

Differential Response of a Maize Hybrid and its Parental Lines to Salinity Stress

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Abstract

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We studied differences in salt stress response between two parental maize genotypes and their hybrid of the first filial (F₁) generation. We determined their membrane stability index in addition to the content of photosynthetic pigments, superoxide radical, Na⁺, proline and sugars and the activities of ascorbate peroxidase, catalase and glutathione reductase. The F₁ hybrid, known as Pusa Early Hybrid Makka 3 (PEHM 3), showed a higher membrane stability index and chlorophyll content and lower superoxide radical and Na⁺ content than its parents, the sensitive CM 142 line and the tolerant CM 213 line. This heterosis allows the conclusion that the hybrid might be better equipped to cope with salinity stress than both parental lines, although it did not show the best antioxidant protection or the highest sugar or proline content.

Keywords: antioxidant enzymes; Na⁺; proline; salinity stress; sugars; *Zea mays* L.

Nearly 20% of the world's cultivated area and nearly half of the world's irrigated land are affected by salinity (ZHU 2001). Taking into consideration the expected changes in rainfall throughout the world, studying the effects of salinity induced stress will doubtless be one of the most important fields of the future agricultural research. Soil salinity imposes ion toxicity, osmotic stress, nutrient deficiency, increase of reactive oxygen species (ROS) and oxidative stress in plants. Ion toxicity is the result of replacement of K⁺ by Na⁺ in biochemical reactions. For several enzymes K⁺ acts as a co-factor and high K⁺ concentration is also required for the binding of tRNA to the ribosomes (TESTER & DAVENPORT 2003). Increases in activities of antioxidant enzymes, such as superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT) and glutathione

reductase (GR) under salinity stress have been observed in whole leaf extracts from plant genotypes of various species (HERNÁNDEZ *et al.* 1999; SAIRAM *et al.* 2005). The other characteristic response of plants to salinity stress is the synthesis of various osmolytes for maintaining ionic homeostasis, e.g. proline, mannitol, pinitol, ononitol, and sorbitol in cytosol. Proline also has a role in cellular homeostasis, including redox balance and energy status, it can act as a signalling molecule, influences cell proliferation or cell death and triggers specific gene expression, which can be essential for plant recovery (SZABADOS & SAVOURÉ 2010).

Maize belongs among three primary crops of the world. Pusa Early Hybrid Makka 3 (PEHM 3) hybrid, the F₁ hybrid of parental generation CM 213 and CM 142, is a hybrid which gives average grain yields

of 4.0–5.5 t/ha and shows tolerance to high temperature. The objective of the present investigation was to determine whether this hybrid might also be resistant to salinity stress and to compare its stress response with that of its parental generation.

MATERIAL AND METHODS

We used three maize (*Zea mays* L.) genotypes: parental inbred line CM 213 (female) which is characterised by high proline content under salinity and which was found relatively salinity tolerant, CM 142 (male) which was found relatively salinity sensitive, and PEHM 3 (first filial generation).

Seeds were planted in earthen pots (30 × 30 cm) lined with one layer of polyethylene bags filled with 10 kg of air-dried sandy-loam soil and farm yard manure at a 3:1 ratio. The fertilization of each pot corresponded to 100, 60 and 60 kg/ha of N, P and K, respectively. Pots were placed on the experimental plot of Indian Agricultural Research Institute in New Delhi (28.7°N, 77.2°E) during the summer-rainy season. The plants were subjected to three treatments: control without any salinity treatment, and two sets with the application of NaCl in two concentrations: 250mM in salinity 1 (S1) and 375mM in salinity 2 (S2) in a single application at the plant age of 18 days. The actual soil salinity levels were 1.07 (control), 21.23 (S1) and 31.84 (S2) dS/m at the time of the first sampling and 1.08 (control), 22.33 (S1) and 31.94 (S2) dS/m at the time of the second sampling. The sampling took place as follows: the first sampling 15 days after salt application (at the plant age of 32 days) and the second sampling when 50% of control plants showed tasseling (at the plant age of 48 days). Third leaves from the top were used for the sampling for all biochemical experiments.

