

Differential Response of Antioxidative Enzymes to Various Abiotic Stresses in *Pennisetum glaucum* Seedlings¹

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Abstract—Antioxidative enzyme activities and their isozyme patterns under water-deficit, salinity, high and low temperature stresses were studied in the seedlings of *Pennisetum glaucum* (L.) R.Br. It was observed that under water-deficit stress glutathione reductase (GR) was the key enzyme while in case of high temperature stress, GR along with catalase played a major role. Superoxide dismutase was found to be the main enzyme under low temperature stress. Co-ordinated higher expression of all the antioxidative enzymes was observed under salt stress. This study revealed the operation of different enzymatic antioxidative mechanisms under various abiotic stresses that will aid in understanding the metabolic basis of stress tolerance in pearl millet.

Keywords: *Pennisetum glaucum*, abiotic stresses, normalized membrane stability index, antioxidative enzymes, isozymes

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INTRODUCTION

Abiotic stresses such as drought, salinity and extreme temperatures are the major constraints in agriculture that adversely affect crop growth and yield. One of the metabolic changes in plants under these harmful stress conditions is the accumulation of reactive oxygen species (ROS) which are inevitable by-products of normal cell metabolism.

To combat against these cellular damages, plants are equipped with antioxidative (AOX) defense mechanisms that function as an extremely efficient cooperative system. The major ROS detoxification mechanisms include superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR), ascorbate peroxidase (APX) and guaiacol peroxidase (GPX) which are located in various sub-cellular compartments of chloroplasts, mitochondria and peroxisomes within the plant cell. SOD, the front-line enzyme in ROS detoxification, scavenges superoxide dismutating it to O₂ and H₂O₂. The major enzymatic cellular scavengers of H₂O₂ are CAT, APX and GPX. APX functions as a fine regulator of intracellular H₂O₂, while CAT located exclusively in the peroxisomes functions as a bulk remover of excess of H₂O₂ produced under stress conditions. GR, the last enzyme of the ascorbate/glutathione cycle scavenges singlet oxygen,

superoxide and hydroxyl radicals [1]. Changes in the subcellular distribution of antioxidative enzyme activities along with their isozymes result in a more efficient protection strategy than an increase in the enzyme activity alone.

The level of the AOX enzymes activities enhance under different stresses depends on the capacity of the plant species to withstand against that particular stress treatment [2]. In addition, a tremendous biological diversity among different plant species necessitates the identification of crop specific mechanisms. Earlier workers showed a correlation between abiotic stress tolerance and antioxidative responses in different plant systems, e.g. finger millet [3]. There are very few reports on the antioxidative mechanisms operating under various abiotic stresses in pearl millet [4]. These antioxidative enzymes and their isozymes demonstrate the importance of ROS detoxification for cellular survival and their roles in imparting tolerance in plants under abiotic stresses.

Pennisetum glaucum (Pearl millet) is an important cereal crop endowed with climate resilience and is tolerant to abiotic stresses [5], and hence it is a good model for understanding the metabolic basis of stress tolerance in terms of antioxidative enzymes. Although, the role of AOX enzymes under different stresses has been studied individually in many crop plants, till date there are very few reports available on comparison of these responses under four different abiotic stresses. A comparison of these responses under various stresses enables us to identify the stress

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Abbreviations: AOX—antioxidative; APX—ascorbate peroxidase; CAT—catalase; GPX—guaiacol peroxidase; GR—glutathione reductase; SOD—superoxide dismutase.

specific and common components related to the relative ability of pearl millet to cope with these abiotic stresses. Therefore, the present study was aimed to elucidate the mechanism of antioxidative defense system under water-deficit, salinity, high and low temperature stresses in pearl millet.

MATERIALS AND METHODS

Plant growth and stress treatments. Seeds of pearl millet [*Pennisetum glaucum* (L.) R. Br.] ICMH 356 and its parents ICMR 356(♂) and ICMB 88004(♀) used in the present study were obtained from International Crops Research Institute for Semi-Arid Tropics (ICRISAT, Patancheru, Telangana, India). Seeds were sown in pots (18.5 cm diameter and 16 cm height) and then maintained at 28°C under optimal conditions in a glass house with regular watering. Fifteen-days-old seedlings with uniform growth were subjected to various stress treatments.

For water-deficit stress treatment, the seedlings were taken, washed with water to remove the soil particles from the roots, pat dried and were placed in the desiccator equilibrated with 25% glycerol.

The seedlings were initially acclimatized for high temperature stress by gradually increasing the temperature from 37 to 45°C by increasing 2°C per hour. The samples were then subjected to high temperature stress at 45°C.

For the imposition of salt stress, seedlings were administered with 0.25 M NaCl solution instead of water.

