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ORIGINAL PAPER

Polyamine metabolism influences antioxidant defense mechanism in foxtail millet (*Setaria italica* L.) cultivars with different salinity tolerance

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Abstract

Key message **Polyamines can regulate the expression of antioxidant enzymes and impart plants tolerance to abiotic stresses.**

Abstract A comparative analysis of polyamines, their biosynthetic enzymes at kinetic and at transcriptional level, and their role in regulating the induction of antioxidant defense enzymes under salt stress condition in two foxtail millet (*Setaria italica* L.) cultivars, namely Prasad, a salt-tolerant, and Lepakshi, a salt-sensitive cultivar was conducted. Salt stress resulted in elevation of free polyamines due to increase in the activity of spermidine synthase and *S*-adenosyl methionine decarboxylase enzymes in cultivar Prasad compared to cultivar Lepakshi under different levels of NaCl stress. These enzyme activities were further confirmed at the transcript level via qRT-PCR analysis. The cultivar Prasad showed a greater decrease in diamine oxidase and polyamine oxidase activity, which results in the accumulation of polyamine pools over cultivar Lepakshi. Generation of free radicals, such as $O_2^{\cdot-}$ and H_2O_2, was also analyzed quantitatively. A significant increase in $O_2^{\cdot-}$ and H_2O_2 in the cultivar Lepakshi compared with cultivar Prasad was recorded in overall pool sizes. Further, histochemical staining showed lesser accumulation of $O_2^{\cdot-}$ and of H_2O_2 in the leaves of cultivar Prasad than cultivar Lepakshi. Our results also suggest the ability of polyamine oxidation in regulating the induction of antioxidative defense enzymes, which involve in the elimination of toxic levels of $O_2^{\cdot-}$ and H_2O_2, such as Mn-superoxide dismutase, catalase and ascorbate peroxidase. The contribution of polyamines in modulating antioxidative defense mechanism in NaCl stress tolerance is discussed.

Keywords Salt stress · Antioxidants · Histochemical assay · Polyamines · *Setaria italica* L. · Spermidine synthase · *S*-Adenosylmethionine decarboxylase · Polyamine oxidase

Introduction

Polyamines (PAs) are small aliphatic low-molecular weight polycationic nitrogenous compounds that are ubiquitous in higher plants; the most common polyamines found in all living cells are putrescine (Put), spermidine (Spd) and spermine (Spm). In plants, Put is derived from either arginine (Arg) or ornithine (Orn), via the arginine decarboxylase (ADC; EC 4.1.1.19) or the ornithine decarboxylase (ODC; EC 4.1.1.17) pathways. Spd and Spm biosyntheses require the concerted action of spermidine synthase (SPDS; EC 2.5.1.16)/*S*-adenosyl-L-methionine decarboxylase (*S*-AMDC; EC 4.1.4.50) and spermine synthase (SPMS; EC 2.5.1.22)/*S*-AMDC, respectively (Paschalidis et al. 2009). PAs occur as free molecular bases (free form), or are often conjugated with small molecules (phenolic acids) or bound to macromolecules such as nucleic acids and proteins (Groppa and Benavides 2008).

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They have been implicated in a wide range of biological processes in plant growth and development, including senescence, environmental stress and infection by pathogenic fungi and viruses (Groppa and Benavides 2008).

PAs increase survival of various plants under salt stress (Legocka and Kluk 2005), drought (Yamaguchi et al. 2007; Kubis 2008), flooding (Yiu et al. 2009), chilling (Gao et al. 2009), osmotic and acidic stress (Capell et al. 2004), radiation-induced oxidative stress (Katerova and Todorova 2009) and heavy metal stress (Wang et al. 2007; Zhao and Yang 2008).

Although there is increasing evidence supporting the stress-induced accumulation of polyamines in several plant species, the maintenance of accumulated polyamines at constant levels throughout the stress periods is thought to be a very important phenomenon. Changes in polyamine content and catabolism have been shown to occur in interaction between plants and stressful environments (Panagiotis et al. 2008; Walters 2003). Diamine oxidase (DAO) and polyamine oxidase (PAO) are thought to play key roles in maintaining constant levels of polyamines in plants throughout the stress period. These enzymes catalyze the breakdown of PAs allowing the generation of excess level of H_2O_2 leading to oxidative stress (Tisi et al. 2011; Paola et al. 2011). Rea et al. (2004) have transformed *Nicotiana tabacum* with *MPAO* and *PcuAO* genes isolated from *Zea mays* and *Pisum sativum*, respectively, and demonstrated that both types of transgenic plants (*MPAO* and *PcuAO*) produced elevated levels of H_2O_2 in the presence of exogenous substrates (Spd and Put). Recently, Goyal and Asthir (2010) also reported an increase in the PAO and DAO activities leading to a decline in the levels of endogenous PAs in wheat genotypes under high temperature stress.

The detrimental damage caused by H_2O_2 is combated by defense mechanism in plants involving enzymatic and non-enzymatic antioxidant systems. The enzymatic antioxidant system involves catalase (CAT), superoxide dismutase (SOD), glutathione reductase (GR) and different peroxidases. Involvement of PAs in oxyradical detoxification has been particularly studied in relation to ozone (O_3) pollution (Bouchereau et al. 1999 and reference there in). However, the mechanisms of antioxidant action are poorly understood. It was shown that involvement of PAs in ROS scavenging is based on their ability to form soluble conjugates with various phenol derivatives (Bouchereau et al. 1999). It cannot also be ruled out, however, that functioning of PAs in oxidative stress is mediated by H_2O_2 produced during their oxidative degradation and plays a signal role (Kuznetsov and Shevyakova 2007). Further, one of the manifestations of the PAs antioxidant effect is their ability to regulate the expression of genes encoding antioxidant enzymes. The capacity of PAs to induce expression of antioxidant genes was demonstrated for Spd in the case of peroxidase in tobacco plants by Hiraga et al. (2000) and cadaverine in the case of SOD in the roots of the halophyte *M. crystallinum* by Aronova et al. (2005). To this direction, we hypothesize that PAs have a physiological role in stress tolerance of plants and expression levels of some enzymes involved in polyamine biosynthesis and lead to the increase in overall pool size of polyamines. Further, we ask whether PAs are able to regulate the expression of antioxidant enzymes to ameliorate the oxidative damage caused by free radicals generated during degradation of polyamines by catalyzed action of polyamine oxidase.

Foxtail millet (*Setaria italica* L.) is an important stress-tolerant small millet. It serves as food for many people in arid and semi-arid regions such as the Rayalaseema region of Andhra Pradesh, India. Previously, our studies demonstrated the differences in salt tolerance among local cultivars of foxtail millets, classifying the cultivar Prasad as salt tolerant and cultivar Lepakshi as salt susceptible (Sreenivasulu et al. 1999). In the present investigation, we report the accumulation of PAs and their contribution in regulating the expression of antioxidant genes in foxtail millet seedlings subjected to salinity stress.

Materials and methods

Plant material and salinity treatments

Foxtail millet seeds (*S. italica* L.) cultivar Prasad (salt tolerant) and cultivar Lepakshi (salt sensitive) were surface sterilized with 0.1 % sodium hypochlorite solution for 5 min, thoroughly rinsed with distilled water and allowed to germinate in Petri plates lined with filter paper moistened with Hoagland half-strength nutrient solution. NaCl solutions at 0 (unstressed), 100, 150 and 200 mM (stressed) were used for treatments. The Petri plates were kept in a growth chamber at mean temperature, 25 ± 4.0 °C and relative humidity 60 ± 10.0 % for 7 days. In each treatment, 25 seedlings were pooled and analyzed for different parameters and results were calculated from five independent biological triplicates.