Membrane stability index (MSI) was estimated as described in SAIRAM *et al.* (1997). 0.1 g of leaves was taken into 10 ml of double distilled water in two sets. One set was subjected to 40°C for 30 min and conductivity was measured (C1). The other set was kept in a boiling water bath for 10 min and its conductivity was then recorded (C2). MSI was calculated as follows:

$$\text{MSI} = [1 - (C_1/C_2)] \times 100$$

Chlorophyll and carotenoid contents were estimated by extracting 0.05 g of the fresh leaf material

in 5 ml of dimethyl sulfoxide (HISCOX & ISREALTAM 1979). The samples were heated at 65°C for 4 h. Absorbance of the extracts was recorded at 645, 665 and 470 nm, and chlorophyll and carotenoid contents were calculated as per standard methods (LICHTENTHALER & WELLBURN 1983).

Superoxide radical generation was assayed as the increase in absorbance due to formazan formation in the presence of SOD inhibitor diethyl dithiocarbamate (CHAITANYA & NAITHANI 1994). Absorbance was recorded at 560 nm (Specord 200, AnalytikJena, Jena, Germany).

For the estimation of Na⁺, samples were prepared by digesting 1 g of dry leaf material in 100 ml Pyrex digestion tubes with 10 ml of 2:1 (v/v) mixture of nitric acid to perchloric acid, and allowed to stand overnight. After the preliminary digestion, the tubes were placed in a block digester, where temperature was raised to 150°C for 1 h and subsequently to 235°C. Volume was made up to 100 ml with distilled water. Na⁺ content was estimated using flame spectrophotometer (TANDON 1995).

Both free proline and total sugars contents were measured spectrophotometrically (Specord Bio-200, AnalytikJena, Jena, Germany). Free proline content in the leaves was determined by the method of BATES *et al.* (1973), the absorbance was read at 520 nm against toluene blank. For estimation of total sugars the method of DUBOIS *et al.* (1951) was used. Absorbance was measured at 490 nm.

Enzyme extract for APX, CAT and GR assays was prepared by first freezing of leaf samples in liquid nitrogen to prevent proteolytic activity followed by grinding with 10 ml of extraction buffer (0.1M phosphate buffer, pH 7.5, containing 0.5mM EDTA and 1mM ascorbic acid). Brie was passed through four layers of cheesecloth and filtrate was centrifuged for 10 min, 15 000 × g at 4°C. APX activity was assayed by recording the decrease in optical density due to ascorbic acid consumption at 290 nm (NAKANO & ASADA 1981). CAT activity was determined by measuring the decomposition of H₂O₂ at 240 nm as described by AEBI (1984). GR was assayed as the increase in absorbance at 412 nm due to the formation of a coloured complex by reduced glutathione with 5,5-dithiobis-2-nitrobenzoic acid according to the method of SMITH *et al.* (1988). Protein content was determined by the method of BRADFORD (1976) with standard curves prepared using bovine serum albumin.

Sampling took place between 7 and 8 a.m. of the local time. We used two individual groups of

samples; each was comprised of the mixture of five leaves per each group. Each measurement was assayed in triplicate. The results are thus the mean of 3×2 ($n = 6$) observations in all the cases. For the analysis of differences between the genotypes, the data were subjected to one-way analysis of variance followed by Tukey's test with the probability level of 0.05 as the statistically significant one.

For gene induction of Δ^1 -pyrroline-5-carboxylate synthetase (P5CS), a different set of 30 days old plants was used. These plants were subjected to salinity treatment (250mM NaCl) for 24 h. The youngest leaves from control and treated plants were used; one sample included the mixture of 3 leaves from 3 plants. Total RNA from leaf tissue was extracted using Trizol reagent (GibcoBRL). One μ g of total RNA was reverse transcribed using gene specific degenerate primers for P5CS expression and Qiagen one step RT-PCR kit. Actin gene was used for standardization of PCR conditions (Table 1). Linear amplification for semi-quantitative RT-PCR was obtained with 35 cycles (Genie 32 Thermal Block, Bioneer, Daedejon, Korea) as follows: initial PCR activation step: 15 min at 95°C; reverse transcription: 30 min at 50°C; denaturation: 1 min at 94°C; annealing: 1 min at 60°C; extension: 1 min at 72°C; final extension: 10 min at 72°C. The amplification products were electrophoresed on 1.2 % agarose gel at 120 V in buffer containing 0.4M Tris-borate and 0.001M EDTA (pH 8.0) using a known concentration of DNA ladders. Gels were stained with ethidium bromide and visualized on Uvi Pro Gel Documentation System (Uvitec, Cambridge, England). The quantity of PCR product was analysed after scanning using Multi Gauge software (Fuji Film, Kio, Japan).