For low temperature stress, the seedlings were exposed to 4°C. For water-deficit and high temperature stresses, the samples were collected at 0, 6, 12, 24 and 48 h after stress treatment while, for salt and low temperature stresses, the samples were collected at 0, 12, 24, 48 and 72 h. Well watered seedlings served as control. Since the time intervals were not uniform in all the stresses, normalization of MSI was done to arrive at a common indicator. For normalization, MSI at 0 h was considered as 100% and MSI of the subsequent time intervals were calculated as follows. Normalized MSI (%) = MSI at a given time interval × 100/MSI at 0 h.

Stress quantification. Stress induced was quantified in terms of relative water content (RWC), proline accumulation, chlorophyll content, leaf membrane stability index (MSI) and lipid peroxidation. RWC was determined in the leaf tissues [6]. RWC was calculated with the following equation:

$$\text{RWC}(\%) = [(\text{FW} - \text{DW})/(\text{TW} - \text{DW})] \times 100, \quad (1)$$

where FW is fresh weight of the leaf tissue taken, TW is the turgid weight after rehydration and DW is the dry weight after oven drying.

Proline accumulation was estimated according to Bates et al. [7]. Chlorophyll content was determined

with acetone method [8]. Leaf membrane stability index (MSI) was recorded as described by Sairam [9] and calculated with a formula:

$$\text{MSI} = [1 - (C_1/C_2)] \times 100, \quad (2)$$

where C_1 and C_2 are the electric conductivities recorded at 40 and 100°C, respectively. For calculation of normalized MSI values, MSI at 0 h was considered as 100% and subsequent values at different time intervals were calculated. Lipid peroxidation was determined by estimating the amount of malondialdehyde (MDA) content recorded at an absorbance of 532 nm and the non-specific absorption at 600 nm was subtracted. The MDA content recorded was then calculated with the extinction coefficient of 155 mM cm⁻¹ [10].

In vitro assay of antioxidative enzymes. For in vitro assay of antioxidative enzymes, viz., SOD, CAT, GR, APX and GPX, the enzyme extract was prepared by homogenizing the leaf tissue in 100 mM potassium phosphate buffer (pH 7.5) and 0.5 mM EDTA under chilled conditions. The homogenate was centrifuged at 10000 rpm for 20 min at 4°C and the supernatant was used as the source of enzyme. For the determination of APX the homogenization buffer was applied except that it also contained 5 mM ascorbate. Soluble protein content in the enzyme extract was determined with bovine serum albumin as standard. Activities of all the AOX enzymes, except SOD were monitored in kinetics for 3 min in the spectrophotometer and expressed as enzyme units/(mg protein min).

The activity of SOD was assayed by decrease in absorbance of formazone formation in the reaction of nitro-blue tetrazolium chloride with superoxide radicals at 560 nm [11]. One unit of SOD activity was defined as the quantity of enzyme required to cause 50% inhibition. CAT activity was assayed from the rate of H₂O₂ decomposition as measured by the decrease in absorbance at 240 nm [12]. One unit of CAT is defined as the amount of enzyme that decomposes 1 μmol of H₂O₂ per min at pH 7.0. GR activity was estimated by recording the increase in absorbance in the presence of oxidized glutathione and DTNB (5,5-dithiobis-2-nitrobenzoic acid) [13]. One unit of GR activity may be referred as the amount of enzyme required to cause the oxidation of 1.0 μmol of NADPH at pH 7.5. APX activity was assayed from the rate of H₂O₂ decomposition as measured by the decrease in absorbance at 290 nm [14]. Activity of APX was expressed by the decrease in ascorbic acid content with a standard curve (20–100 mg/mL). GPX activity was determined according to Chance and Machly [15] by monitoring the formation of tetra guaiacol at 470 nm. One unit is the amount of enzyme which catalyzes the conversion of 1 μmol of H₂O₂ per min.

Native PAGE for staining isozyme patterns of SOD, APX and GPX. The separation of isozymes of SOD, APX and GPX was carried out on native polyacryl-

amide gels containing 10% resolving gel (pH 8.8) and 4% stacking gel (pH 6.8) under chilled conditions according to the procedure of Laemmli [16] except SDS. A protein concentration of 50 µg was loaded into each well and then electrophoresed at 100 V through the stacking gel for 15 min and 120 V through the separating gel for 60 min. After electrophoresis, SOD isozymes were stained according to the modified photochemical method of Beauchamp and Fridovich [17]. The gel was first soaked in 25 mL of 1.23 mM NBT for 15 min, briefly washed, then soaked in 30 mL of 100 mM potassium phosphate buffer (pH 7.0) containing 28 mM TEMED and 0.02 mM riboflavin for another 15 min in dark. The gel was briefly washed with distilled water and illuminated for 15 min to initiate the photochemical reaction. For carrying out native PAGE of APX isozymes, the carrier buffer was supplemented with 2 mM ascorbate and the gels were pre-run for 30 min to allow ascorbate to enter the gel prior to the application of samples [18]. After the separation, the gels were incubated in 50 mM potassium phosphate buffer (pH 7.0) containing 2 mM ascorbate for 30 min followed by incubation in phosphate buffer containing 4 mM ascorbate and 2 mM H₂O₂ for 20 min. The gels were washed with buffer in 1 min and then soaked in potassium phosphate buffer (pH 7.8) containing 28 mM TEMED and 2.45 mM NBT for 15 min with gentle agitation [18, 19]. GPX isozymes were stained by incubating the gel in 50 mM sodium acetate buffer (pH 5.0) containing 330 µL of guaiacol and the reaction was initiated with 30% H₂O₂ [19, 20]. The isozymes were visualized and photographed.

