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Evaluation of Multiple Salinity Tolerance Indices for Screening and Comparative Biochemical and Molecular Analysis of Pearl Millet [*Pennisetum glaucum* (L.) R. Br.] Genotypes

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Abstract

Salinity is a major constraint for plant growth, development and yield worldwide. Evaluation of a large number of germplasms in salt-stressed environments may help identify superior salt-tolerant genotypes. The present study dissects the genetic diversity of 33 pearl millet genotypes (landraces and inbred lines) for salinity tolerance through in vitro screening at the seedling stage. Our results revealed a significant reduction in total biomass and shoot growth of the salt-sensitive genotypes upon exposure to 150 mM NaCl, in contrast to the tolerant genotypes showing better growth characteristics. A significant differential effect of salt treatment on morphological traits was observed by analysis of variance (ANOVA), confirming substantial genetic diversity among all genotypes for salt tolerance. The genotypes were clustered into three groups based on multiple stress indices. The genotypes were also evaluated using principal component analysis (PCA) to identify the key contributing traits for stress tolerance. Based on these results, a total of four contrasting genotypes were selected for further biochemical and molecular analysis. Physiological studies confirmed that salt tolerance might be due to the higher content of osmolytes and the activity of antioxidant enzymes. Similarly, gene expression profiling of catalase *(CAT)*, glutamate dehydrogenase *(GDH)*, glutathione reductase *(GR),* and nitrate reductase (*NR)* revealed a profound increase in *NR* and *GDH* transcript levels in the tolerant genotypes, suggesting their major role as reactive oxygen species (ROS) scavengers under salinity. The overall findings of this study could be utilized further for candidate gene mining through "omics" approaches, aiming toward development of salinity resilient crop plants.

Keywords Antioxidant enzymes · Gene expression profiling · Germplasm screening · Pearl millet · Salinity stress tolerance · Stress indices

Introduction

Salinity stress is among the key abiotic stresses that critically impede plant development. Almost 20% of the world's irrigated land is affected by salinity (FAO 2011), which is a prime cause of the reduction in crop productivity in the arid and semi-arid regions. Therefore, it is necessary to develop salinity tolerance in the major crop plants to provide global food security for the growing population. The average yield losses of 15–90% has been well documented in major crops due to salinity stress. For instance, maize, wheat, and cotton exhibited 55%, 28%, and 15% grain yield loss under moderate soil salinity, while up to 55% and 93% yield losses were observed in cotton and tef under high saline conditions (Tadele 2018; Zörb et al. 2019). Very little information is available on millets regarding salt stress responses and yield losses, as compared to other crops. In Finger millet, a

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significant reduction in grain yield by 23–27% was estimated (Krishnamurthy et al. 2014). In a recent study, 12.90–22.43% reduction in pearl millet grain yield was observed under salinity levels of 8–12 dS m^{-1} (Yadav et al. 2020). Pearl millet [*Pennisetum glaucum* (L.) R. Br.] is a major C4 cereal crop, mainly grown in semi-arid and arid regions of Asia and Africa. It is ranked as the sixth economically important crop plant (Shivhare and Lata 2017). It can survive under adverse environmental conditions, and poor nutrient-deficient soil. Although high salinity is known to impact its growth and productivity in several arid zones (Krishnamurthy et al. 2007). An average reduction of ~ 3–4 folds in shoot biomass productivity and grain yield was reported in 15 accessions of pearl millet (Krishnamurthy et al. 2011). In another study, 11 pearl millet lines showed a significant reduction of 19.1% and 41.3% in biomass and grain yield, respectively under salinity (Toderich et al. 2018). Whereas a mean reduction of 47–86% in grain yield and 51% in fodder yield was observed under high saline conditions in pearl millet (Choudhary et al. 2019; Kulkarni et al. 2006; Ribadiya et al. 2018).

The differential responses of plants toward salinity stress rely upon their genetic make-up and the environment. Therefore, screening a large number of genotypes is essential to select the superior genotypes with greater stress tolerance. The candidate genes could be identified from those potential genotypes, and transferred to other salt-sensitive crops by plant breeding or transgenic approaches (Jha 2019). Germplasm screening for salinity stress tolerance has been performed in several plant species, viz. rice, wheat, maize, sorghum, etc. (Morton et al. 2019), but only a few candidate genes have been identified for stress tolerance, owing to the complex nature of salinity stress (Jha 2018; Lakra et al. 2018). Large genotypic variation has been observed in pearl millet toward salinity stress tolerance. A wide range of pearl millet breeding lines has been evaluated extensively for salt tolerance (Krishnamurthy et al. 2007; Mukhopadhyay et al. 2005; Ribadiya et al. 2018; Toderich et al. 2018; Yakubu et al. 2010). Typically, the landraces and wild relatives of a crop species exhibit genetic diversity and are known to harbor novel genes for environmental adaptation and other agronomic important traits. Therefore, these genotypes can be used as valuable genetic resources for developing abiotic stress tolerance (Hoang et al. 2016; Manga 2015; Quan et al. 2018). Despite having a wide genetic diversity and a large germplasm collection available at the National repositories, limited reports are available for the identification and selection of superior genotypes for abiotic stress tolerance in pearl millet (Shivhare and Lata 2017; Yadav 2010).

The present study aimed to screen 33 landraces and inbred lines of pearl millet for salinity stress tolerance under in vitro conditions, as field screening is not a powerful approach due to the high degree of variability in the applied salt concentrations among the plots in a single field (Krishnamurthy et al. 2007). To our knowledge, this is the first report describing the evaluation of these pearl millet germplasms for salinity stress tolerance under the hydroponic system. Since salt stress tolerance during the early vegetative stage plays a pivotal role in crop setting under the saline environment (Mukhopadhyay et al. 2005), germplasm screening was performed at early seedling growth stage to select and identify the most tolerant genotypes of pearl millet. Various stress indices are in widespread use for selecting genotypes based on their performance under a stressed environment (Morton et al. 2019; Singh et al. 2015). On account of that, one of the objectives of this study was development of effective screening criterion by evaluating multiple stress indices, viz. stress tolerance index (STI), stress susceptibility index (SSI), tolerance index (TOL), and salt tolerance (ST). Furthermore, physiological and molecular analyses of the selected contrasting genotypes were performed in the present study to identify the molecular mechanism and genes involved in salinity responses in pearl millet.