Quantification of free polyamines

Free polyamines were quantified by direct dansylation as described by Smith and Best (1977). Frozen seedlings (0.1 g of powder) were homogenized in 1 ml of cold 5 % perchloric acid (PCA) and allowed to stand for 1 h at room temperature. The homogenates were centrifuged for 20 min at 12,000×*g*, and the resultant supernatant fraction used for dansylation with 0.4 ml of 75 mM dansyl chloride in acetone in the presence of 0.4 ml saturated sodium

carbonate. Dansylation was carried out in vial hermetically sealed for 18 h at room temperature. 1 ml of 2.0 mM proline was added to destroy the excess of dansyl chloride. Dansylated polyamines were extracted from the mixture three times with 3.0 ml of toluene. The toluene phase was dried 40 °C under a stream of air. Once derivatized, the samples were cleaned by adding 0.6 ml of 5.0 mM KOH in methanol according to Seiler and Knodgen (1979). The mixtures were left to stand for 45 min at 40 °C, and then 1.5 ml of an aqueous mixture containing 200 mg of KH_2PO_4 and 200 mg of Na_2HPO_4 was added. Polyamines were extracted again three times in 3.0 ml toluene, as described above. The organic phase was dried and dry residues were redissolved in 200 µl of methanol to be chromatographed. After dansylation, the PAs were separated on thin layer chromatography (TLC) plates with cyclohexane/ethyl acetate mixture (5/4 V/V) in the dark. Individual dansylated PA bands were identified by comparing Rf values of dansylated Put, Spd and Spm standards. The dansylated PA bands were scraped off and eluted into 2 ml of ethyl acetate. After stirring for 2 min and centrifugation at 12,000×*g* for 5 min, polyamine levels were quantified with a fluorescence spectrophotometer at an excitation of 350 nm and a measuring emission of 495 nm.

Preparation of enzyme extracts

One gram of 7-day-old seedlings were homogenized in 50 mM phosphate buffer (pH 7.8) and centrifuged at 10,000×g for 20 min at 4 °C. The resultant supernatant was passed through Sephadex-G25 (0.8 × 18 cm) to remove the polyphenolic contents and the filtrate collected and used as an enzyme source for assay of DAO. For SPDS and PAO, 1 g of 7-day-old seedlings was homogenized in 100 mM phosphate buffer (pH 8.0) containing 20 mM sodium ascorbate, 1 mM pyridoxal-5′-phosphate, 10 mM DTT, 0.1 mM Na_2EDTA and 0.1 mM PMSF (phenylmethylsulfonyl fluoride) and the homogenate centrifuged at 23,000×*g* for 60 min at 4 °C. The resultant supernatant was passed through Sephadex-G25 (0.8 × 18 cm) to remove the polyphenolic contents and the filtrate collected and used as an enzyme source for assay of SPDS and PAO.

Assay of spermidine synthase

Spermidine synthase activity was assayed by the method described by Yoon et al. (2000). The reaction mixture containing the enzyme extract, 100 mM Tris–HCl (pH 8.0), 30 µM putrescine, 25 µM decarboxylated *S*-adenosylmethionine and 20 µM adenine, was incubated at 37 °C for 30 min. The reaction product (5′-deoxy-5′-methylthioadenosine) was quantified via HPLC (SCL-10AVP) equipped with a fluorescence detector (RF-10AXL, Shimadzu, Japan) and a reverse phase column (µBondapak C18, Waters, USA). 1,7-Heptanediamine was used as the internal standard.

Proteins in the extract were quantified as described by Lowry et al. (1951) using BSA as a standard.

Assay of diamine oxidase enzyme activity

Diamine oxidase was assayed according to Naik et al. (1981) with slight modification. The reaction mixture contained 50 mM phosphate buffer (pH 7.8), 10 mM putrescine, 0.1 mM pyridoxal phosphate and enzyme extract in a total volume of 4 ml. After incubation at 30 °C for 60 min, the reaction was terminated by adding 1 ml of trichloroacetic acid and after 30 min of incubation; the total content was centrifuged at 5,000*g* for 15 min. To the supernatant, 1 ml of ninhydrin reagent (25 mg dissolved in 6 ml of acetic acid and 0.4 ml of phosphoric acid) was added and incubated for 30 min in a boiling water bath to develop the color. Finally after adding 1 ml of acetic acid, the absorbance was measured at 510 nm in a spectrophotometer. In reference, trichloroacetic acid was added prior to the addition of the enzyme solution. One unit of DAO was defined as an increase of A_{510} per hour.

Assay of polyamine oxidase enzyme activity

Polyamine oxidase activity was assayed as described by Liu and Liu (2004). The reaction mixture (3.0 ml) contained 0.1 ml crude enzyme extract, 2.5 ml of 100 mM sodium phosphate buffer (pH 6.5), 0.2 ml 4-aminoantipyrine/*N*,*N*′-dimethylaniline and 0.1 ml horseradish peroxidase (250 U/ml). The reaction was initiated by the addition of 0.1 ml 200 mM spermidine for the determination of PAO activity. A 0.01 change in the absorbance value at 555 nm was regarded as one enzyme activity unit.

Estimation of superoxide anion content

Levels of $O_2^{\cdot-}$ were detected based on their ability to reduce nitro blue tetrazolium (NBT) as described in the method of Doke (1983). Seedlings (100 mg) were cut into fragments and immersed in 10 mM potassium phosphate buffer, pH 7.8, containing 0.05 % (w/v) NBT and 10 mM NaN_3, and left for 1 h at room temperature. After incubation, 2 ml of the reaction solution was heated at 85 °C for 15 min and cooled rapidly. Optical density was measured at 580 nm in a spectrophotometer (Shimadzu 1800, Japan) and the $O_2^{\cdot-}$ content was expressed as an increase of absorbance/g dry weight.

Estimation of hydrogen peroxide content

H_2O_2 levels were determined as the method described by Messner and Boll (1994). Fresh seedlings (400 mg) were homogenized in a pre-chilled mortar with 3 ml (w/v) of 100 mM potassium phosphate buffer, pH 7.0; containing 10 % (w/v) polyclar (removes phenolic substances, interfering with the formation of the blue color). For H_2O_2 determination, 1.5 ml extract was taken in an aliquot and to this 50 µl horseradish peroxidase (1 mg^{-1} w/v, dissolved in 100 mM potassium phosphate buffer, 60 units mg^{-1}) and 50 µl 50 mM (w/v) ABTS (2,20-azino-bis(3-ethylobenzo-thiazoline-6-sulfonic acid) diammonium salt) solution were added. Absorbance at of 415 nm was measured after 3 min in a spectrophotometer (Shimadzu 1800, Japan) and compared with the standard curve consisting of freshly prepared 0–30 nM H_2O_2 solutions in 100 mM potassium phosphate buffer, pH 7.0.

In situ histochemical localization of $O_2^{\cdot-}$ and H_2O_2

In situ accumulation of $O_2^{\cdot-}$ and H_2O_2 was detected by histochemical staining with nitro blue tetrazolium (NBT) and diaminobenzidine (DAB) according to Romero-Puertas et al. (2004) with minor modification. For $O_2^{\cdot-}$ detection, the leaves of control and stressed seedlings were excised and immersed in NBT solution (1 mg ml^{-1}) prepared in 10 mM phosphate buffer (pH 7.8) at room temperature. The immersed leaves were illuminated for 2 h until the appearance of dark spots, characteristic of blue formazan precipitates. For localization of H_2O_2, another set of leaves was sampled and immersed in DAB solution (1 mg ml^{-1}, pH 3.8) that was freshly made in 10 mM phosphate buffer (pH 7.8) and incubated at room temperature for 8 h in the light until brown spots were visible, which are derived from the reaction of DAB with H_2O_2. For both staining methods, the leaves were then bleached in concentrated ethanol to visualize the blue and brown spots and kept in 70 % ethanol for image capturing by a digital camera.

Assay of superoxide dismutase activity

Superoxide dismutase (EC 1.15.1.1) activity was assayed by following the method described by Beauchamp and Fridovich (1971). Seedlings (200 mg) were homogenized in a pre-chilled mortar with 4 ml (w/v) of 50 mM Na-phosphate buffer, pH 7.0, containing 1 % (w/v) polyvinylpolypyrrolidone (PVPP), 1 mM Na_2EDTA and 0.5 M (w/v) NaCl. Homogenates were centrifuged at 17,000×*g* for 25 min at 4 °C. The reaction mixture contained 50 mM sodium phosphate buffer, pH 7.8, 0.1 mM (w/v) Na_2EDTA, 13 mM (w/v) methionine, 25 mM (w/v) NBT, 2.4 mM (w/v) riboflavin and 0.03 ml enzyme extract. The addition of riboflavin and the placement of tubes under fluorescent lamps ensuring an irradiation intensity of 185 µM m^{-2} s^{-1} started the reaction of blue formazan accumulation. Tubes without the enzyme developed maximum color. Absorbance at a wavelength of 560 nm was recorded and 1 unit of activity was estimated as the enzyme quantity reducing absorbance to 50 % in comparison to that of tubes lacking the enzyme.