RESULTS AND DISCUSSION

Decreasing MSI reveals the malfunctioning of the cellular membranes by increasing their permeability to ions and electrolytes. From this point

of view, F_1 generation PEHM 3 showed the best response during the first sampling whereas there was no difference among parental lines (Table 2). During the second sampling, CM 142 showed the worst membrane damage (Table 2). Although PEHM 3 showed the highest MSI in S2 during the second sampling, no statistically significant difference occurred between parental CM 213 and F1 PEHM 3 (Table 2).

Total chlorophyll content is considered as a parameter reflecting salt tolerance (SRIVASTAVA *et al.* 1988). CM 142 showed the lowest chlorophyll content during both samplings in both salinity levels (Table 2). PEHM 3 had significantly higher chlorophyll content in S2 during both samplings.

Carotenoid reduction due salinity stress has been observed e.g. in wheat and in maize (ABD EL SAMAD 1993; KHOLOVA *et al.* 2009) and this trend was confirmed in our experiments as well. CM 142 had the lowest carotenoid content in S2 during both samplings (Table 2). Carotenoids can directly deactivate singlet oxygen and they can also quench the excited triplet state of chlorophyll, thus indirectly reducing the formation of ROS (FOYER & HARBINSON 1994). From this point of view, CM 142 showed weak low molecular protection against superoxide radicals compared to the other two cultivars in the higher salinity level. PEHM 3 had higher carotenoid content compared to CM 213 with the exception of S1 in the second sampling, where the content was insignificantly higher in CM 213 (Table 2).

CM 142 accumulated the highest concentration of superoxide radical ions during both samplings (Table 2). This content was lower in PEHM 3 compared to CM 213, however the difference was not significant (with the exception of S1 during the first sampling, where significance was proved). It could be a crucial parameter in salt stress response because the superoxide radical was implicated as an agent accelerating lipid peroxidation, membrane damage, cellular toxicity and single breaks in DNA (FRIDOVICH 1986).

Table 1. Primer sequences for Δ^1 -pyrroline-5-carboxylate synthetase (P5CS) and for actin

Gene	Source		Primers
P5CS	maize	forward	GCATCAACTAAGATACCTGTCCTC
		reverse	GTGTTGTTAAGAGACCTTCAACAC
Actin	maize	forward	TCAGGTGATGGTGTGAGCCACA
		reverse	CACCGATCCAGACACTGTACTTC

Table 2. Effect of two salinity levels (250mM NaCl – S1 and 375mM NaCl – S2) on the membrane stability index (%), contents of total chlorophyll and carotenoids (mg/g fresh weight), superoxide radical ($\Delta A_{560}/s/kg$ fresh weight), Na^+ ions ($\mu mol/g$ dry weight), proline ($\mu mol/g$ dry weight) and total sugars ($\mu mol/g$ dry weight) and the activities of ascorbate peroxidase (APX) ($\Delta A_{290}/s/\mu g$ total protein), catalase (CAT) ($\Delta A_{240}/s/\mu g$ total protein) and glutathione reductase (GR) ($\Delta A_{412}/s/\mu g$ total protein)); within each row, the same letters indicate no significant difference among treatments at the $P < 0.05$ level