Materials and Methods

Plant Material and Experimental Set-up for Salinity Stress Tolerance Screening

Germplasms for a total of 33 genotypes of pearl millet were collected from the National Bureau of Plant Genetic Resources (NBPGR), Jodhpur, and the International Crop Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, Hyderabad, as shown in Table 1. Seeds of different genotypes were surface sterilized and germinated on filter paper moistened with distilled water in closed Petridishes at 28 ± 2 °C. Germinated seedlings were transferred to half strength of Hoagland solution (pH 5.6) for further growth and development. Salinity screening of different pearl millet genotypes was performed at the seedling stage using the salinized nutrient solution in the hydroponic system following the International Rice Research Institute (IRRI) standard protocol for rice (Gregorio et al. 1997). Non-salinized and salinized setups were maintained in the greenhouse at 25 ± 5 °C at 60 ± 5% relative humidity. Fifteen-day-old seedlings of pearl millet were subjected to a range of salt concentrations (50, 75, 100, and 150 mM NaCl) for 7, 14, and 21 days by growing the plants in NaCl-supplemented Hoagland's medium (Supplementary Fig. S1). Culture media were replaced every alternate day to maintain a stable salt concentration and pH of the medium. An unstressed control (0 mM NaCl) was maintained in each case under similar growth conditions. Seedlings were measured after 7, 14, and 21 days of salt treatment to calculate the relative growth rate (RGR) [relative shoot length (RSL), relative fresh weight (RFW)] according to the following formula:

Table 1 Detailed information of the 33 genotypes of pearl millet

S.No	Accession Code	Genotype	Obtained by
1	2	IC 285172	NBPGR
2	3	IC 285173	NBPGR
3	4	IC 285175	NBPGR
4	5	IC 285176	NBPGR
5	6	IC 285177	NBPGR
6	7	IC285178	NBPGR
7	8	IC285185	NBPGR
8	9	IC 325750	NBPGR
9	10	IC 325765	NBPGR
10	11	IC 325776	NBPGR
11	12	IC 325794	NBPGR
12	13	IC 325825	NBPGR
13	14	IC 329028	NBPGR
14	15	IC 329031	NBPGR
15	16	IC 329041	NBPGR
16	17	IC 370482	NBPGR
17	18	IC 370487	NBPGR
18	19	IC 370507	NBPGR
19	20	IC 420309	NBPGR
20	21	IC 420312	NBPGR
21	22	IC 420314	NBPGR
22	23	IC 420315	NBPGR
23	24	IC 420317	NBPGR
24	25	IC420319	NBPGR
25	26	IP 17196	NBPGR
26	27	IP 17224	NBPGR
27	28	IP 17276	NBPGR
28	29	IP 17319	NBPGR
29	30	IP 17399	NBPGR
30	31	PRC2-18933	ICRISAT
31	32	ICMB-90111B-P6	ICRISAT
32	33	863B-P2	ICRISAT
33	34	841B- P3	ICRISAT

RGR = SL or FW in a known concentration of salt/ SL or FW in absence of salt × 100.

At least 20 independent biological replicates (individual seedlings) for each sample (control v/s treatment) were used for the analysis. Individual genotype was scored for salinity tolerance based on seedling growth parameters. Different stress indices were calculated based on the phenotypic analysis for each genotype, using the following formulae:

Stress Tolerance Index (STI) = (Yp x Ys)/ $(Xp)^2$ (Fernandez 1992).

Tolerance Index (TOL) = Yp – Ys (Rosielle and Hamblin 1981).

Salt Tolerance (ST) = Y_{salt} at T2 / $Y_{control}$ at T2 (Genc et al. 2007).

Stress Susceptibility Index (SSI) = (1 – Ys/Yp)/SI (Fischer and Maurer 1978).

Stress Intensity (SI) = [1-(Xs/Xp)] (Fischer and Maurer 1978).

Here,

Yp = Growth related trait for each genotype under control condition.

Ys = Growth related trait for each genotype under stress condition.

Xp = Average of the observed trait for all genotypes under control condition.

Xs = Average of the observed trait for all genotypes under stress condition.

Survival rate or vigor score was depicted by visual scoring of salt injury in individual genotypes on the scale of 1–9 (1 for most tolerant and 9 for most sensitive), according to Standard Evaluation System (SES), IRRI protocol (Gregorio et al. 1997). A similar ranking of genotypes was performed based on multiple stress indices.

Biochemical and Molecular Analysis

Salinity Treatment

Two salt-tolerant and two sensitive genotypes of pearl millet were selected for further biochemical and molecular analysis. For these studies, 15-day-old plantlets of pearl millet were subjected to salt stress by growing the plants in 150 mM NaCl-supplemented medium for 72 h, under conditions as described earlier. Since the seedlings of pearl millet showed a noticeable stress phenotype at 150 mM NaCl concentration, we have selected this salt concentration and time point for further studies. Our earlier studies exhibited complete suppression of plant growth at concentrations higher than150 mM NaCl, leading to the death of the seedlings. After three days of treatment, seedlings were harvested and immediately frozen in liquid nitrogen for further experiments. Control was maintained in each experiment under non-stressed conditions (0 mM NaCl).

Seed Germination Assay

To determine the effect of salinity on seed germination ability of the contrasting genotypes of pearl millet, seeds from the four genotypes were surface sterilized using 0.1% $HgCl_2$ and germinated on moistened filter paper under increasing salt concentrations (100, 150, 200, and 250 mM NaCl) at 28 ± 2 °C. The filter paper moistened with distilled water served as control (0 mM NaCl) under similar conditions. At least 50 seeds per replicate were used for each treatment, and three independent biological replicates were analyzed for every salt concentration. The

emergence of radical was considered as the initiation of seed germination. The percent germination was calculated based on the following formula:

% Seed germination = No. of germinated seeds under salt stress/ Total no. of seeds *100.