Assay of catalase activity

Catalase (EC 1.11.1.6) activity was measured according to the method of Beers and Sizer (1952), with minor modifications. The reaction mixture (1.5 ml) consisted of 100 mM phosphate buffer (pH 7.0), 0.1 µM Na_2 EDTA, 20 mM H_2O_2 and enzyme extract. The reaction was started by the addition of enzyme extract. The decrease of H_2O_2 was monitored at 240 nm and quantified by its molar extinction coefficient (36 M^{-1} cm^{-1}).

Assay of ascorbate peroxidase activity

Ascorbate peroxidase (EC 1.11.1.11) was assayed according to Nakano and Asada (1981). Frozen seedlings (200 mg) were extracted in 50 mM Tris–HCl (pH 7.8) buffer with the addition of purified sea sand and polyvinylpolypyrrolidone (PVPP) and centrifuged at 10,000×*g* for 20 min at 4 °C. The resulting supernatant was passed through the Sephadex G-25 to remove the phenolic contents and the protein fraction was used as a source for ascorbate peroxidase assay. The reaction mixture contained 50 mM Na-phosphate buffer (pH 7), 0.5 mM ascorbate, 0.1 mM hydrogen peroxide, 0.1 mM EDTA and enzyme extract. The reaction was started by the addition of hydrogen peroxide and the change in absorbance was measured at 3 min in a spectrophotometer at 290 nm. Enzyme activity was calculated by using the molecular extinction coefficient 2.8 $mmol^{-1}$ cm^{-1}.

Estimation of lipid peroxidation products

Lipid peroxidation products were extracted as described by Johnston et al. (2008). 200 mg of frozen powered tissue was homogenized in a pre-chilled micro-centrifuge tube by adding 1 ml of cold extraction buffer (50 mM KH_2PO_4, 1 mM Na_2EDTA, 1 mM $CaCl_2$, 1 mM KCl, pH 7.0) and maintained on ice for 20 min; samples were vortexed at 5 min intervals. The homogenate was centrifuged at 12,000×*g* for 10 min (at 4 °C), and the supernatant removed to fresh tubes and stored on ice.

Assays for total MDA content was performed according to Gerard-Monnier et al. (1998). The reaction mixture

Table 1 The primer sequences used in quantitative real-time polymerase chain reaction analysis

Gene name	Forward primer nucleotide sequence (5′–3′)	Reverse primer nucleotide sequence (5′–3′)
Actin	TCTCCTTGTATGCAAGTGGTCG	ACCAGCGAGATCCAAACGAAGG
Ft_Spdsyn	TCGTGTCCGACTTCATGTTGGTGA	TAAACGGTTTCTCCACGAGTGCCT
Ft_Catalase	TCAAGATCGGTGGAGCGAATCACA	AGGTCTTGGTAACATCAAGCGGGT
Ft_MnSOD	TATCATGCAGCTCCACCACCAGAA	TTCCTTGGTGGGAGCCAGATTCTT
Ft_SAMDC	TCTGTTGCCGTGACCATCTTC	AGTTTCGTCCCCCAGGTCTT
Ft_PAO	AACGGCGGCAAGATGAAC	GGAGCTTGAGGGTGGAGTTG

containing 0.65 ml of activated 1-methyl-2-phenylindole and 0.2 ml of sample or standard (0–100 mM tetraethoxypropane) was taken into 2 ml micro-centrifuge tubes, vortex mixed, and 0.15 ml of 37 % (v/v) HCl was added and incubated at 45 °C for 60 min. Finally, the reaction was stopped by immersion in ice. The optical density was measured at 586 nm and total MDA content was expressed in μmol g^{-1} FW.

Quantitative RT-PCR analysis

Applied Biosystems (StepOne) real-time PCR system was used for qRT-PCR analyses (http://www.appliedbiosystems.com) of spermidine synthase, *S*-adenosylmethionine decarboxylase, polyamine oxidase, catalase and superoxide dismutase; all primers were validated to facilitate the ΔΔCt for gene expression analysis (Livak and Schmittgen 2001). Primer sequences from 5′ to 3′ are listed in Table 1. The *FtActin* gene was used to normalize the expression of target genes. Diluted cDNA (1 μl) was used in a 14/15 μl reaction mixture along with the SYBR Green PCR Master Mix (2×; Applied Biosystems, http://www.appliedbiosystems.com). Melting curve analysis was performed after the PCR reaction to determine the specificity of the PCR products. PCR reactions were performed with the following program: 95 °C (10 min); 95 °C for 30 s and 60 °C for 1 min, for 40 cycles. At least five independent biological replicates were analyzed for quantification of gene expression.

Statistical analysis

Experiments were performed independently in five biological sets and 25 seedlings for treatment, unless otherwise specified. All measurements were done on five samples from two to five independent experiments. Each treatment was evaluated using ANOVA (Student's *t* and Tukey–Kramer honestly significant difference (HSD) tests; JMP 8.0 software; http://www.jmp.com/). Data presented are mean ± SD of five replicates. Twenty-five leaves from each treatment were utilized in each independent experiment of in situ histochemical analysis.

Results

Free polyamine content

Total polyamine content increased with the increase of NaCl concentrations from 100 to 200 mM (Fig. 1a). However, the increase of free polyamines was more pronounced in cultivar Prasad when compared with cultivar Lepakshi. While the increase in free polyamine content in cultivar Prasad was 3.8-fold at 200 mM NaCl, it was only 2.6-fold in cultivar Lepakshi in comparison to the respective controls. Among the individual polyamine species, putrescine was found to be the most abundant polyamine in seedlings under control conditions and with NaCl treatments (100, 150 and 200 mM). With the increase of NaCl concentration, free putrescine concentration per unit weight taken was significantly increased in both cultivars (Fig. 1b), but the magnitude of increase was 3.5-fold in cultivar Prasad and 2.4-fold in cultivar Lepakshi at 200 mM NaCl. Similarly, the concentrations of spermidine and spermine displayed a remarkable rise with the increase in NaCl concentration (Fig. 1c, d). However, the rise in spermidine and spermine was 5.5- and 6.5-fold, respectively, in cultivar Prasad, whereas it was 3.6- and 4.2-fold, respectively, in cultivar Lepakshi at 200 mM NaCl stress.

Spermidine synthase assay

Spermidine synthase activity, which was assayed in both control and stressed seedlings of both cultivars, increased gradually with increasing NaCl concentration compared to control seedlings (Fig. 2a). NaCl caused increase in spermidine synthase activity in stressed seedlings compared to control seedlings and this increase was linearly increased with increase in NaCl concentration. Spermidine synthase activity was increased by 4.5- and 3.3-fold in cultivar Prasad and Lepakshi, respectively, at 200 mM NaCl treatments.

Diamine oxidase assay

When foxtail millet seedlings were grown under normal conditions, we observed no detectable differences

