18±0.24	5.18±0.24	4.27±0.59	1.35±0.26	2.95±0.32	4.95±0.41	2.81±0.09
	PHEM 3	5.10±0.30	8.60±0.63	8.60±0.63	10.50±0.44	4.20±0.48	9.68±0.44	12.41±0.25	14.74±0.35

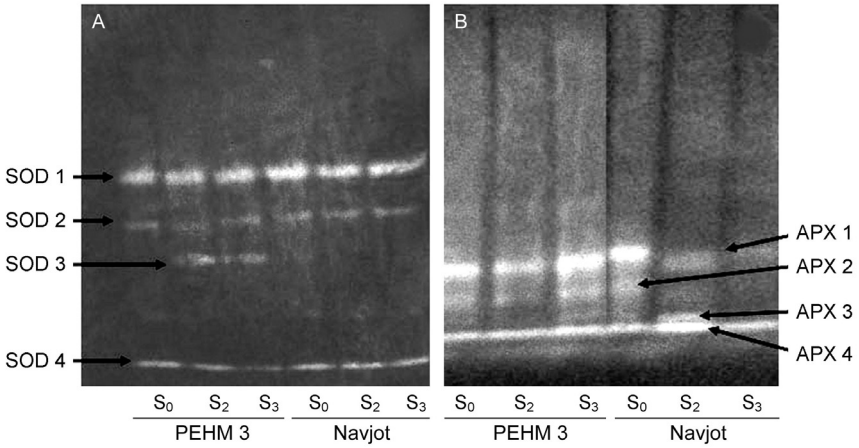

Fig. 1. Effect of salinity levels on superoxide dismutase (*A*) and ascorbate peroxidase (*B*) activities on native-PAGE in PEHM 3 (tolerant) and Navjot (susceptible) maize genotypes at different salinity levels at anthesis stage.

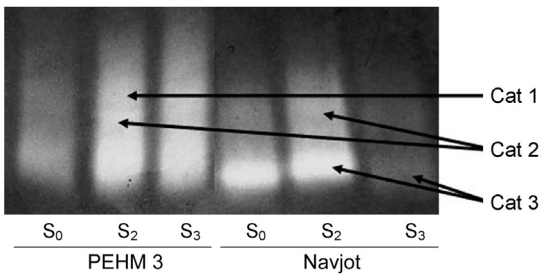

Fig. 2. Effect of salinity levels on catalase activity on native-PAGE in PEHM 3 (tolerant) and Navjot (susceptible) maize genotypes at different salinity levels at anthesis stage.

APX, GR and CAT (Table 4) increased at all salinity levels in PEHM 3 at both the stages. PEHM 3 also showed higher APX, GR and CAT activity than Navjot at all salinity levels. Navjot showed decline in the activity of all three enzyme at S_3 at both the stages. While APX and CAT activities were higher at anthesis, GR was higher at vegetative stage. In the case of SOD-native-PAGE, PEHM 3 showed 4 bands at S_2 and S_3, and only three in control plants and Navjot under all the treatments (Fig. 1). Ascorbate peroxidase activity bands in PEHM 3 showed increase in intensity with salinity, while in case of Navjot the band intensity decreased with increasing salinity. In all treatments of PEHM 3 and control of Navjot only three APX bands (APX 1, 2, 4) were visualized. However, at S_2 in Navjot an additional APX band (APX 3) was visible, with the concurrent disappearance of APX 2. At S_3 only two bands, APX 1 and 4 were visible in Navjot (Fig. 1). In the case of catalase, the bands are not very well defined. However, in case of Navjot two bands, one bright one (CAT 3) at S_0 and S_2, and a second (CAT 2) little less bright only at S_2

were apparent, while in case of PEMH 3, CAT 1, CAT 2 and CAT 3 were present at S_2 and S_3. CAT activity staining increased with increasing salinity in case of PEHM 3, while in case of Navjot it decreased at S_3 (Fig. 2).

Discussion

Salinity is one of the important abiotic stresses, which affects crop productivity. Unlike drought, salinity stress is an intricate phenomenon, which includes osmotic stress, specific ion effect, nutrient deficiency, *etc*., thereby affecting various physiological and biochemical mechanisms associated with plant growth and development.

The decrease in RWC and Chl content under salinity stress in the maize genotypes is in confirmation of already reported results (Sairam *et al*. 2002, Gadallah 1999). Tolerant genotype PEHM 3 retained higher RWC and Chl content and lower Chl *a*/*b* ratio at all salinity levels and at both the stages. Srivastava *et al.* (1988) reported Chl content as one of the parameters of salt tolerance in crop plants. Hernandez *et al.* (1995) observed higher Chl degradation in NaCl sensitive pea cultivar as compared to tolerant one. Chl *a*/*b* ratio increased under salinity stress suggesting more damage to Chl *b* than Chl *a* under salt stress. Comparatively greater total Chl content and lower Chl *a*/*b* ratio was observed at anthesis. Salinity stress induced reduction in Car contents in wheat has been reported by Abd el Samad (1993). PEHM 3 retained higher Car content than Navjot under salt stress. Car are responsible for quenching of singlet oxygen (Knox and Dodge 1985), hence higher Car content in PEHM 3 may determine its relative tolerance.