Proline Estimation

Quantitative estimation of free proline content was performed according to Bates et al. (1973) using the acid-ninhydrin method. A 0.25 g of fresh plant leaf sample (stressed and unstressed seedlings from the selected contrasting genotypes) was extracted with 5 ml of 3% sulphosalicylic acid and centrifuged at 4 °C at 10,000 rpm for 10 min. The supernatant was mixed with acetic acid and ninhydrin reagent (each 2 ml). The reaction mixture was heated for 1 h in a boiling water bath and the reaction was arrested by quick cooling in ice. A 4 ml of toluene was added to the reaction mix, mixed and the absorbance of the upper layer was measured at 520 nm. Proline concentration was calculated using a standard curve prepared with D-proline.

Total Soluble Sugar Content

Total soluble sugar was analyzed using the Anthrone reagent as described by Roe (1955). A 0.1 g of plant sample (stressed and unstressed seedlings from sensitive and tolerant genotypes) was homogenized with 5 ml of 80% ethanol. The mixture was centrifuged and an equal volume of 80% ethanol was added to 0.5 ml supernatant, followed by the addition of 4 ml of Anthrone reagent. The mixture was heated for 5 min in a boiling water bath and absorbance was taken at 620 nm.

Assays for Antioxidant Enzymes

Catalase Activity Assay

Catalase activity was determined by the initial rate of disappearance of H_2O_2 at 240 nm (Aebi, 1984). A 0.5 g of plant sample (stressed and unstressed seedlings from the selected contrasting genotypes) was homogenized with 3 ml of Sodium phosphate buffer (pH 7.0, 0.1 M) and centrifuged at 10,000 rpm for 15 min at 4 °C. A 1.5 ml of extraction buffer and 1 ml of H_2O_2 (30 mM) were added to 0.5 ml of supernatant, and decomposition of H_2O_2 was measured by taking absorbance at 240 nm. One unit of catalase activity is equivalent to 1 µmol of H_2O_2 decomposed per min under standard conditions.

Peroxidase Activity Assay

Peroxidase activity (POX) was measured using the guaiacol method of Honold and Stahmann (1968). A 0.5 g of plant sample was homogenized with 3 ml of 0.1 M Citrate buffer (pH 5.0) and centrifuged at 10,000 rpm for 15 min at 4 °C. The reaction mixture (3 ml) contained 1.5 ml of extraction buffer, 0.025 ml enzyme extract, and 1.175 ml distilled water. A 0.150 ml of H_2O_2 (200 mM) and 0.150 ml guaiacol were added to the reaction mixture just before taking absorbance, which was taken at 470 nM for 3 min with 15 s of interval. One unit of peroxidase (guaiacol) activity is equivalent to the amount of the enzyme catalyzing the formation of 1 µmol of GDHP guaiacol dehydrogenation product per min by oxidation of guaiacol.

RNA Isolation and Quantitative Real-Time PCR (qRT-PCR)

RNA from control and treated seedlings of the selected contrasting genotypes of pearl millet was isolated from 100 mg of plant tissues using Tri-reagent (Sigma Aldrich, USA) according to the manufacturer's instructions. After DNase treatment, cDNA was synthesized from 2 µg RNA using the iScript™ cDNA Synthesis Kit (Bio-Rad, USA), and further utilized as a template for qPCR analysis using SYBR Green master mix and CFX96 Real-Time PCR system (Bio-Rad, USA). Primers used for the real-time PCR analysis have been synthesized using gene sequence from transcriptome data of the selected genotypes of pearl millet (Jha et al., unpublished data) by online oligo design tool (IDT) and analyzed for specificity, *Tm,* and other parameters by oligo analyzer. The polyubiquitin (Ub) was used as an endogenous control for data normalization. The specificity of the amplification was verified by melt-curve analyses. The relative transcript level was calculated by using $2^{-\Delta\Delta Ct}$, where ΔCt denotes the difference between Ct (cycle threshold) values of a target gene and the endogenous control (Ub in this case) in the same sample, and $\Delta\Delta Ct$ is the difference between the ΔCt value of a treatment sample and the untreated control sample (Agarwal et al. 2009). The data of quantitative real-time PCR presented as mean ± standards errors of at least three independent biological replicates along with three technical replicates for each sample.

Statistical Analysis

All results were displayed as mean ± standard deviation (SD) of at least 20 replicates for phenotypic analysis, in three independent experiments. Data were analyzed by one-way ANOVA using the statistical software IBM SPSS

Statistics 20.0. The post-hoc Duncan's multiple range test (DMRT) was used to compare the treatment mean values, with significance at $p < 0.05$. Multivariate cluster analysis of various genotypes was performed with SPSS 20.0 based on Ward's algorithm, and principal component analysis (PCA) analysis was performed using XLSTAT (Microsoft Excel). The correlation study was performed using the Pearson correlation method. For physio-biochemical and molecular analysis, three independent biological replicates along with three technical replicates were analyzed for each sample, and results were displayed as mean ± standard deviation (SD). The post-hoc DMRT and student's t-test were used to compare the treatment mean values ($p < 0.05$), for physiological and qRT-PCR data analysis, respectively.

Results

Screening for Salt Stress Tolerance in Pearl Millet Genotypes and Analysis of Growth Parameters

A total of 33 genotypes of pearl millet (landraces and inbred lines) obtained from NBPGR and ICRISAT were screened for salt stress tolerance over a range of salt concentrations (details of all accessions are shown in Table 1). In vitro grown seedlings were treated with different concentrations of salt (0 to 150 mM NaCl), to which plants exhibit salt stress phenotype, but can survive the treatment. Set-up for the hydroponic system has been standardized for pearl millet following IRRI standard protocol. For analysis of genotype × salinity level treatment combinations, phenotypic characters such as relative shoot length and relative fresh weight were recorded at 7, 14, and 21 days after salt treatment. A large variation in salt tolerance levels were detected among various pearl millet genotypes (Supplementary Fig. S2), which exhibited a significant reduction of 28–83% (7d), 17–100% (14d), 44–100% (21d) in relative fresh weight, and of 15–63% (7d), 31–100% (14d), 26–100% (21d) in relative shoot length, after treatment with 150 mM NaCl (Supplementary Tables S1 and S2).