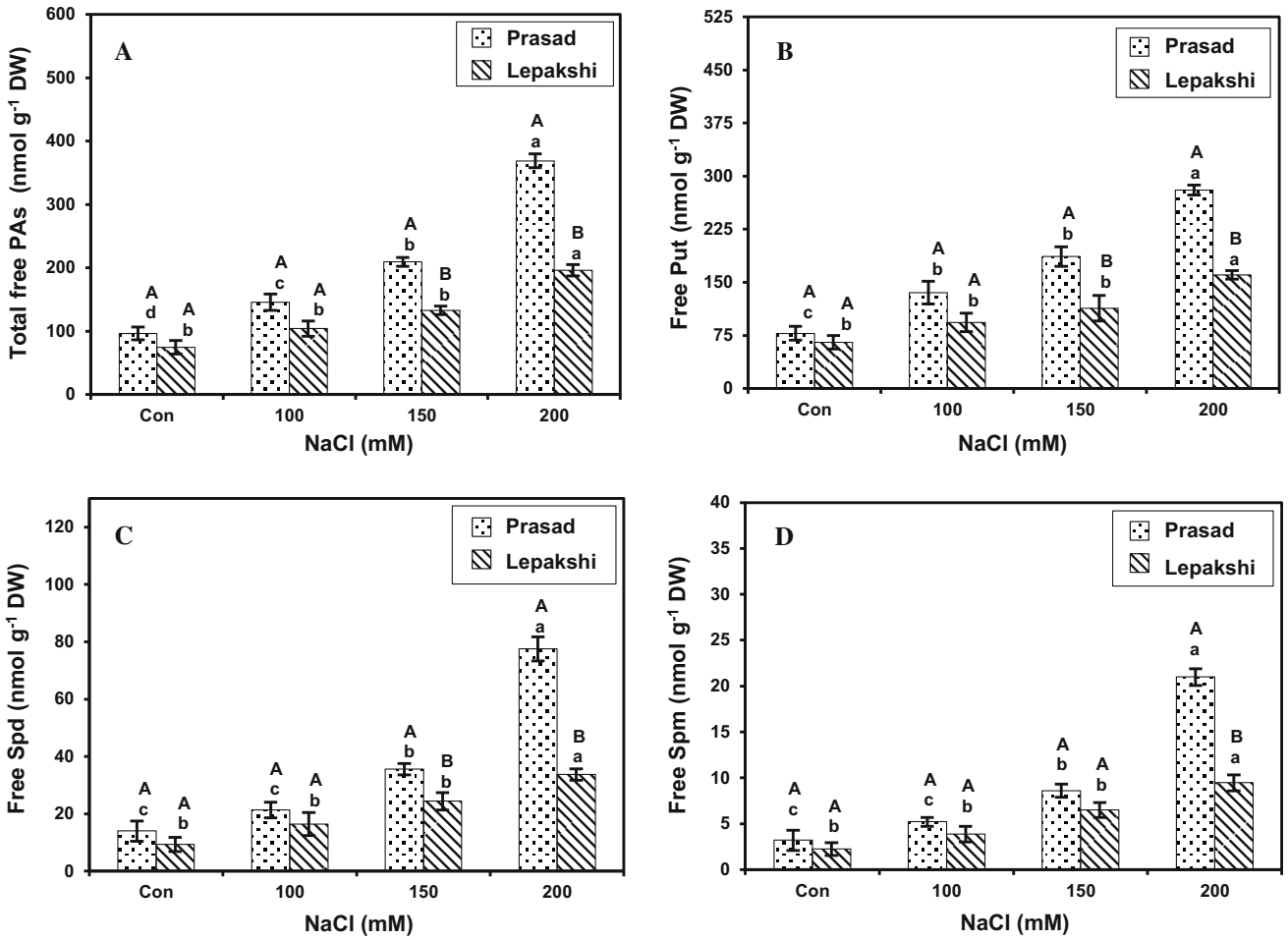

Fig. 1 Levels of free polyamines in two cultivars of foxtail millet seedlings subjected to different levels of NaCl stress for 7 days. **a** Total free polyamines, **b** free putrescine, **c** free spermidine, **d** free spermine. *Error bars* indicate SD ($n = 5$). *Different uppercase letters* indicate significant differences between cultivars in response to treatments (Student's *t* test; JMP 8.0 software), and *lowercase letters* indicate significant differences within the cultivar in response to treatments (Tukey–Kramer HSD test; JMP 8.0 software)

in diamine oxidase activity (Fig. 2c). However, the diamine oxidase activity was gradually raised with the application of different concentrations of NaCl. Nevertheless, the raise in DAO activity was more significant with the application of 200 mM NaCl, 3.2-fold in the cultivar Prasad and 2.39-fold in the cultivar Lepakshi.

Polyamine oxidase assay

Polyamine oxidase activity consistently increased with the increase in concentration of NaCl in both cultivars studied (Fig. 2d), but cultivar Lepakshi registered a 5.9-fold increase in polyamine oxidase activity that is almost double that in cultivar Prasad that registered threefold. Nevertheless, polyamine oxidase showed a negative trend with total free polyamines and their biosynthetic enzymes, i.e., spermidine synthase in cultivar Lepakshi that registered the least values for both.

Levels of $O_2^{\cdot-}$ and H_2O_2

We quantified $O_2^{\cdot-}$ and H_2O_2 in both cultivars in unstressed and stressed conditions. From Fig. 3a, it can be seen that the pools of $O_2^{\cdot-}$ increased by 3.5-fold with application of 200 mM NaCl in the sensitive cultivar Lepakshi, but only 2.3-fold in the tolerant cultivar compared to their respective controls. Moreover, this increase in the levels of $O_2^{\cdot-}$ was also reflected in the histochemical studies. Similarly, NaCl application resulted in an accumulation of H_2O_2 by twofold in the tolerant cultivar and 3.2-fold in the sensitive cultivar (Fig. 3c), which is also a positive indicator of the rapid degradation of the polyamine pool by the increased catabolic action of oxidases as reflected in in situ localization.

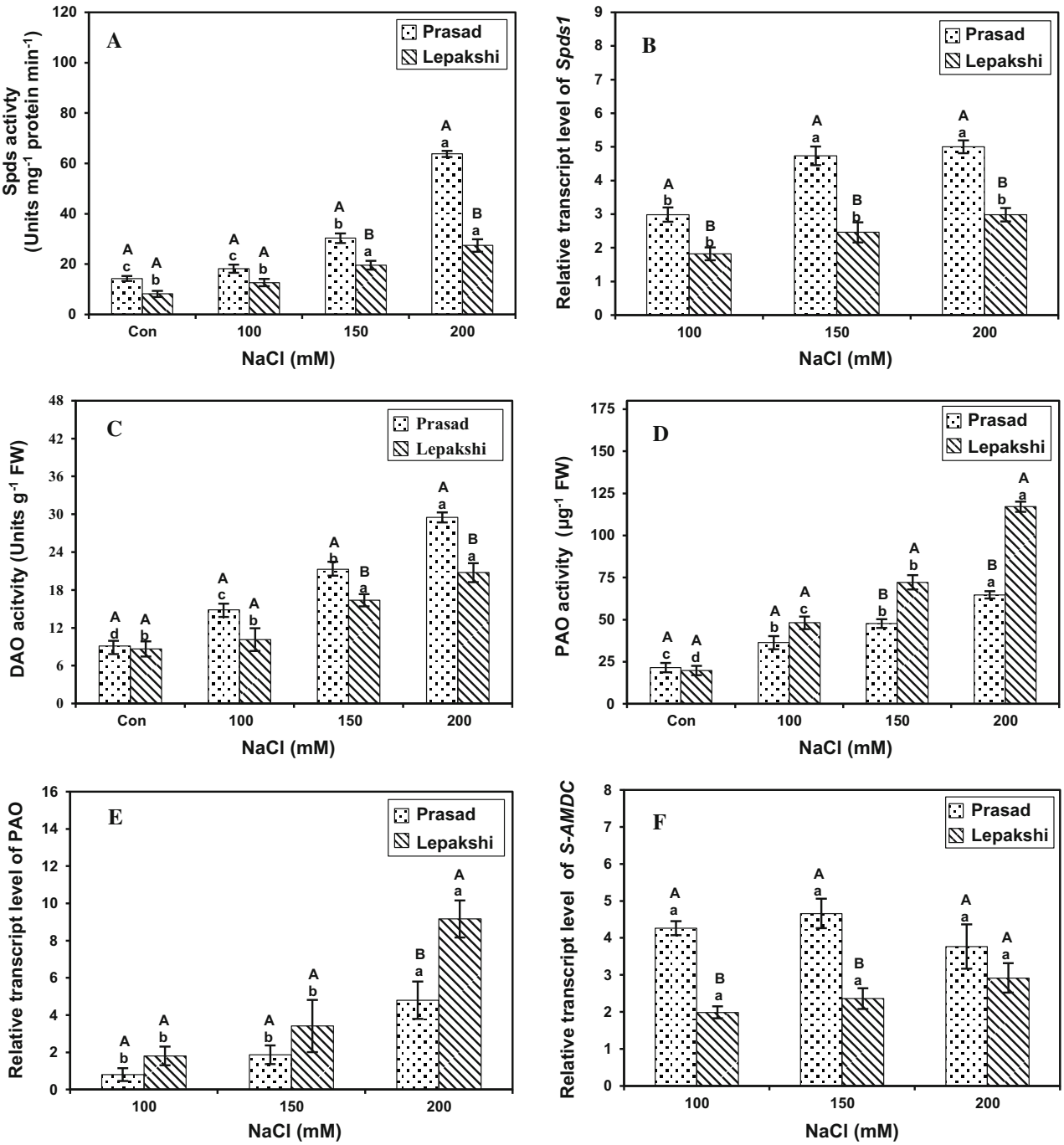

Fig. 2 NaCl stress-induced modulation in polyamine biosynthetic enzymes and transcript levels in two cultivars of foxtail millet. **a** Spds activity, **b** relative transcript levels of *Spds1*, **c** DAO activity, **d** PAO activity, **e** relative transcript levels of *PAO* and **f** relative transcript levels of *S-AMDC*. All RT-PCR expression assays were performed and analyzed three times in independent biological experiments. Actin transcripts were used as internal control. *Error bars* indicate SD ($n = 5$). *Different uppercase letters* indicate significant differences between cultivars in response to treatments (Student's *t* test; JMP 8.0 software), and *lowercase letters* indicate significant differences within the cultivar in response to treatments (Tukey–Kramer HSD test; JMP 8.0 software)