Statistical analysis. There were three replicates for each treatment and the experiment was repeated at least twice. The standard error and ANOVA were done according to standard statistical procedure [21].

RESULTS

Stress Quantification

Relative water content, membrane stability index and chlorophyll content decreased gradually while proline accumulation increased in the hybrid and parents of pearl millet when subjected to water-deficit, salt (NaCl), high and low temperature stresses. The variations among the genotypes, treatments and their interactions were statistically significant (Figs. 1a–d). The percent decline in RWC was more under water-deficit (32%) and high temperature stresses (22–31.5%) compared to that under salt and low temperature (21.4–25.8%) stresses. Proline accumulation gradually increased and peaked at the end of the stress treatments. A remarkable increase ranging from 87–89% was observed under high temperature, NaCl and low temperature stresses as compared to a moderate increase of 63.4% under water-deficit stress. The decrease in chlorophyll content was higher in salt (NaCl) stress (52.3%) compared to the other three

stresses (29–44.9%). Membrane damage as indicated by their higher MDA and lower MSI values was higher under salt and low temperature stresses (75%) compared to water-deficit and high temperature stresses (66–69%). The hybrid performed better in compare with its male and female parents in terms of all the stress indicators under all the four stresses.

Activities of Antioxidative Enzymes under Various Abiotic Stresses

The activities of various AOX enzymes studied, viz., SOD, CAT, GR, APX and GPX increased gradually with the increase in time intervals in pearl millet hybrid and parents under all the four stresses (Fig. 2). However, their activities varied with stress treatments, time intervals and the genotypes. AOX enzyme activities were significantly higher in the hybrid in compare with male and female parents, respectively (Fig. 2). The SOD activities were found to be higher under NaCl and low temperature stresses (61.4–65.9%) as compared to water-deficit and high temperature (50.8–55.3%) stresses. CAT activity was higher under high temperature (57.2%) and NaCl stresses (52.5%) in compare with water-deficit and low temperature. GR activity increased under water-deficit and high temperature stresses in compare with NaCl and low temperature stresses. The levels of GPX and APX were almost similar under all the four stresses and ranged between 54–68.9 and 50–59%, respectively.

Isozyme Patterns of SOD, APX and GPX under Various Abiotic Stresses

Isozyme patterns of SOD, APX and GPX viewed on native gels indicated variation in the number as well as their expression in pearl millet hybrid and parents. Since equal amounts of protein extracts were electrophoresed on a non-denaturing gel, the band intensity equates to the total enzyme activity. Fe SOD1 was differentially expressed under NaCl and high temperature stresses. Cu-Zn SOD isoform was differentially expressed in all the stresses (Fig. 3). Induction of two new isozymes was observed under high temperature stress at 48 h only in the hybrid (Fig. 3b).

The isoforms of APX observed on gel were designated as APX1 to APX5 in the order of their increasing migration (Fig. 4). The isozyme profiles of APX revealed the expression of five different isoforms (APX1 to APX5) under water-deficit and salt, while only three isoforms (APX1, APX3 and APX4) were determined under low and high temperature stresses. APX1 and APX4 expressions were found to be prominent in all the four stresses. APX5 isoform expression was higher in water-deficit and salt (NaCl) stresses.

Isozyme pattern of GPX revealed the induction of six different isoforms under all the four stress treatments (Fig. 5). The expression of isoforms GPX1, GPX2, GPX5 and GPX6 was higher in all the four