The differential effect of salinity on morphological characters of various pearl millet genotypes after treatment with 150 mM NaCl was determined by analysis of variance (ANOVA), which indicated a highly significant difference in all the observed traits in both treatments and genotypes (Table 2). The genotype x treatment interactions were also significant at probability level $p < 0.01$. The mean square values due to salt treatment were found highly significant for all the investigated traits at different time intervals, indicating the presence of considerable variations among genotypes for salinity tolerance (Table 2).

Since the genotypes exhibited distinct variability in all observed traits after 14 days of treatment with 150 mM NaCl, we have selected this time period for the calculation of stress indices and further analysis. These genotypes exhibited differences for various stress tolerance indices (STI, ST, SSI, TOL), and ranked based on those multiple stress indices along with scoring for visual injury due to salt stress (Table 3, Supplementary Table S3). The highest STI values were obtained for IC 285173, IC 285176, IC 325825, IC 329041, IC 370482, IC 370507, and IC 420315 (ranked as 1–3), revealing that these genotypes exhibited a lesser reduction in the observed growth parameters and a higher tolerance for the imposed salt stress; whereas the lowest STI value was found for IC 285172, IC 285175, IC 285177, IC 325765, IC 370487, IC 420309, IC 420317, IP 17224, IP 17276, IP 17399, PRC2-18933, 863B-P2 and 841B- P3 (ranked as 9), indicating that these genotypes exhibited the higher salt sensitivity. Similar results were obtained for ST (Table 3, Supplementary Table S3). On the other hand, IC 285172, IC 285175, IC 325765, IC 370487, IC 420309, IC 420317, IP 17224, IP 17276, IP 17399, PRC2-18933, 863B-P2 and 841B- P3 (ranked as 9) showed the highest SSI (and/or TOL) values, and considered as the salt-sensitive genotypes, in contrast to the salt-tolerant genotypes IC 285176, IC285178 and IC 325825 (ranked as 1–3), having lowest SSI (and/or TOL) values. Similar results were obtained from visual scoring of salt induced injury and survival in pearl millet genotypes, confirming IC 285172, IC 285175, IC 285177, IC 325765, IC 370487, IC 420309, IC 420317, IP 17224, IP 17276, IP 17399, PRC2-18933, 863B-P2 and 841B- P3 (ranked as 9) with minimum survival rate under stress as salt-sensitive, and IC 285176, IC285178, IC 325750, IC 325776, IC 325794, IC 325825, IC 329041, IC 370482 and

Table 2 ANOVA of morphological traits for salinity stress tolerance in the 33 genotypes of pearl millet after treatment with 150 mM NaCl. Mean Square values are displayed for each trait

Source of Variance	*df*	7d FW	14d FW	21d FW	7d SL	14d SL	21d SL
Genotypes (G)	32	1.442**	1.160**	0.752**	388.5912**	254.726**	174.874**
Treatment (T)	1	34.098**	134.371**	242.424**	13,730.5922**	62,231.478**	105,455.277**
G X T	32	0.380**	0.798**	0.885**	65.0652**	231.208**	153.324**
Error	413	0.106	0.250	0.311	21.4422	25.805	24.140

**significant at $p < 0.01$probability level

Table 3 Ranking of the 33 genotypes of pearl millet on the basis of standard visual scoring system and various stress indices calculated by using fresh weight data after 14 days of treatment with 150 mM NaCl

Accession code	Genotype	STI[a]	TOL[b]	SSI[c]	ST[d]	Survival (%)
2	IC 285172	0 [9]	2.189333 [8]	1.230815 [9]	0 [9]	0 [9]
3	IC 285173	0.579143506 [2]	1.279333 [4]	0.782113 [4]	0.353654 [3]	12.5 [8]
4	IC 285175	0 [9]	2.736667 [9]	1.210054 [9]	0 [9]	0 [9]
5	IC 285176	1.421369786 [1]	0.684359 [2]	0.373411 [2]	0.691409 [1]	56 [1]
6	IC 285177	0 [9]	1.644 [6]	1.210054 [9]	0 [9]	0 [9]
7	IC285178	0.174905073 [6]	0.511 [2]	0.650197 [3]	0.462671 [2]	50 [2]
8	IC285185	0.101783851 [7]	0.899 [3]	0.973893 [7]	0.195166 [7]	16.6666667 [6]
9	IC 325750	0.10383141 [7]	0.700091 [2]	0.883282 [6]	0.270047 [4]	50 [2]
10	IC 325765	0 [9]	0.907143 [3]	1.210054 [9]	0 [9]	0 [9]
11	IC 325776	0.182775885 [6]	0.833 [3]	0.841381 [5]	0.304674 [3]	50 [2]
12	IC 325794	0.11042186 [7]	0.918571 [3]	0.967742 [7]	0.200249 [7]	52.5 [1]
13	IC 325825	0.486903245 [3]	0.213556 [1]	0.216911 [1]	0.820742 [1]	58 [1]
14	IC 329028	0.161599155 [6]	0.924643 [3]	0.904397 [6]	0.252598 [5]	40 [3]
15	IC 329031	0.053718097 [8]	0.858571 [3]	1.050928 [8]	0.131503 [8]	33.3333333 [4]
16	IC 329041	0.602620791 [2]	1.568 [6]	0.855439 [5]	0.293057 [4]	50 [2]
17	IC 370482	0.595668171 [2]	2.003333 [7]	0.94693 [6]	0.217448 [6]	48.3333333 [2]
18	IC 370487	0 [9]	1.805833 [7]	1.210054 [9]	0 [9]	0 [9]
19	IC 370507	0.51896841 [2]	1.469286 [5]	0.859193 [5]	0.289955 [4]	20 [5]
20	IC 420309	0 [9]	1.101429 [4]	1.210054 [9]	0 [9]	0 [9]
21	IC 420312	0.361329395 [4]	1.369231 [5]	0.900835 [6]	0.255542 [5]	12.5 [8]
22	IC 420314	0.243539755 [5]	1.276905 [4]	0.945856 [6]	0.218336 [6]	33.3333333 [4]
23	IC 420315	0.442441618 [3]	1.287 [4]	0.838632 [5]	0.306947 [3]	14.2857143 [7]
24	IC 420317	0 [9]	1.345 [5]	1.210054 [9]	0 [9]	0 [9]
25	IC420319	0.241014877 [5]	1.55 [6]	1.008378 [8]	0.166667 [7]	20 [5]
26	IP 17196	0.116249133 [7]	0.690833 [2]	0.856648 [5]	0.292058 [4]	50 [2]
27	IP 17224	0 [9]	0.926923 [3]	1.210054 [9]	0 [9]	0 [9]
28	IP 17276	0 [9]	1.248182 [4]	1.210054 [9]	0 [9]	0 [9]
29	IP 17319	0.188305935 [6]	0.937143 [3]	0.881016 [6]	0.27192 [4]	40 [3]
30	IP 17399	0 [9]	2.084167 [8]	1.210054 [9]	0 [9]	0 [9]
31	PRC2-18933	0 [9]	1.588333 [6]	1.210054 [9]	0 [9]	0 [9]
32	ICMB-90111B-P6	0.1056854 [7]	0.722 [2]	0.891489 [6]	0.263265 [4]	33.3333333 [4]
33	863B-P2	0 [9]	1.455385 [5]	1.210054 [9]	0 [9]	0 [9]
34	841B- P3	0 [9]	1.742 [6]	1.210054 [9]	0 [9]	0 [9]