In situ histochemical localization of $O_2^{\cdot-}$ and H_2O_2

Histochemical staining was employed to reveal in situ accumulation of $O_2^{\cdot-}$ and H_2O_2, two important representatives of reactive oxygen species. Under control condition, there was no detectable difference between tolerant and sensitive cultivars in the accumulation of $O_2^{\cdot-}$ radicals (Fig. 3b). However, conspicuous differences were observed between the cultivars with the increase in the intensity of stress, in which cultivar Lepakshi showed more

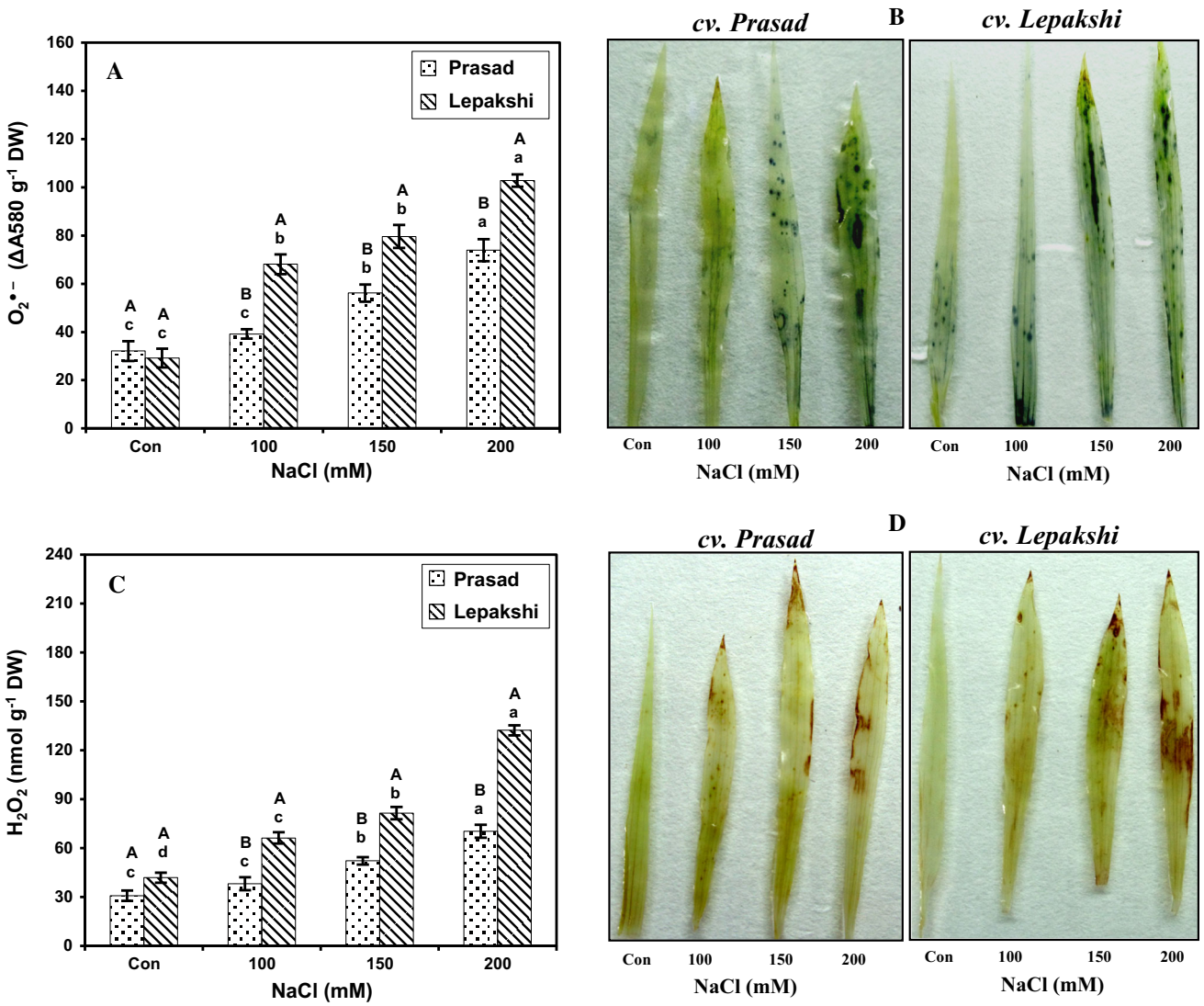

Fig. 3 Effect of NaCl stress on free radical levels. **a**, **c** Levels of $O_2^{\cdot-}$ and H_2O_2 radicals, respectively. **b**, **d** In situ histochemical localization of $O_2^{\cdot-}$ and H_2O_2, respectively, in two cultivars of foxtail millet seedlings subjected to different levels of NaCl stress. *Error bars* indicate SD ($n = 5$). *Different uppercase letters* indicate significant differences between cultivars in response to treatments (Student's *t* test; JMP 8.0 software), and *lowercase letters* indicate significant differences within the cultivar in response to treatments (Tukey–Kramer HSD test; JMP 8.0 software)

local blue spots (Fig. 3b, indicator of $O_2^{\cdot-}$) than cultivar Prasad. Similarly, from Fig. 3d, it can be seen that the tolerant cultivar Prasad did not have any local brown spots (indicator of H_2O_2), but following the NaCl stress course local spots gradually increased; the sensitive cultivar Lepakshi, showed a few local spots even in control condition and they significantly increased up on 150 mM.

Antioxidative enzyme activities

To understand the ability of PAs in activating the antioxidative defense mechanisms, we assayed antioxidative enzymes such as superoxide dismutase, catalase and ascorbate peroxidase from both cultivars under control and stressed conditions with the hypothesis in mind that these antioxidative enzymes must play a role in the elimination of the toxic levels of free radicals generated during polyamine catabolism. There was a slight increase in superoxide dismutase activity of the tolerant cultivar under control condition, which gradually increased during the course of NaCl stress and was significant with 200 mM NaCl (4.3 fold). The sensitive cultivar Lepakshi showed a threefold increase in superoxide dismutase activity compared to the tolerant cultivar, which had 4.3-fold increase in superoxide dismutase activity at 200 mM NaCl stress (Fig. 4a), suggesting a possible reason for elevated levels