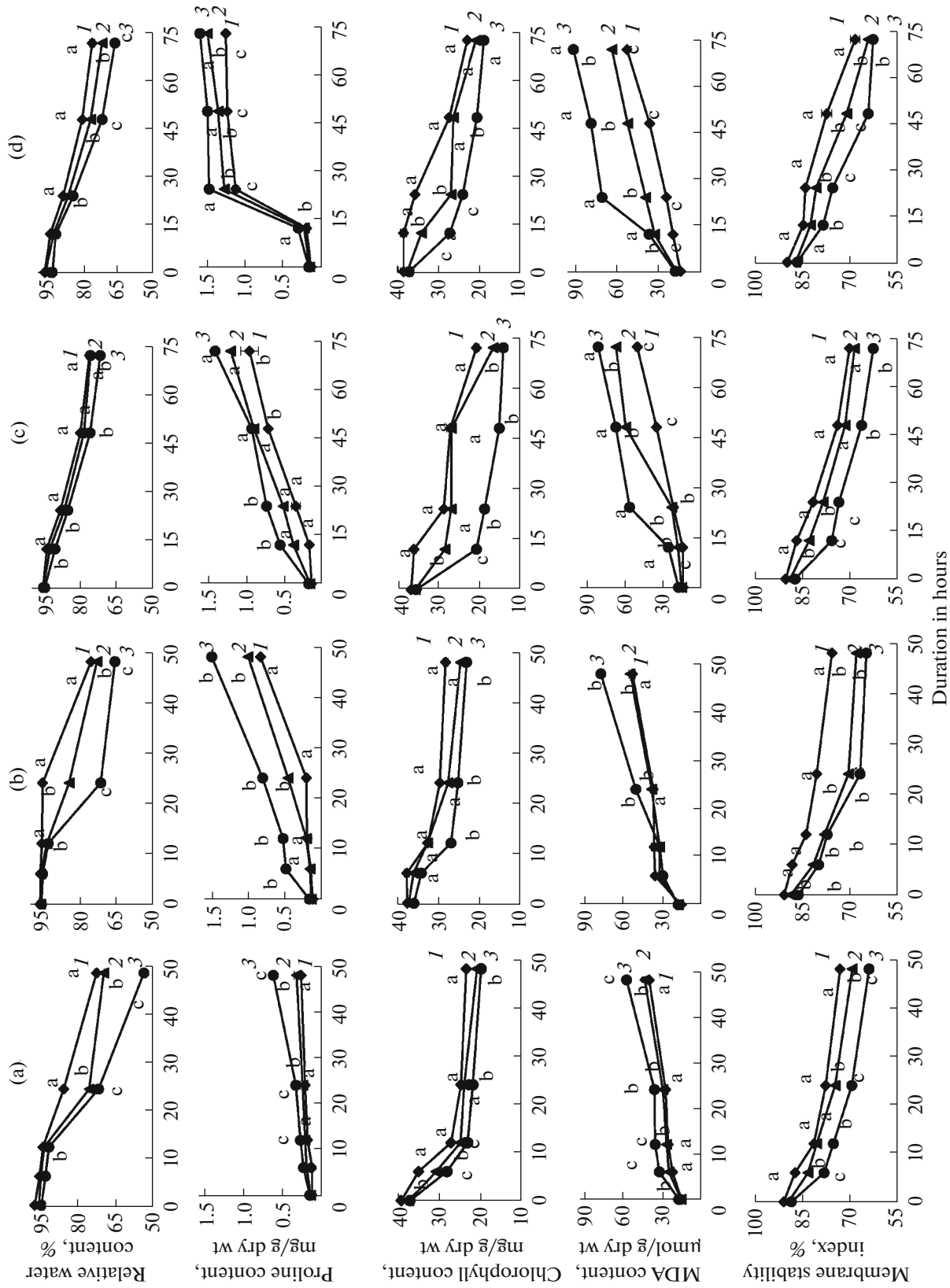


Fig. 1. Effect of water-deficit (a), high temperature (b), salt (NaCl) stress (c) and low temperature stress (d) on relative water content, proline accumulation, chlorophyll content, malondialdehyde content and membrane stability index in pearl millet hybrid and parents at various time intervals. 1—ICMH 356; 2—ICMB 88004. Values are means \pm SD ($n = 3$). Bars marked with the same letters are not significantly different ($P \leq 0.05$) based on LSD tests.