Note: The numbers in parentheses denote the rank of the genotype for each stress index

[a]Stress Tolerance Index

[b]Tolerance Index

[c]Stress Susceptibility Index

[d]Salt Tolerance

IP 17196 (ranked as 1–2) with highest survival rate under stress as salt-tolerant genotypes (Table 3, Supplementary Table S3).

Correlation Analysis

The Pearson correlation coefficient (r) was calculated to demonstrate the association among various stress indices, survival rate, and observed traits (Table 4). The results showed a weak non-significant correlation between the morphological traits under the stressed condition (Ys) and control unstressed condition (Yp), indicating the fact that optimum growth and larger biomass in a non-stressed environment does not necessarily result in a better phenotype under a stressed condition. For example, IC 370487 selected in this study as a "salt-sensitive" genotype showed a good

Table 4 Correlation matrix (Pearson) for various stress indices derived in the present study

	Yp[a]	Ys[b]	STI[c]	TOL[d]	SSI[e]	ST[f]	Survival %
Yp[a]	1						
Ys[b]	0.256 [ns]	1					
STI[c]	0.426[**]	0.957[**]	1				
TOL[d]	0.789[**]	−0.391[*]	−0.202 [ns]	1			
SSI[e]	0.035 [ns]	−0.910[**]	−0.753[**]	0.612[**]	1		
ST[f]	−0.032 [ns]	0.910[**]	0.753[**]	−0.609[**]	−1.000[**]	1	
Survival %	−0.168 [ns]	0.671[**]	0.532[**]	−0.586[**]	−0.797[**]	0.796[**]	1

[ns]non-significant

[*]significant at $p < 0.05$

[**]significant at $p < 0.01$

[a]Growth related trait for each genotype under control condition

[b]Growth related trait for each genotype under stress condition

[c]Stress Tolerance Index

[d]Tolerance Index

[e]Stress Susceptibility Index

[f]Salt Tolerance

phenotype and higher biomass under unstressed conditions, but it exhibited a maximum reduction in relative fresh weight and relative shoot length after imposing salt stress (Supplementary Table S2). On the other hand, a significant negative association was found between Ys and TOL or SSI (Table 4), indicating that the lower values of TOL and SSI are associated with salt tolerance under a stressed environment. Conversely, Ys had a significant positive correlation with STI, ST, and survival rate, indicating that a higher value of these stress indices is a suitable predictor of stress tolerance (Table 4). Moreover, the TOL and SSI values showed a significant positive correlation between each other, and are negatively correlated with ST and survival rate. The STI has shown a significant positive correlation with ST and survival rate, while negatively correlated with SSI and TOL (Table 4). The results indicated that these stress indices can distinct between salt-sensitive and tolerant genotypes, and could be used as selection criteria for tolerant genotypes of pearl millet under salinity stress.

Multivariate Cluster Analysis

In the present study, cluster analysis using Ward's algorithm and squared Euclidean distance categorized 33 pearl millet genotypes into three groups (Fig. 1, Supplementary Fig. S3). Members of each cluster are shown in Table 5 and Supplementary Table S4. Based on this analysis, members of cluster-I, cluster-II, and cluster-III were identified as salt-sensitive, moderately tolerant, and highly tolerant genotypes, respectively (Fig. 1). The clustering was performed based on various stress indices as described above, after imposing salinity stress for 14 days. The classification based on relative fresh weight (Fig. 1, Table 5) was generally consistent with the relative shoot length (Supplementary Fig. S3, Supplementary Table S4). The phylogenetic distance and variability were minimum within a cluster, as compared to maximum genetic distance and dissimilarity between two clusters. The results were in good correlation with the phenotypic observations.

Principal Component Analysis

In addition to cluster analysis, principal component analysis (PCA) based on various stress tolerance indices and survival rate was performed to detect superior genotype among all pearl millet genotypes under study. The analysis has grouped the variables into two main components that accounted for 89.54% of the total variability in the dataset and had eigenvalue > 1 (Fig. 2). The biplot diagram showed that the first principal component (PC1 or F1) accounted for maximum variability in the dataset (i.e., 72.46%), and had a strong positive correlation with STI, ST, survival rate, and relative fresh weight (RFW). The results indicated that these indices can identify the tolerant genotype that executes well under salinity-stressed conditions. In contrast, PC1 is negatively correlated with SSI and TOL. It can be concluded that the traits, which contributed more positively to the first principal component (STI, ST, RFW, and survival rate) were the best indicator of salinity stress tolerance in pearl millet genotypes under study.