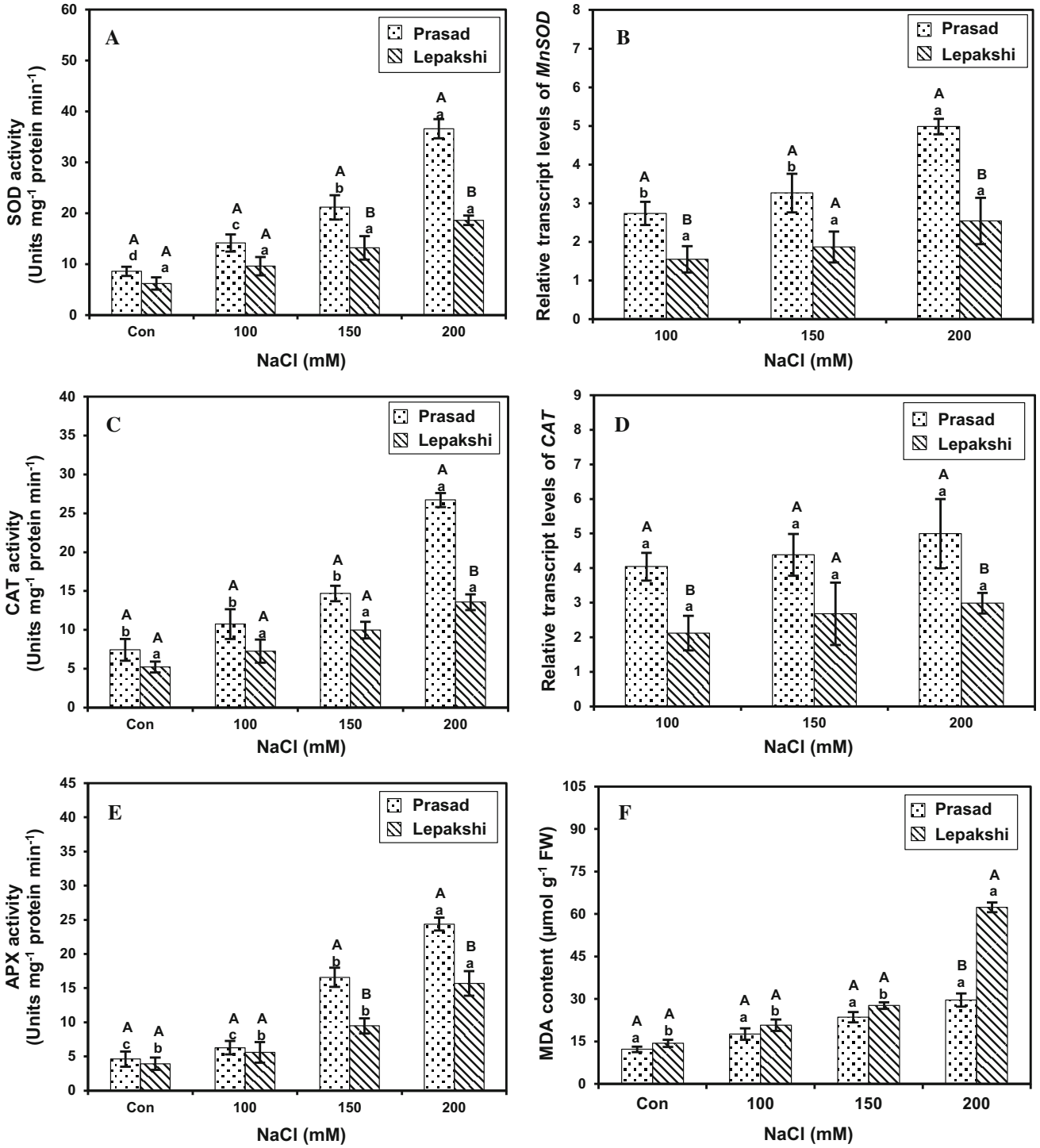

Fig. 4 Modulations in antioxidant enzymes, transcript levels and MDA content in two cultivars of foxtail millet. **a** SOD activity, **b** relative transcript levels of *MnSOD*, **c** CAT activity, **d** relative transcript level of CAT, **e** APX activity and **f** MDA content. All RT-PCR expression assays were performed and analyzed at three times in independent biological experiments. Actin transcripts were used as internal control. *Error bars* indicate SD ($n = 5$). *Different uppercase letters* indicate significant differences between cultivars in response to treatments (Student's *t* test; JMP 8.0 software), and *lowercase letters* indicate significant differences within the cultivar in response to treatments (Tukey–Kramer HSD test; JMP 8.0 software)

of $O_2^{\cdot-}$ during stress conditions. Similarly, the activity of catalase was elevated more in the tolerant cultivar Prasad (3.6 fold) than in the sensitive cultivar Lepakshi (2.6 fold) at 200 mM NaCl stress (Fig. 4c). Catalase activity showed a linear relation with a drop in H_2O_2 levels in the tolerant cultivar, but a non-linear relation with elevated levels of

H_2O_2 in the sensitive cultivar as evidenced from Figs. 3 and 4. As expected, an additional member of antioxidant enzymes, the ascorbate peroxidase, which followed the catalase, enhanced upon NaCl application, being more significant in tolerant cultivar Prasad (5.3 fold) at 200 mM, while cultivar Lepakshi registered only fourfold compared to the untreated condition (Fig. 4e).

Contents of lipid peroxidation products

The content of lipid peroxidation products, malondialdehyde, increased gradually, but substantially in the stressed seedlings compared to unstressed seedlings in both cultivars at all regimes (Fig. 4f). Nevertheless, cultivar Lepakshi registered higher levels of lipid peroxidation products (4.35 fold) compared to cultivar Prasad (2.4 fold), on application of 200 mM NaCl. As expected, lipid peroxidation products had a positive relationship with increasing polyamine oxidase enzyme activity and free radical pools (Figs. 2d, 3a, c).

Relative transcript levels of *SPDS1* and *S-AMDC*

To understand the transcript levels of endogenous *SPDS1* and *S-AMDC*, we performed quantitative RT-PCR analysis for foxtail millet seedlings subjected to unstressed and NaCl-stressed conditions. In parallel to the endogenous polyamine pools, the relative expression of *SPDS1* and *S-AMDC* transcripts was found to be greater in the tolerant compared to the sensitive cultivar (Fig. 2b, f): the relative expression of *SPDS1* showed 1.5-fold increase in Prasad and 0.2-fold increase in Lepakshi with 200 mM NaCl. The relative expression of *S-AMDC* in cultivar Prasad showed little change with increasing salt concentration, although it was maximal with 150 mM NaCl and decreased by 200 mM NaCl stress (Fig. 2f). The endogenous levels of *S-AMDC* transcripts in Lepakshi gradually increased with increasing severity of stress; however, transcript levels were relatively less than in the tolerant cultivar.

Relative transcript levels of PAO

Endogenous levels of polyamine oxidase transcripts were analyzed in unstressed and stressed foxtail millet cultivars. As expected from polyamine oxidase enzyme activity, PAO transcript levels were not increased much in cultivar Prasad, whereas in cultivar Lepakshi there was an almost 4.5-fold increase by 200 mM NaCl (Fig. 2e).

Relative transcript levels of antioxidant enzymes

To check the expression of antioxidant enzymes, we analyzed transcript levels of *MnSOD* and CAT in foxtail millet cultivars subjected to NaCl stress. *MnSOD* transcripts were found to be higher in cultivar Prasad compared to cultivar Lepakshi; the transcripts increased slightly with increasing NaCl in both cultivars (Fig. 4b). Similarly, *CAT* transcripts were increased both in cultivar Prasad and Lepakshi, but the magnitude of increase was less in Lepakshi than Prasad (Fig. 4d). Both (*MnSOD* and *CAT*) transcripts reflected the changes in enzyme activity (Fig. 4a, c).

Discussion

In the present investigation, PA accumulation under salt stress was associated with stress tolerance, corroborating previous works where salt stress results in increasing endogenous polyamine levels in various species (Legocka and Kluk 2005; Zapata et al. 2004). These authors found that PA levels changed with salinity and in most cases putrescine decreased, while spermidine and/or spermine increased. The spermidine + spermine/putrescine ratio increased in different plant species such as spinach, lettuce, melon, pepper, broccoli, beetroot and tomato, with increased salinity tolerance (Zapata et al. 2004). In the present investigation, the total PA levels significantly increased following salt stress, with the increase in PA level being comparatively more significant in the tolerant cultivar Prasad than in the sensitive cultivar Lepakshi. More specifically, the spermidine + spermine/putrescine ratio was greater in the tolerant cultivar than in the sensitive cultivar, which could be the reason for the better performance of cultivar Prasad over cultivar Lepakshi.

There is increasing evidence for the role of individual polyamines in plants under stress conditions. In plants, putrescine is required for stress tolerance; spermidine is essential for the maintenance of plant growth, whereas spermine has a pivotal role in signal transduction (Takahashi and Kakehi 2010). As an example, a loss-of-function mutation of *ADC2* in *Arabidopsis* showed reduced putrescine levels and its stress tolerance was restored by exogenously supplied putrescine (Urano et al. 2005). These findings suggest a direct protective role of putrescine in abiotic stress tolerance. Further, it is also clear that putrescine is an important precursor for the synthesis of higher polyamines. According to a model based on studies using transgenic plants with altered putrescine levels, Capell et al. (2004) suggested that the putrescine levels must exceed a certain threshold level to enhance the synthesis of spermidine and spermine under stress; such synthesis is necessary for recovery from stress. It has been shown that double mutants of *Arabidopsis ADC1* and *ADC2*, which could not produce PAs, died at the embryo stage under salt stress (Urano et al. 2005). Moreover, putrescine titers is an indication that stress-induced damage

varied in different cultivars. There was a significant difference in the putrescine concentrations after NaCl application to seedlings and was very significant with 200 mM NaCl. During NaCl stress, putrescine can bind to antioxidant enzymes such as superoxide dismutase or be conjugated to small antioxidant molecules allowing them to permeate to sites of oxidative stress within cells, thereby alleviating NaCl-induced membrane injury as previously suggested by Bouchereau et al. (1999).