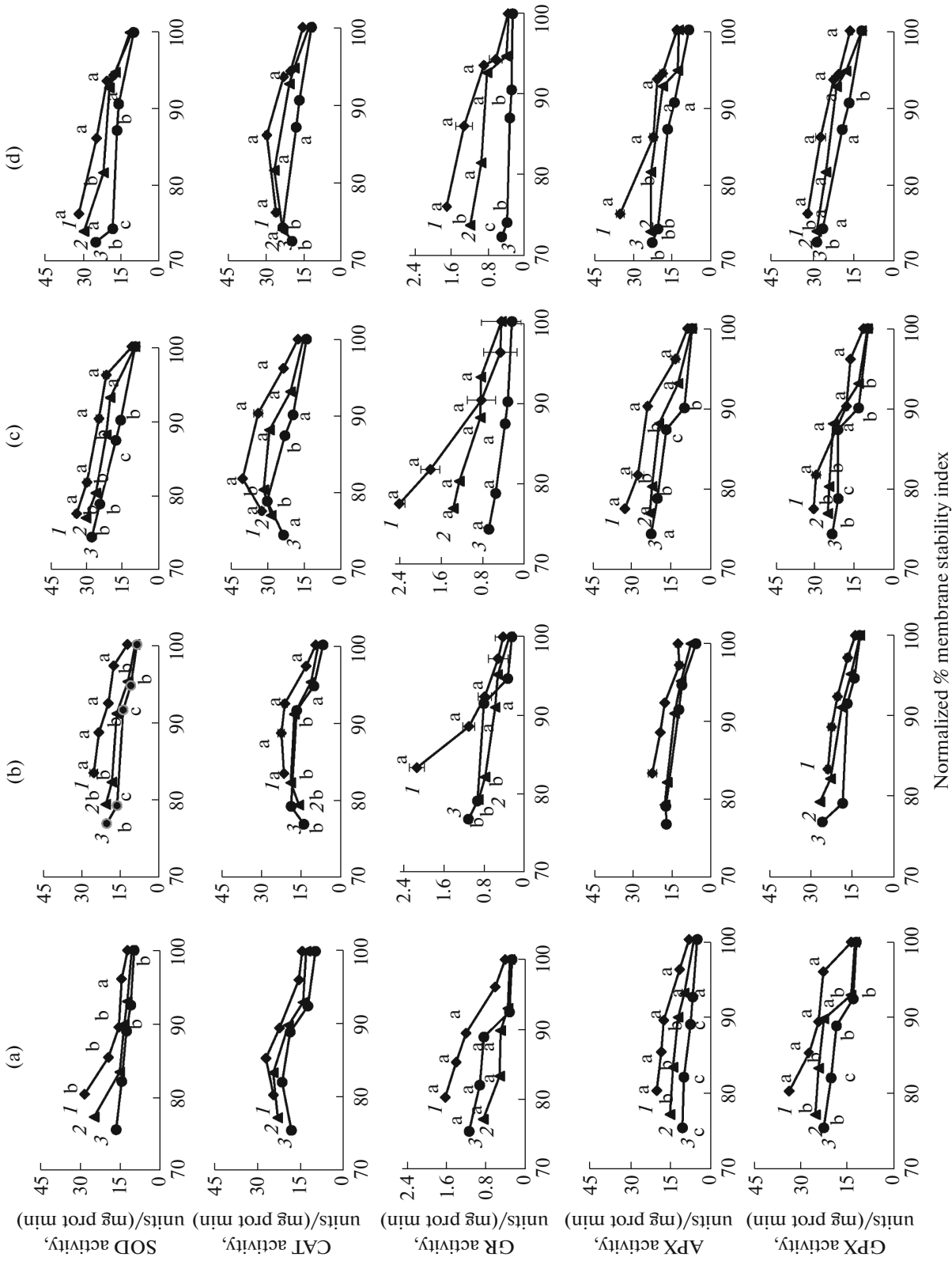


Fig. 2. Effect of water-deficit (a), high temperature (b), salt (NaCl) stress (c) and low temperature stress (d) on activities of superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR), ascorbate peroxidase (APX) and guaiacol peroxidase (GPX) in pearl millet hybrid and parents. 1—ICMH 356; 2—ICMR 356; 3—ICMB 88004. Values are means ± SD (*n* = 3). Bars marked with the same letters are not significantly different (*P* ≤ 0.05) based on LSD tests.

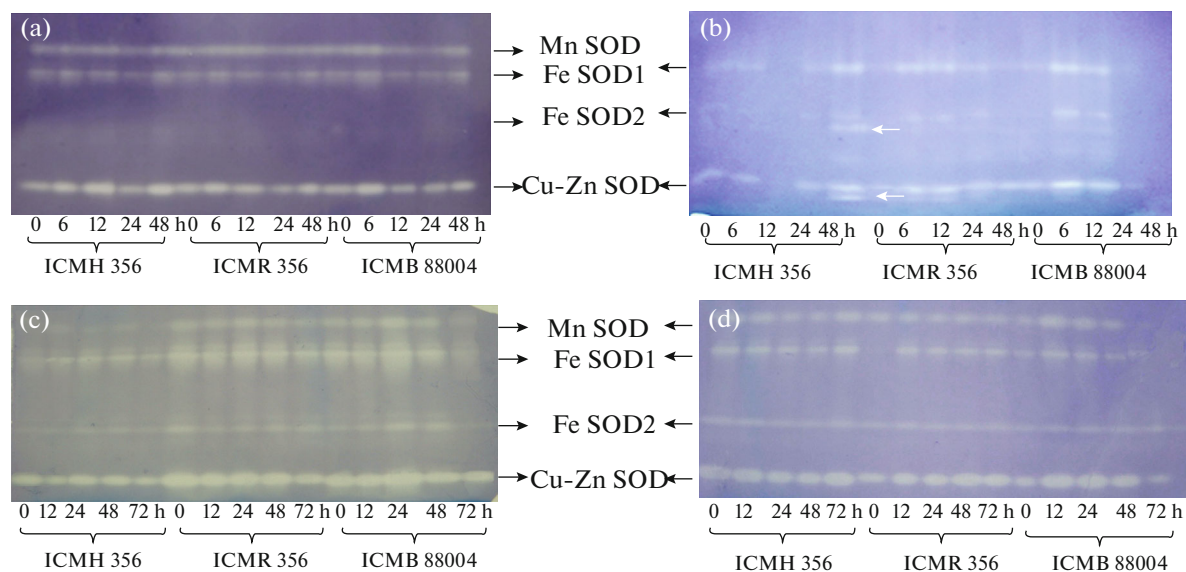


Fig. 3. Isozyme patterns of superoxide dismutase (SOD) in pearl millet hybrid and parents when subjected to water-deficit (a), high temperature (b), salt (NaCl) stress (c) and low temperature stress (d). Arrows in the gel picture indicates induction of new isoform.