	S ₀			S ₁			S ₂		
	CM 213	CM 142	PEHM 3	CM 213	CM 142	PEHM 3	CM 213	CM 142	PEHM 3
After 15 days of treatment									
Membrane stability index	91.08 ± 0.84 ^b	90.83 ± 1.83 ^b	89.45 ± 1.36 ^b	81.62 ± 0.52 ^a	80.96 ± 0.36 ^a	89.85 ± 1.25 ^b	79.93 ± 2.55 ^a	78.10 ± 1.06 ^a	88.41 ± 0.18 ^b
Total chlorophyll content	3.67 ± 0.07 ^b	3.21 ± 0.27 ^b	3.50 ± 0.19 ^b	3.27 ± 0.40 ^b	2.99 ± 0.22 ^b	3.55 ± 0.38 ^b	1.12 ± 0.10 ^a	0.74 ± 0.35 ^a	3.42 ± 0.47 ^b
Carotenoids content	0.36 ± 0.05 ^b	0.42 ± 0.13 ^b	0.44 ± 0.01 ^b	0.34 ± 0.03 ^b	0.27 ± 0.19 ^a	0.40 ± 0.07 ^b	0.23 ± 0.03 ^a	0.13 ± 0.09 ^a	0.38 ± 0.04 ^b
Superoxide radical content	0.25 ± 0.05 ^a	0.25 ± 0.03 ^a	0.28 ± 0.02 ^a	0.44 ± 0.04 ^b	0.59 ± 0.10 ^b	0.36 ± 0.06 ^a	0.45 ± 0.06 ^b	0.65 ± 0.11 ^b	0.43 ± 0.08 ^b
Na ⁺ content	47.3 ± 5.2 ^a	56.7 ± 6.0 ^a	46.0 ± 6.8 ^a	106.0 ± 3.8 ^b	160.7 ± 6.5 ^c	100.0 ± 3.6 ^b	182.7 ± 9.0 ^c	225.3 ± 7.3 ^d	164.7 ± 11.6 ^c
Proline content	11.59 ± 1.72 ^a	18.49 ± 1.87 ^b	15.0 ± 1.63 ^b	71.68 ± 4.84 ^d	28.93 ± 6.49 ^c	35.26 ± 3.93 ^c	71.68 ± 4.84 ^d	28.93 ± 6.49 ^c	35.26 ± 3.93 ^c
Total sugar content	666 ± 38 ^b	661 ± 39 ^b	309 ± 27 ^a	1222 ± 60 ^c	741 ± 79 ^b	871 ± 44 ^b	1336 ± 120 ^c	760 ± 89 ^b	1130 ± 83 ^c
APX activity	8.04 ± 0.52 ^a	7.22 ± 0.49 ^a	6.72 ± 0.39 ^a	7.58 ± 0.82 ^a	10.73 ± 0.42 ^b	10.19 ± 1.09 ^b	9.69 ± 0.47 ^b	11.56 ± 1.95 ^b	15.35 ± 1.08 ^c
CAT activity	2.22 ± 0.33 ^b	1.89 ± 0.50 ^b	1.09 ± 0.33 ^a	3.63 ± 0.07 ^c	2.11 ± 0.08 ^b	1.81 ± 0.13 ^b	3.86 ± 0.10 ^c	5.76 ± 0.27 ^d	3.86 ± 0.40 ^c
GR activity	2.14 ± 0.62 ^b	1.62 ± 0.09 ^a	1.47 ± 0.29 ^a	2.61 ± 0.07 ^b	4.08 ± 0.12 ^c	1.92 ± 0.06 ^a	4.27 ± 0.07 ^c	3.89 ± 0.09 ^c	2.64 ± 0.08 ^b
After 31 days of treatment									
Membrane stability index	91.97 ± 1.20 ^c	91.10 ± 2.12 ^c	92.63 ± 2.02 ^c	90.62 ± 1.70 ^c	88.99 ± 1.13 ^c	91.77 ± 1.79 ^c	77.82 ± 1.32 ^b	61.87 ± 0.92 ^a	81.95 ± 2.79 ^b
Total chlorophyll content	4.23 ± 0.13 ^b	3.62 ± 0.45 ^b	3.33 ± 0.19 ^b	2.69 ± 0.26 ^b	2.18 ± 0.22 ^b	2.13 ± 0.26 ^b	1.53 ± 0.22 ^a	0.97 ± 0.07 ^a	2.61 ± 0.28 ^b
Carotenoids content	0.43 ± 0.05 ^b	0.45 ± 0.02 ^b	0.58 ± 0.07 ^c	0.36 ± 0.06 ^b	0.33 ± 0.08 ^b	0.33 ± 0.03 ^b	0.23 ± 0.07 ^a	0.13 ± 0.09 ^a	0.38 ± 0.06 ^b
Superoxide radical content	0.20 ± 0.02 ^a	0.20 ± 0.08 ^a	0.18 ± 0.06 ^a	0.23 ± 0.03 ^a	0.25 ± 0.08 ^a	0.17 ± 0.02 ^a	0.23 ± 0.02 ^a	0.53 ± 0.15 ^b	0.19 ± 0.02 ^a
Na ⁺ content	47.9 ± 3.0 ^b	58.7 ± 5.2 ^c	38.7 ± 3.5 ^a	154.7 ± 15.7 ^e	209.3 ± 27.7 ^f	100.0 ± 12.5 ^d	211.3 ± 18.6 ^f	289.3 ± 13.7 ^g	160.0 ± 9.3 ^e
Proline content	19.50 ± 2.74 ^b	12.50 ± 1.38 ^a	10.63 ± 1.66 ^a	39.47 ± 3.15 ^c	22.59 ± 4.23 ^b	26.82 ± 3.30 ^b	59.80 ± 3.51 ^d	27.78 ± 3.89 ^b	35.36 ± 5.33 ^c
Total sugar content	563 ± 51 ^b	588 ± 39 ^b	385 ± 33 ^a	875 ± 89 ^c	830 ± 92 ^c	571 ± 47 ^b	1129 ± 61 ^d	747 ± 55 ^c	1090 ± 56 ^d
APX activity	6.37 ± 0.58 ^b	2.19 ± 0.48 ^a	4.71 ± 0.03 ^b	7.35 ± 0.14 ^c	5.85 ± 1.06 ^b	9.86 ± 0.92 ^c	8.70 ± 0.16 ^c	10.54 ± 0.07 ^c	15.16 ± 0.65 ^d
CAT activity	1.98 ± 0.23 ^a	3.10 ± 0.35 ^b	2.98 ± 0.97 ^b	3.63 ± 0.12 ^c	3.86 ± 0.22 ^c	2.56 ± 0.65 ^b	4.58 ± 0.12 ^c	2.14 ± 0.32 ^a	3.10 ± 0.22 ^b
GR activity	3.20 ± 0.20 ^b	3.66 ± 0.18 ^b	2.03 ± 0.18 ^a	3.80 ± 0.18 ^b	4.99 ± 0.20 ^c	2.84 ± 0.14 ^b	6.00 ± 0.27 ^d	3.61 ± 0.14 ^b	3.52 ± 0.08 ^b