Similar studies have been conducted to illustrate the role of spermidine and spermine in plants. Bouchereau et al. (1999) demonstrated that spermidine and spermine inhibited chilling injury by retarding lipid peroxidation and preserving membrane integrity; also, spermidine and spermine might interact with membranes by either stabilizing molecular complexes of thylakoid membranes or inhibiting the transbilayer movement of phospholipids. In the present investigation, we registered elevated levels of both spermidine and spermine upon NaCl application, and this elevation was very significant in tolerant cultivar Prasad compared to cultivar Lepakshi (Fig. 1). Further, each single mutant of *SPDS1* and *SPDS2*, spermidine synthase encoding genes of *Arabidopsis,* shows no growth defects, but embryo development of the double mutant is arrested indicating the requirement of spermidine during the course of embryogenesis (Imai et al. 2004). Also, Imai et al. (2004) demonstrated no requirement of spermine under normal conditions in a loss-of-function mutant of *SPMS* in *Arabidopsis*, but the existence of a polyamine metabolon (a large protein complex containing both spermidine synthase and spermine synthase) is probably responsible for the efficient production of spermine in plant cells (Panicot et al. 2002). Yamaguchi et al. (2007) has shown that *spms* mutant of *Arabidopsis* appears to be more sensitive to drought and salinity stress than the wild type. This phenotype might be related to the fact that inward potassium currents across the plasma membrane of guard cells are blocked by intracellular polyamines (Takahashi and Kakehi 2010). Their specificity for selectively blocking outward Na^+ channels (vs. the K^+ channels) in the tonoplast membrane apparently helps the vacuole to contain Na^+ within it, thus changing the effective K^+/Na^+ ratio in the cytoplasm under stressful conditions. Blocking of ion channels by polyamines in plants has also been reported previously for vacuolar cation channels in barley and red beet (Dobrovinskaya et al. 1999; Bose et al. 2011; Zepeda-Jazo et al. 2011), and for non-selective cation channels in pea mesophyll cells (Shabala et al. 2007). Similarly, in the present investigation we registered exalted level of spermidine synthase activity that were associated with increased spermidine and spermine levels in cultivar Prasad compared to cultivar Lepakshi (Fig. 2a). Perhaps, the presence of excess spermidine and spermine might help in regulating inward potassium currents or in stabilizing molecular complexes of thylakoid membranes and/or in inhibiting the transbilayer movement of phospholipids, thereby contributing tolerance to cultivar Prasad, while cultivar Lepakshi failed in accumulating sufficient levels of spermidine and spermine and showed less tolerance to stress.

Further, Ioannidis et al. (2006) reported that putrescine can increase the light energy utilization through stimulation of photophosphorylation. Also, Ioannidis and Kotzabasis (2007) reported that putrescine is an efficient stimulator of ATP synthesis in comparison to spermidine and spermine, but spermidine and spermine are efficient stimulators of non-photochemical quenching. They found that spermidine and spermine are efficient uncouplers of photophosphorylation at high concentrations. Furthermore, the effectiveness of PSII efficiency restoration and stacking of thylakoids was higher when the polycationic character of the amine was high, suggesting the importance of chloroplastic PAs in the photosynthetic membrane function. In the present investigation also, the increased spermidine + spermine/putrescine ratios might contribute to the effective photophosphorylation and better performance of the cultivar Prasad under NaCl stress conditions. Results in the present investigation further underline the role of PAs in the functional maintenance of photosynthetic membrane integrity.

As transcriptional regulation is responsible for polyamine synthesis, the relative expression of polyamine biosynthetic genes such as *SPDS1* and S*AMDC* was assayed via qRT-PCR using unstressed and stressed samples of both the cultivars. The expression relative to the controls of *SPDS1* transcripts was increased fivefold in cultivar Prasad compared to twofold in cultivar Lepakshi (Fig. 2b). *SAMDC* in cultivar Prasad exhibited maximum expression at 150 mM NaCl, followed by a decline at 200 mM NaCl, but was higher than that of cultivar Lepakshi (Fig. 2f). Based on these studies, there exists a strong correlation between the transcript levels of *SPSD1* and S*AMDC* and elevated polyamine pools in foxtail millet. Previously, incongruence between gene expression and polyamine biosynthesis was noticed and assumed to be common, as such a phenomenon has been frequently reported (Liu and Moriguchi 2007). Our results are in concurrence with Bagni et al. (2006) who reported the up-regulation of two genes of each *SAMDC* (*SAMDC1* and *SAMDC2*) and *SPDS* (*SPDS1* and *SPDS2*) with the application of NaCl during the vegetative and reproductive stages in *Arabidopsis*.

Changes in polyamines and their catabolism have been shown to occur in incompatible interaction between plants and stress environments (Walters 2003). The catabolic action of diamine oxidase and polyamine oxidase is

thought to play a pivotal role in oxidation of polyamines that leads to lower titers, with the concomitant production of H_2O_2 (Goyal and Asthir 2010). H_2O_2 may acts as structural defense signal molecule, but high levels of H_2O_2 is cytotoxic leading to oxidative stress. Earlier, Ha et al. (1997) correlated the programmed cell death in animal cells to an increase of polyamine oxidase. Recently, Rodriguez et al. (2009) and Goyal and Asthir, (2010) reported an increase in polyamine oxidase activity following the exposure of maize to NaCl and wheat genotypes to high temperature. In the present study, we followed the activities of two PA catabolic enzymes, DAO and PAO, under unstressed and stressed conditions in both cultivars (Fig. 2c, d). NaCl stress caused an apparent increase in DAO and PAO activities in the sensitive cultivar compared to the tolerant cultivar, supporting the idea that a decline in their activity could contribute to tolerance in cultivar Prasad by maintaining the elevated PA titers and consequently lower levels of free radicals, while enhanced amine oxidase activities in cultivar Lepakshi caused rapid depletion of PAs. In addition, relatively lower PA biosynthetic enzymes activities and rapid catabolic activity of these two enzymes are partly responsible for lower levels of PAs, supports less tolerance of cultivar Lepakshi to NaCl stress. Although we studied the activities of the two amine oxidases, only the differential activity of PAO was further confirmed following the assay of maize polyamine oxidase gene via qRT-PCR analysis at the mRNA level. As expected, we observed a positive correlation between PAO activity and PAO transcripts with a steady increase in cultivar Prasad and a rapid increase in cultivar Lepakshi following NaCl stress (Fig. 2d, e).