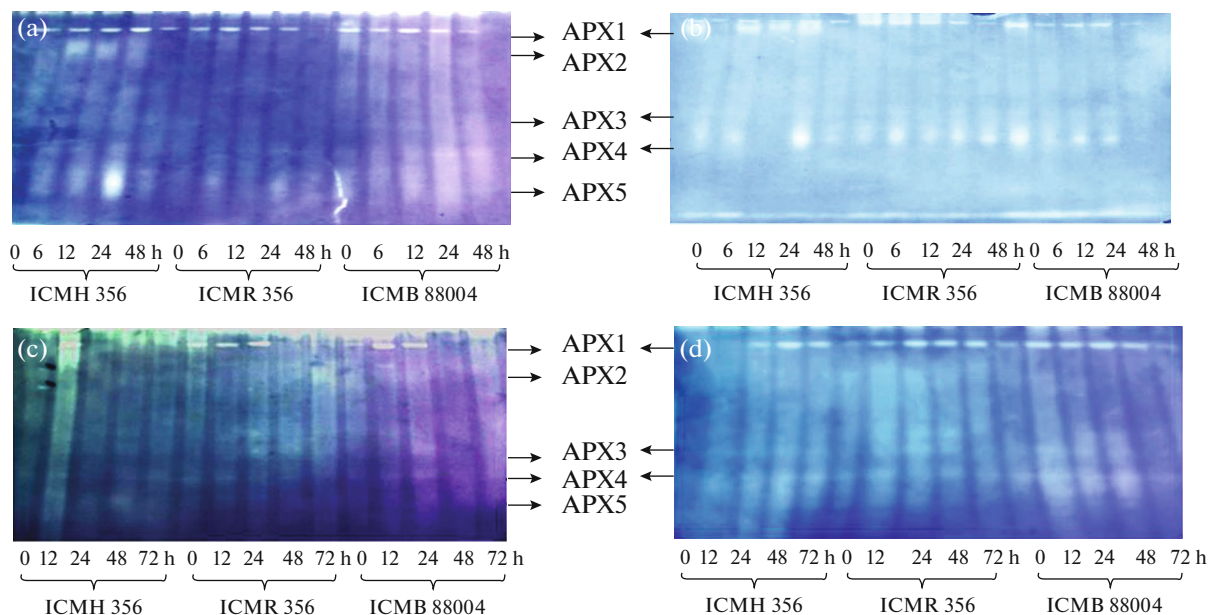


Fig. 4. Isozyme patterns of ascorbate peroxidase (APX) in pearl millet hybrid and parents when subjected to water-deficit (a), high temperature (b), salt (NaCl) stress (c) and low temperature stress (d).

stresses and also showed differential expression. Isoform GPX3 was induced under all the four stresses only in the male parent. Isoform GPX4 was expressed in the hybrid and female parent under all four stresses indicating maternal inheritance. Induction of a new isoform of GPX was observed under salt (NaCl) and under low temperature stresses (Figs. 5c and 5d).

DISCUSSION

The results of this study revealed several important aspects on the enzymatic antioxidative mechanisms under water-deficit, salt (NaCl), high and low temperature stresses in pearl millet (Fig. 6). Based on the stress response in terms of relative water content, chlorophyll, proline and malondialdehyde contents as well