Differential Physiological and Biochemical Responses of the Contrasting Pearl Millet Genotypes

Based on the results of screening, we have selected some highly salt-tolerant and sensitive genotypes of pearl

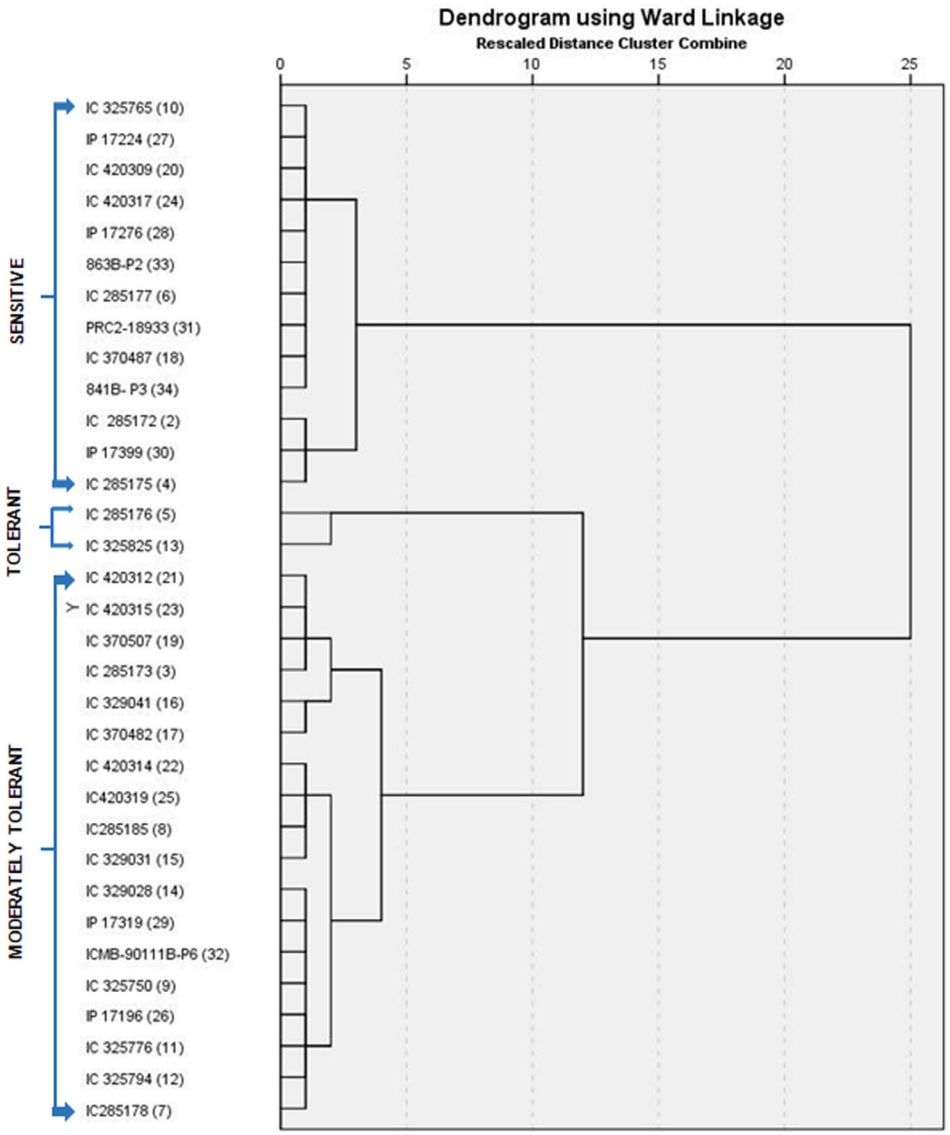

Fig. 1 Dendrogram showing clustering of the 33 Pearl millet genotypes using Ward's linkage. The clustering was performed based on various stress tolerance indices (STI, TOL, SSI, ST, calculated from fresh weight data) and survival rate after 14 days of salt treatment at 150 mM NaCl concentration

Table 5 Categorization of the 33 genotypes of pearl millet for salinity tolerance on the basis of fresh weight recorded after 14 days of treatment with 150 mM NaCl

Cluster	Cluster membership in dendrogram	Salt response
I	IC 285172 (2), IC 285175 (4), IC 285177 (6), IC 325765 (10), IC 370487 (18), IC 420309 (20), IC 420317 (24), IP 17224 (27), IP 17276 (28), IP 17399 (30), PRC2-18933 (31), 863B-P2 (33), 841B- P3 (34)	Sensitive
II	IC 285173 (3), IC285178 (7), IC285185 (8), IC 325750 (9), IC 325776 (11), IC 325794 (12), IC 329028 (14), IC 329031 (15), IC 329041 (16), IC 370482 (17), IC 370507 (19), IC 420312 (21), IC 420314 (22), IC 420315 (23), IC420319 (25), IP 17196 (26), IP 17319 (29), ICMB-90111B-P6 (32)	Moderately Tolerant
III	IC 285176 (5), IC 325825 (13)	Tolerant

millet (two from each category) for further physiological, biochemical, and molecular analysis (Fig. 3). The IC 285176 and IC 325825 were the best performer under salt stress with the highest scoring and exhibited a minimum decrease in the observed traits after 14 days of treatment with 150 mM NaCl (30.8% and 17.9% in relative fresh