High concentrations of Na^+ cause osmotic imbalance, membrane disorganization, reduction in growth and inhibition of cell division and expansion (MAHAJAN & TUTEJA 2005). High Na^+ levels also lead to reduction in photosynthesis and to higher production of ROS (GREENWAY & MUNNS 1980; YEO 1998; MAHAJAN & TUTEJA 2005). Na^+ accumulation in tomato under saline conditions is under genetic control, attributable to additive effects – dominance has a small influence (FOOLAD *et al.* 1997; FLOWERS 2004). In our experiment, CM 142 accumulated the significantly highest Na^+ concentration during both samplings (Table 2). During the first sampling, PEHM 3 had insignificantly lower Na^+ content compared to CM 213 (Table 2) and during the second sampling PEHM 3 showed significantly lower Na^+ content compared to CM 213 in both salinity levels (Table 2). This implies that Na^+ accumulation could be one of the important physiological selection traits for plant breeders.

It is well known that proline concentration increases under salinity up to 100 times the normal level, which makes up to 80% of the total amino acid pool (THOMAS *et al.* 1992). After 24 h of salinity treatment, the gene expression of Δ^1 -pyrroline-5-carboxylate synthetase, bifunctional enzyme catalysing the first two steps of proline biosynthesis, was highest in CM 213. After the normalization of PCR product C5CS to actin gene values, the highest expression occurred in CM 213 (26), while the values in CM 142 and PEHM 3 were identical (9) (Figure 1). The proline content was significantly highest in CM 213 in both salinity levels during both samplings (Table 2), while PEHM 3 produced lower proline

concentrations. CM 142 showed the lowest proline concentration. An increase in the proline content was reported during high salinity conditions (e.g. YOSHIBA *et al.* 1995; KHOLOVA *et al.* 2009; RADYUKINA *et al.* 2011). On the other hand, the proline content was not correlated with salt tolerance in barley (CHEN *et al.* 2007; WIDODO *et al.* 2009; SZABADOS & SAVOURÉ 2010) and similar results were discussed in the review by ASHRAF and HARRIS (2004). In addition, LIU and ZHU (1997) reported that *Arabidopsis* mutant sensitive to salt stress had a higher proline content compared to the less sensitive control. They suggested that the proline content is not a factor limiting salt tolerance in this plant and that proline accumulation is a symptom of stress injury rather than an indicator of stress tolerance (LIU & ZHU 1997). In our experiment, the higher proline content in CM 213 was not also correlated with physiological responses, which were worse in parental CM 213 compared to F_1 PEHM 3 (e.g. chlorophyll content in S2).