To date, numerous reports have been addressed the complexity of interaction between PAs and ROS, particularly when plants are under stressful conditions (Gill and Tuteja 2010; Velarde-Buendía et al. 2012; Pottosin et al. 2014; Minocha et al. 2014). Generally when cellular PA contents are increased, their catabolism also increased which leads to the enhancement of ROS levels especially H_2O_2 and superoxide ($O_2^{\cdot-}$), various antioxidant systems (enzymes and metabolites); hence their role in preventing the cellular damage is customary. As oxidation of PAs by amine oxidases generating H_2O_2, we followed the evaluation of $O_2^{\cdot-}$ and H_2O_2 via spectrophotometric estimation as well as in situ histochemical localization. Levels of $O_2^{\cdot-}$ and H_2O_2 linearly followed the activities of the two amine oxidases in both the cultivars (Figs. 2c, d, 3a, c). Nevertheless, cultivar Prasad was found to maintain lower levels of $O_2^{\cdot-}$ and H_2O_2 than cultivar Lepakshi during stress. Further, levels of $O_2^{\cdot-}$ and H_2O_2 were reflected at tissue level. In situ histochemical staining revealed no detectable differences in the free radicals observed in controls of both the cultivars, but remarkable differences were seen in both the cultivars following NaCl stress. Leaves of cultivar Prasad contained remarkably less blue and brown spots, an indication of $O_2^{\cdot-}$ and H_2O_2, respectively, than leaves of cultivar Lepakshi, which had more spots suggesting a substantial increase in $O_2^{\cdot-}$ and H_2O_2 following the NaCl stress (Fig. 3b, c). The low occurrence of spots in cultivar Prasad is positively correlated with the decline in DAO and PAO activities and lower titers of $O_2^{\cdot-}$ and H_2O_2, indicating the possible source of $O_2^{\cdot-}$ and H_2O_2 during stress conditions. Nevertheless, the elevated free radicals showed a negative trend with titers of polyamines in both cultivars. Further our results are in concurrence with Verma and Mishra (2005) who reported that in *Brassica juncea* PAs reversed the salinity induced increase of $O_2^{\cdot-}$ and H_2O_2 levels and MDA content. Rodriguez et al. (2009) were able to demonstrate the role of PAO in the contribution of $O_2^{\cdot-}$ and H_2O_2 in maize leaves under saline conditions and attributed this phenomenon as a possible source of excess levels of $O_2^{\cdot-}$ and H_2O_2. Further, dehydration stress-induced accumulation of $O_2^{\cdot-}$ and H_2O_2 at tissue level was exemplified by Shi et al. (2010) in citrus leaves by employing histochemical staining. They suggested that pretreatment of *citrus* leaves with spermine effectively ameliorated the generation of $O_2^{\cdot-}$ and H_2O_2 and conferred dehydration tolerance of in vitro *citrus* plants. Interestingly, the lower $O_2^{\cdot-}$ and H_2O_2 was concurrent with the significantly higher endogenous free polyamines in the tolerant cultivar Prasad, which makes it tempting to speculate that the accumulation of endogenous free polyamines below a threshold level might contribute to the generation of $O_2^{\cdot-}$ and H_2O_2 in the sensitive cultivar Lepakshi. More importantly, this lower titer of $O_2^{\cdot-}$ and H_2O_2 cultivar Prasad might be due to the reason that elevated levels of spermidine and spermine at cellular level could mitigate lipid peroxidation by down-regulating the NAD(P)H-oxidase/NAD(P)H peroxidase activity (Gill and Tuteja 2010). This is regarded as a potential source of $O_2^{\cdot-}$ and H_2O_2 generation, thus causing oxidative injuries in plant tissues. As a general rule, during oxidation of putrescine by DAO in addition to generating H_2O_2, it also generates Δ^1-pyrroline, an intermediate precursor of proline biosynthesis, as well. Our previous study (Veeranagamallaiah et al. 2007) demonstrated that P5CR and proline biosynthesis was elevated in NaCl-treated 5-day-old foxtail millet seedlings. This is another line of evidence that the substrate Δ^1-pyrroline for P5CR during proline accumulation might be supplied through putrescine oxidation by DAO. Further, it can also be pointed that glutamate is a common precursor in the biosynthesis of proline and polyamines (Kuznetsov and Shevyakova 1999, 2007). In the case of polyamines, glutamate is a

more distant precursor converted firstly into ornithine or arginine, which in turn serves as substrates for two enzymes, ornithine decarboxylase and arginine decarboxylase, respectively. It was reported that osmo-induced proline accumulation in rapeseed leaves was suppressed by polyamines (Larher et al. 1998). Such facts served a basis for hypothesis that a competition for a common precursor takes place between metabolic pathways of proline and polyamine biosyntheses (Larher et al. 1998; Tonon et al. 2004). Although this hypothesis seems logical, it is difficult to consider it as a universal one, because usually the contents of proline and polyamines in plants differ by an order of magnitude.

The effect of free radicals was also reflected on lipid peroxidation products. Lipid peroxidation products were evaluated via estimation of MDA content. The MDA content was shown to be lower in cultivar Prasad compared to cultivar Lepakshi (Fig. 4f). Our results were in concurrence with the previous work done by various workers in different species (Verma and Mishra 2005; Kubis 2008; Yiu et al. 2009; Rodriguez et al. 2009) under several abiotic stresses. Kubis (2008) reported an enlargement in the level of oxidative stress due to 2.2-fold increase in $O_2^{\cdot-}$ and 2.6-fold increase in H_2O_2 during water deficit conditions, and on watering they observed a significant drop in these ROS. Stress-induced accumulation of $O_2^{\cdot-}$ and H_2O_2 was also observed in Welsh onion during waterlogging (Yiu et al. 2009), with an increase in the generation rate of $O_2^{\cdot-}$ and H_2O_2 by 290 and 240 %, respectively. They also observed a rapid drop in the levels of $O_2^{\cdot-}$ and H_2O_2 following the exogenous application of spermidine and spermine. In fact, in the present investigation the lowered MDA content in tolerant cultivar Prasad is due to the increased levels of spermidine and spermine which reduce the rate of lipid peroxidation.

Although the role of PAs in augmenting antioxidant-based defense systems, by inhibition of production of $O_2^{\cdot-}$ and H_2O_2 and their scavenging, to impart tolerance against stresses that are potent inducers of these free radicals causing oxidative damage to the living cells has been reported by several workers (Minocha et al. 2014 and references there in), the effect may constitute only a partial defense mechanism against the excessive production and scavenging of free radicals. Plants also deploy detoxifying enzymes to control the free radical levels, thereby reducing oxidative stress (Arbona and Gomez-Cadenas 2008). Importantly, a clear manifestation of the role of PAs is their ability to regulate the expression of genes encoding antioxidant enzymes. Of the detoxifying enzymes SOD, CAT and peroxidases are thought to play an essential protective role in scavenging free radicals as a first line of defense against oxidative damage. Earlier, a strong coincidence between salt stress-induced polyamine oxidase activity and generation of $O_2^{\cdot-}$ was established. The concerted action of SOD on $O_2^{\cdot-}$ results in the generation of H_2O_2, which is further scavenged by CAT, along with POD and peroxidases (Jaleel et al. 2009). The activities of SOD, CAT and APX were analyzed to gain an insight into the oxidative status in foxtail millet cultivars with or without NaCl treatment. It can be seen that under NaCl stress, these antioxidant enzymes were significantly increased in cultivar Prasad compared to Lepakshi, suggesting the possible reason for lower levels of $O_2^{\cdot-}$ and H_2O_2 and effective tolerance mechanism.

The increased antioxidant enzymes were largely associated with enhanced polyamine accumulation and their successive degradation under stressful condition. Results presented in this study, along with others via either exogenous application of polyamines or genetic transformation for polyamine biosynthetic genes, have collectively shown that polyamines can moderate the activities of antioxidant enzymes under stress (Kubis 2008; Wen et al. 2009). Verma and Mishra (2005) also reported that PAs increased the activities of antioxidant enzymes and carotenoids in leaf tissues of salt-stressed *B. juncea* seedlings. Previously, numerous authors reported that PAs modulate stress-triggered ROS homeostasis and oxidative damage (malondialdehyde, MDA) by activating some antioxidant enzyme activities, including SOD, CAT and POD (Shi et al. 2010, 2013; Wang et al. 2011; Moschou et al. 2012; Tavladoraki et al. 2012). Similarly, our findings suggest that PAs might activate antioxidant enzymes and elevate antioxidant levels, thereby controlling free radical generation, preventing membrane peroxidation and denaturation of biomolecules in cultivar Prasad. Induction of antioxidant enzymes may contribute to the effective elimination of free radicals, leading to minimized oxidative damage and better performance during stress conditions. For instance, the increase in antioxidant activities was associated with less accumulation of $O_2^{\cdot-}$ and H_2O_2, which was also confirmed during in situ histochemical staining. This can at least partially explain the lower free radical formation in the tolerant cultivar Prasad over the sensitive cultivar Lepakshi, with clear differences between unstressed and stressed conditions. Analysis of SOD and CAT-encoding genes at the mRNA level (Fig. 4b, d) supported this conclusion. It was shown that SOD and CAT transcripts were strongly up-regulated and established a linear relation with their respective enzyme activities and PAO activity during stressful conditions. However, it remains unclear how polyamines can regulate the expression of antioxidant enzymes, though one possible way is that polyamines stimulate de novo synthesis of antioxidant enzymes at the translational level (Jaleel et al. 2009).

Conclusion

Our data showed that cultivar Prasad exhibited a higher degree of NaCl tolerance than cultivar Lepakshi. The enhanced tolerance of cultivar Prasad was accompanied by both its lower polyamine oxidase activity and free radical levels. Lower levels of free radicals in cultivar Prasad may be ascribed to an effective antioxidant defense mechanism mediated by SOD and CAT. Modulation of polyamines and their metabolic enzymes could function together to confer the relative stress tolerance of this cultivar. All of these data suggest that polyamines may be used as an efficient protector for the abatement of NaCl stress-induced damage and thereby stress tolerance potential in foxtail millet cultivars. As a whole, this study provides the framework for the better understanding of the polyamine metabolism that governs plant responses to NaCl-induced stress by modulating the antioxidant defense mechanism.

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Conflict of interest The authors declare that they have no conflict of interest.

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