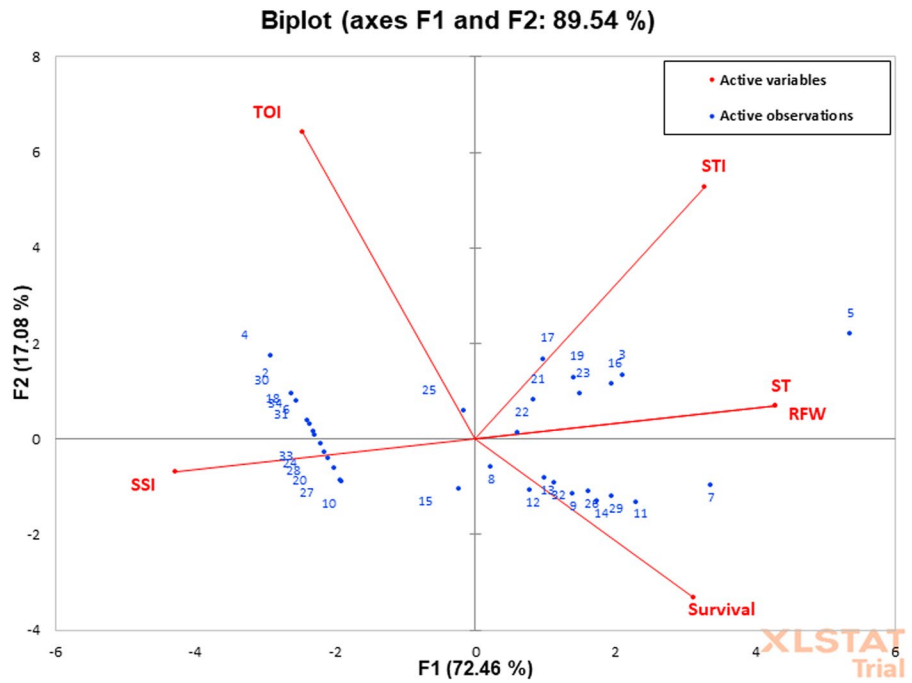

Fig. 2 The Biplot showing Principal Component Analysis (PCA) to examine the importance of various stress indices contributing to salinity tolerance and superior genotypes. The variables used here are Stress Tolerance Index (STI), Tolerance index (TOL), Stress susceptibility index (SSI), Salt Tolerance (ST), Relative Fresh Weight (RFW), and Survival rate

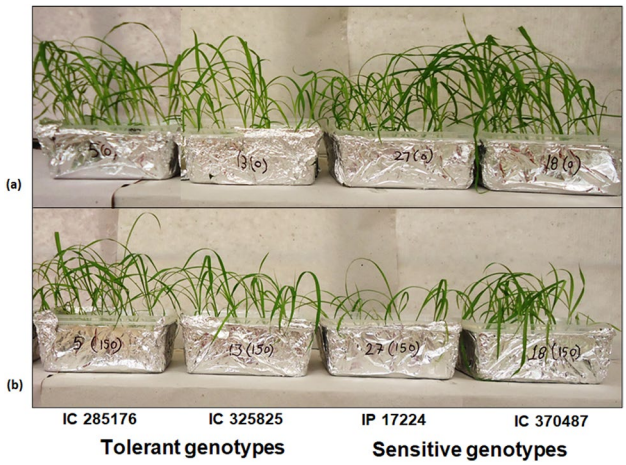

Fig. 3 Phenotype of contrasting genotypes of pearl millet after 3 days of salt treatment at (**a**) 0 mM NaCl, (**b**) 150 mM NaCl concentration

weight, 31.3 and 38.3% in relative shoot length for IC 285176 and IC 325825, respectively), therefore selected as "salt-tolerant genotypes" (Table 3, Supplementary Tables S2, S3). Whereas, IC 370487 and IP 17224 had the worst performance under salt stress with the lowest ranking and exhibited a maximum relative decrease in both traits after 14 days of treatment with 150 mM NaCl (100% in relative fresh weight and relative shoot length for both genotypes), therefore selected as "salt-sensitive genotypes" (Table 3, Supplementary Tables S2, S3).

The seed germination ability of all the four genotypes was analyzed over a range of 0 to 250 mM NaCl. The percentage of germination was found to be reduced for all genotypes at all salt concentrations, but this decline was more profound for the salt-sensitive genotypes (IC 370487 and IP 17224), as compared to the tolerant ones (IC 285176 and IC 325825). At 250 mM NaCl, IC 285176 and IC 325825 exhibited 47% and 37% seed germination, respectively, whereas only 5% and 13% seeds were able to germinate at this salt concentration for IC 370487 and IP 17224, respectively (Fig. 4a).

The hydroponically grown seedlings of all the four genotypes were analyzed for content of osmolytes/compatible solutes and antioxidant enzyme activities, after three days of treatment with 150 mM NaCl, the time point where they exhibited the differential morphological response but did not die due to the imposed stress (Fig. 3). A sudden spurt in the free proline content was observed for all the four genotypes after imposing stress, but the results showed a more significant increase for the tolerant genotypes as compared to the sensitive ones. The genotypes IC 325825 and IC 285176 exhibited ~ 40 and ~ 37-fold increase in the proline accumulation, respectively, in contrast to the ~ 11 and 16-fold increase for IP 17224 and IC 370487, respectively (Fig. 4b) that might not quite enough to withstand the effect of the imposed stress. In contrast, the total soluble sugar content was observed to be significantly elevated (~ 2–3-fold) equally for all the four genotypes, but the basal level of the sugar content was higher in the tolerant genotypes as compared to the sensitive genotypes, resulting in greater