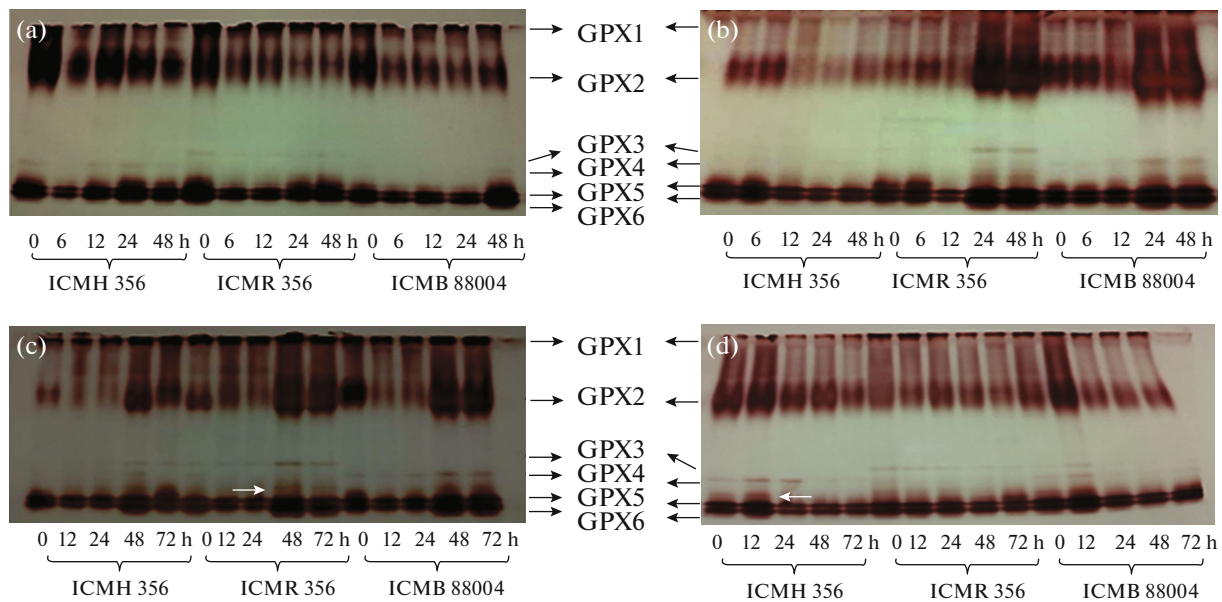


Fig. 5. Isozyme patterns of guaiacol peroxidase (GPX) in pearl millet hybrid and parents when subjected to water-deficit (a), high temperature (b), salt (NaCl) stress (c) and low temperature stress (d). Arrows in the gel picture indicate induction of new isoforms.

as membrane stability index, a gradual and progressive increase in stress intensity from 0 to 48 h in case of water-deficit and high temperature stresses and 0 to 72 h in case of salt (NaCl) and low temperature stresses was established in our experiments. Since, an increased production of ROS and membrane disintegration is central to all abiotic stresses, the levels of various AOX enzyme activities were assessed under different abiotic stresses and normalized MSI was taken as an indicator of the respective stress level. The hybrid and parents differed in the manifestation of stress intensity and its duration. The activity of AOX enzymes are known to be dependent on genotypes and the type of abiotic stress [2]. Based on the induction levels of the activity of AOX enzymes tested as well as the expression pattern of the various isoforms of these enzymes, the schematic diagram Fig. 6 has been drawn.

Under water-deficit stress, GR was the key enzyme responsible for detoxification of ROS generated. GR de novo synthesis is known to be one of the primary responses to mitigate water-deficit stress induced oxidative damage [22]. Increased activity of GR under water-deficit stress was earlier reported in our previous study with pearl millet [23]. Comparative analysis of modulation of AOX defense system has been reported in other crop plants like finger millet and wheat [3, 22]. It is known that SOD has three different isozymes located in different organelles. Fe SOD and Mn SOD are found mostly in chloroplast and mitochondria respectively while Cu-Zn SOD is mostly located in chloroplast, peroxisomes and cytosol [24]. In the present study, among the various isoforms of SOD, Cu-Zn

SOD was differentially expressed indicating its possible role in detoxification of superoxide radical. Other antioxidative enzymes CAT and GPX were induced at a lower level (Fig. 6a).

The antioxidative enzyme GR along with CAT was prominently induced under high temperature stress indicating a possible role for these in ROS detoxification. Catalase is well known to have highest conversion efficiency of all antioxidative enzymes and one molecule of CAT is known to remove about six million molecules of H_2O_2 per min [25], revealing it to be important in bulk detoxification of ROS. Induction of CAT was found to be enhanced under high temperature stress in wheat as well [25]. Induction of new isoforms of Fe SOD and Cu-Zn SOD observed in the present study only in the hybrid indicated their specific role in imparting tolerance to the hybrid (Fig. 6b).

Superoxide dismutase was found to be the main enzyme of antioxidative defense system under low temperature stress in pearl millet hybrid and parents. Increased activity of SOD is often suggested to be correlated with increased tolerance of the plant against various abiotic stresses [26]. The expression levels of GPX were moderate which might be involved in consumption of H_2O_2 generated under low temperature stress. All the isozymes of GPX (1–6) were differentially induced and a new isoform was observed in response to low temperature stress. GPX is known to function as an effective quencher of reactive intermediary forms of O_2 and peroxy radicals under stress conditions [27]. An increase in GPX seemed to be associated with an inactivation of CAT as indicated by its