Accumulation of proline, glycine betaine and soluble sugars under stress protect the cell by balancing the osmotic strength of cytosol with that of vacuole and apoplast (Gadallah 1999, Greenway and Munns 1980). Proline and glycine betaine also stabilize the structure and function of various macromolecules (Rhodes 1987, Smirnoff and Cumbes 1989). A direct consequence of higher osmolyte concentration in tolerant genotype PEHM 3 is the maintenance of comparatively higher MSI and antioxidant enzyme activities.

Greater Na accumulation under salinity stress in Navjot could be one of the reason of its susceptibility to salt stress, while more K and Ca content in PEHM 3 contributed towards its tolerance to salinity stress. Calcium is an important constituent of cell wall and membranes, and it is also involved in signalling pathway leading to induction of antioxidant enzymes (Agarwal *et al*. 2005). Salinity induced increase in Na and depletion of K and Ca contents in case of wheat have been reported earlier (Moustafa *et al.* 1966). Na/K and Na/Ca ratios were less in PEHM 3. Melgar *et al.* (2008) reported increase in K content in citrus and olive and decrease in K/Na ratio under salinity stress. These ratios may be used to predict tolerance or sensitivity in wheat cultivars (Joshi *et al*. 1979).

Heavy metal accumulation under salinity further add to salt toxicity. Contents of Fe, Zn, Cu and Mn increased upto S_2 but declined at S_3. This could be due to damage to carriers, pumps or transporters at high salinity. Hirpara *et al*. (2005) reported salinity induced increase in Zn, Cu and Mn in all parts of *Butea monosperma* with a concurrent decline in Fe content. Turhan and Eris (2005) reported salinity induced increase in the contents of Fe and Mn, and no change in Cu content. Comparatively lower contents of Cu, Mn and Fe in PEHM 3 at S_2 and S_3 and Zn at all salinity levels could contribute to salinity stress tolerance of PEHM 3 as compared to Navjot, which accumulated higher concentration of heavy metals at higher salinity levels.

Salinity treatments caused significant increase in $O_2^{\bullet-}$, H_2O_2 and lipid peroxidation, which were higher in Navjot than PEHM 3. Increase in $O_2^{\bullet-}$ and H_2O_2 contents and lipid peroxidation under salt stress (Ying *et al*. 1995, Wan *et al*. 1995) have been reported earlier. Enhanced levels of $O_2^{\bullet-}$ and H_2O_2, and lipid peroxidation resulted in significant decrease in MSI in both genotypes at both the stages. MSI and extent of lipid peroxidation have been suggested as indices of salt injury/tolerance in *Amaranthus* (Bhattacharjee and Mukherjee 1996). Comparatively higher content of $O_2^{\bullet-}$ and lipid peroxidation at anthesis in the two genotypes is reflected in lower MSI. Lower H_2O_2 accumulation and lipid peroxidation and higher membrane stability have been reported in drought tolerant genotypes of wheat (Sairam *et al.* 2005, Hernandez *et al.* 2000).

The greater activity of SOD, APX, CAT and GR in PEHM 3 than Navjot under increasing salinity stress signifies their role as mechanism of salinity stress tolerance. Navjot not only showed lower activities at S_0, but also decline in APX, GR and CAT activity at S_3. Native-PAGE data also showed appearance of new salinity induced SOD isozyme (SOD 3) at S_2 and S_3 in PEHM 3, while in case of Navjot it was not visualized either in control or salinity treatment. In case of APX, Navjot showed appearance of APX 3 at S_2, which was not present in PEHM 3 or at any other treatments of Navjot, with the concurrent loss of APX 2 at S_2 and S_3. This suggests that APX 2 of Navjot is highly susceptible to salt stress. Further, the salinity induced APX 3 was also completely inhibited by the highest salinity level in Navjot. In contrast, APX 1, 2 and 4 of PEHM were stable and showed increase with increasing salt stress. Similarly in case of CAT, the tolerant genotype PEHM 3 showed enhanced activity at higher salinity, while the susceptible Navjot showed decline even below the control plants.

It is apparent that Navjot, with lower contents of osmolytes, K^+ and Ca^{2+}, and antioxidant enzymes activities, is ill equipped to face salt stress, resulting in lower RWC and higher $O_2^{\bullet-}$ and H_2O_2 contents, lipid peroxidation, and consequently lower membrane stability index and chlorophyll content under salt stress. Further, it

can also be concluded that both constitutive as well as salt induced increase in antioxidant enzymes activities are important for providing protection against ROS. While constitutive levels provide protection from oxidative stress arising from normal oxidative metabolism, the salinity or the abiotic stress induced increase in anti-oxidant activity may provide tolerance against stress induced ROS. The marked difference in the activities of SOD, APX, GR and CAT, and various osmolytes contents in tolerant and susceptible genotypes suggest that these play an important role in the overall mechanism of salinity stress tolerance in maize

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