Although it has been suggested that the saccharide accumulation under salinity could be a useful biomarker for selecting tolerant wheat genotypes (NAUREEN & NAQVI 2010), the situation seems to be much more complicated. ASHRAF and TUFAIL (1995) found that the saccharide content increased significantly in five different sunflower lines with increasing salt concentration in the growth medium and the salt tolerant lines had generally higher saccharide content than the sensitive ones. In contrast, in safflower the pattern of accumulation of sugars differed: the salt tolerant line accumulated sugars similarly to salt sensitive lines (ASHRAF & FATIMA 1995; ASHRAF & HARRIS 2004). It is difficult to evaluate the saccharide content in our experimental plants: although CM 213 showed the highest saccharide content (Tables 2 and 3), the most distinct increase under salinity treatment compared to control was shown by PEHM 3, while control plants contained approximately a half of the saccharide amount compared to that in CM 213

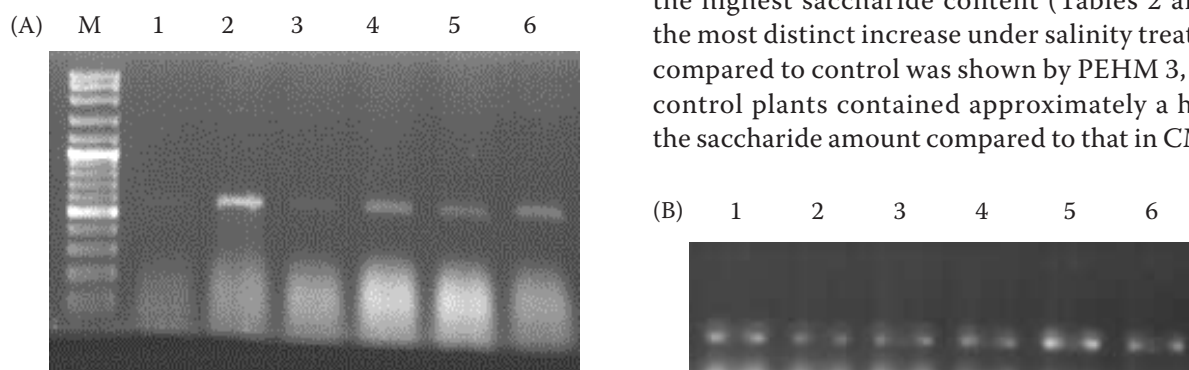


Figure 1. RT-PCR expression analysis of Δ^1 -pyrroline-5-carboxylate synthetase (A) and actin (B) genes under the treatment with 250mM NaCl for 24 h and under control conditions (M – marker; 1 – CM 213 control; 2 – CM 213 salinity; 3 – CM 142 control; 4 – CM 142 salinity; 5 – PEHM 3 control; 6 – PEHM 3 salinity)

and CM 142. Nevertheless, even after comparison of an increase with the corresponding controls, CM 142 showed the lowest increase.

The inheritance of parameters associated with the activities of antioxidant enzymes was reported before (SINGH *et al.* 2010). Considering the high heritability of such parameters, GORJI *et al.* (2011) even suggested to consider antioxidant enzymes as good criterion for selecting triticale genotypes resistant to drought. ASHRAF and OLI (2008) suggested the same criterion for the selection to salt stress resistance in canola. It has also been documented that plants containing high concentrations of antioxidants show considerable resistance to the oxidative damage caused by salinity (e.g. MITTOVA 2000). Our results were not so unequivocal. Although CM 142 was generally characterised by worse physiological parameters (contents of total chlorophyll, Na⁺ and superoxide radical), during the first sampling CM 142 showed the highest APX activity in S1 and CAT activity in S2 (Table 2); and during the second sampling this genotype showed the highest CAT and GR activities in S1 (Table 2). On the other hand, PEHM 3 with the highest MSI and chlorophyll content had the lowest GR activities in both S1 and S2 during both samplings together with the lowest CAT activities in S1 during both samplings; nevertheless APX activity was the highest in this cultivar in S2 during both samplings (Table 2). CM 142 was the only genotype whose activities of CAT and GR decreased during the second sampling in S2 compared to S1 (Table 2).

In our experiment, a heterotic effect occurred after the crossing of comparatively sensitive CM 142 with tolerant CM 213 maize lines. There are no well-defined plant indicators for salinity tolerance that could practically be used by plant breeders for the improvement of salinity tolerance in agricultural crops (ASHRAF & HARRIS 2004). Our results suggest that together with cell protecting factors, superoxide radical and Na⁺ contents along with indicators of stress damage (i.e. MSI) might be considered in maize salinity tolerance improvement breeding programmes.

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