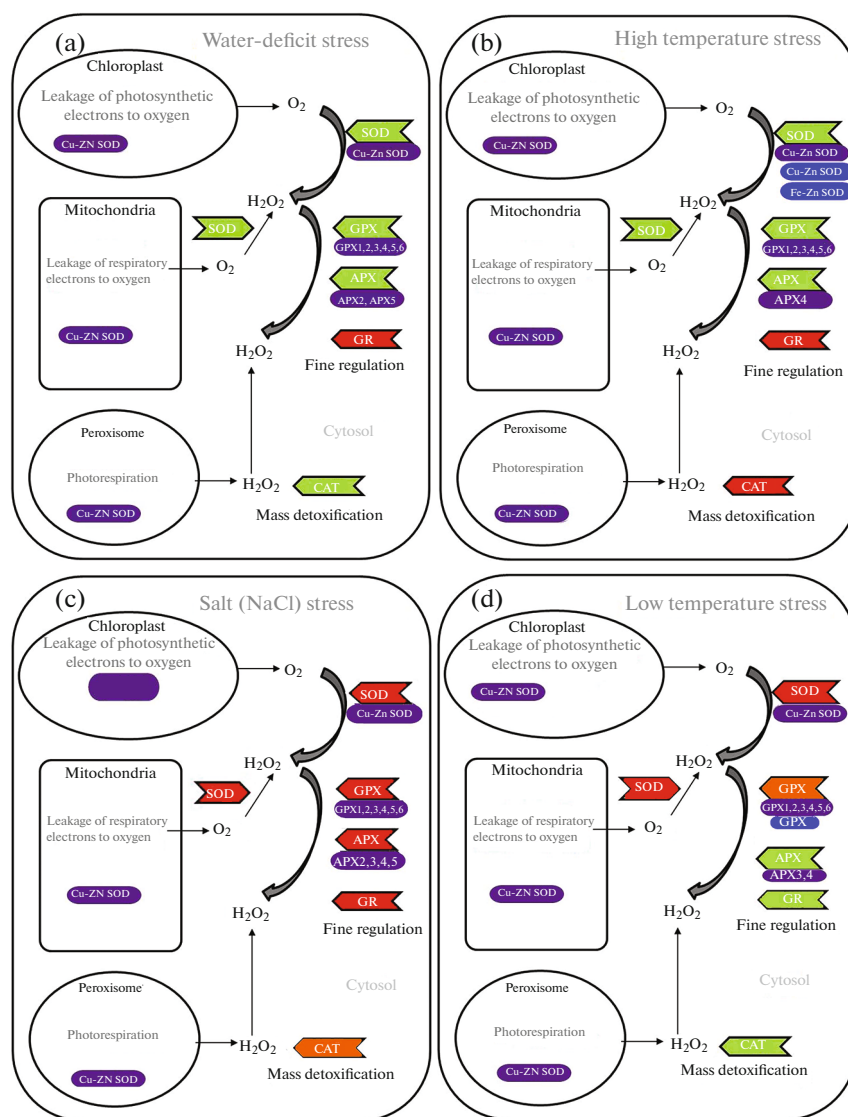


Fig. 6. Schematic diagram representing the antioxidative enzymatic pathways under water-deficit (a), high temperature (b), salt (NaCl) stress (c) and low temperature stress (d) in pearl millet. The coloured boxes in the figure indicate induction levels of AOX enzymes: red—high level; orange—medium level; green—low level; violet—differentially expressed isozymes; blue—newly induced isozymes.

lower level of expression. Earlier reports also suggested that an increase in GPX is associated with CAT inactivation [28]. Novel isozyme expression observed in high and low temperatures display the temperature specific expression (Fig. 6d).

In response to NaCl stress, the antioxidative enzymes SOD, APX, GPX and GR were highly induced while catalase was induced in medium level. The isozymes Fe SOD1 and Cu-Zn SOD were differentially expressed and might have contributed to the enhanced SOD activity. Similarly, APX isozymes APX1 and 4 were expressed higher and might have been resulted in the high APX enzyme activity. All the isozymes of GPX were highly induced. A co-ordinated high expression of all these antioxidative enzymes

under NaCl stress revealed the existence of probably a different mechanism of mass detoxification in compare with other three abiotic stresses (Fig. 6c). The generation of ROS and increased activity of many antioxidative enzymes during NaCl stress have been detected in several plant species [21, 29, 30].

Use of hybrid and parents is well recognized approach for comparative assessment of the pattern of trait expression and inheritance. In the present study, pearl millet hybrid and its parents were used to understand the manifestation of heterosis in AOX metabolic related traits. Various AOX enzyme activities were higher or equivalent to the better parent indicating expression of heterosis in the enzymes of AOX defence system in the hybrid. This approach can be further uti-

lized for fixing of desirable traits for eventually enhancing the stress tolerance.

This study revealed probable specific mechanisms of antioxidative defense system under water-deficit, high temperature, salt (NaCl) and low temperature stresses in pearl millet. The higher constitutive and enhanced activity of SOD, CAT, GR, APX and GPX in the hybrid compared to either of its parents indicates a better protection mechanism against stress-induced oxidative damage in the hybrid over its parents. Specific induction and expression of GPX4 in the hybrid and the female parent revealed the maternal inheritance governing the induction of this isozyme. Understanding the stress specific antioxidative defense system as well as the probable cross talk amongst different abiotic stresses may facilitate in developing strategies for genetic enhancement of abiotic stress tolerance.

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