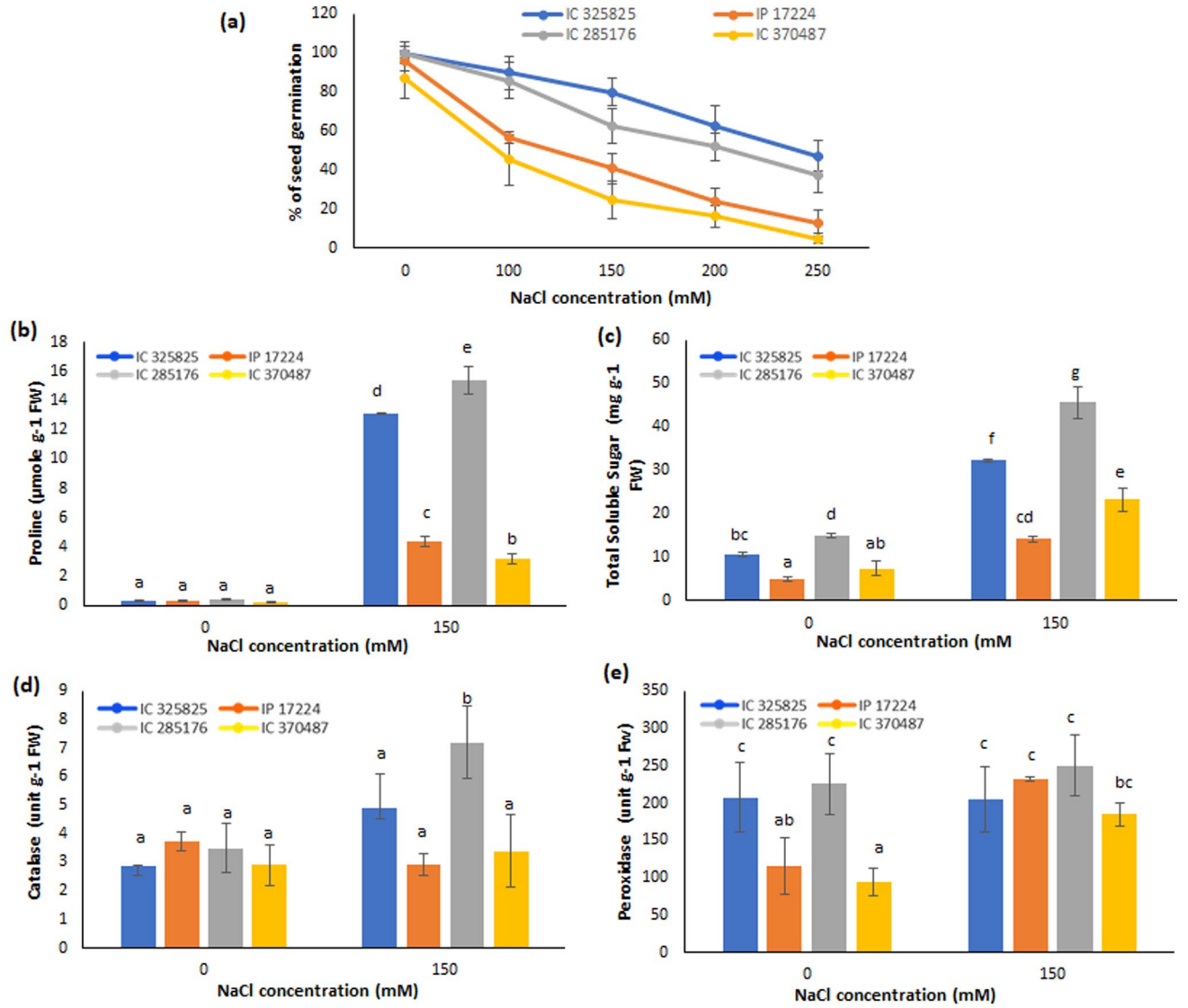

Fig. 4 Effect of salt stress on various physiological and biochemical parameters in stress-tolerant (IC 325825 and IC 285176) and sensitive (IP 17224 and IC 370487) genotypes of pearl millet after three days of NaCl treatment at 0 mM (control) and 150 mM (treatment) concentrations. **a** Seed germination; **b,c** Compatible solutes: **b** proline content; **c** total soluble sugar content; **d,e** Antioxidant enzyme activity: **d** Catalase activity expressed in units per gram FW. One unit is equivalent to 1 µmol of H_2O_2 decomposed per min under standard conditions; **e** Peroxidase (Guaiacol) activity expressed in units per gram FW. One unit is equivalent to the amount of enzyme catalyzing the formation of 1 µmol of GDHP guaiacol dehydrogenation product per min by oxidation of guaiacol. Data represent the mean values ± SD of three independent experiments, each having at least three biological replicates. Different letters on the graphs denote significant differences according to Duncan's multiple range test ($P \leq 0.05$)

sugar accumulation under stress condition (Fig. 4c), providing more salt tolerance.

The modulation of antioxidant enzyme activity after the imposition of salt stress differed significantly among all the four genotypes of pearl millet. The catalase (CAT) activity was increased (~ 1.7–2-fold) for the tolerant genotypes IC 325825 and IC 285176, whereas no significant change for its activity was observed in the sensitive genotypes IP 17224 and IC 370487 under stress condition (Fig. 4d). In contrast to this, the activity of the peroxidase (POX) enzyme exhibited no significant change in the tolerant genotypes, whereas it showed a significant ~ twofold increase in the sensitive genotypes under stress conditions (Fig. 4e). Here it is worthwhile to mention that the native activity of this enzyme was almost double in the tolerant genotypes IC 325825 and IC 285176, as compared to the sensitive genotypes IP 17224 and IC 370487, which remained higher even after exposure to salt stress (Fig. 4e).

Gene Expression Profiling of the Contrasting Pearl Millet Genotypes

The expression of stress-responsive genes in the selected contrasting genotypes of pearl millet subjected to 150 mM NaCl was analyzed by quantitative real-time PCR. For this, the transcript levels of few selected antioxidant genes were measured under control (C) and 150 mM NaCl treatment (T) conditions. The primers for these genes were designed from the sequences obtained from pearl millet salinity transcriptome data (Supplementary Table S5). The sequences of primers used in this study are shown in Supplementary Table S6. The mRNA level of catalase (*CAT*) did not show any significant alteration in the tolerant genotypes IC 325825 and IC 285176 under stress conditions, whereas its expression was significantly reduced to ~ 0.2 to 0.5-fold in the sensitive genotypes IC 370487 and IP 17224 (Fig. 5a). In contrast to this, glutamate dehydrogenase (*GDH*) expression exhibited a significant ~ 3 to 4-fold upregulation in the tolerant genotypes as opposed to the salt-sensitive genotypes, where its transcript level did not change significantly after exposure to salt stress (Fig. 5b). On the other hand, the transcript level of glutathione reductase (*GR*) did not exhibit any significant change in all the four genotypes under stress conditions (Fig. 5c). Similarly, the mRNA level of nitrate reductase (*NR*) did not show significant alteration in the sensitive genotypes, whereas its expression was significantly induced by ~ 2 to 3-fold in the tolerant genotypes after the imposition of salt stress (Fig. 5d).

Discussion

The performance of a plant species under abiotic stress should be analyzed by studying the observed trait variability among several genotypes to select the superior genotypes with greater stress tolerance. Landraces of crops are reported to contain genes for environmental stress tolerance and can be used as a donor for crop improvement programs. However, only a few studies have evaluated the salt tolerance potential of different genotypes of pearl millet (Supplementary Table S7). Early vegetative growth stage has been reported to be more sensitive to salt stress, as compared to the adult stage (Cardamone et al. 2018; Mukhopadhyay et al.

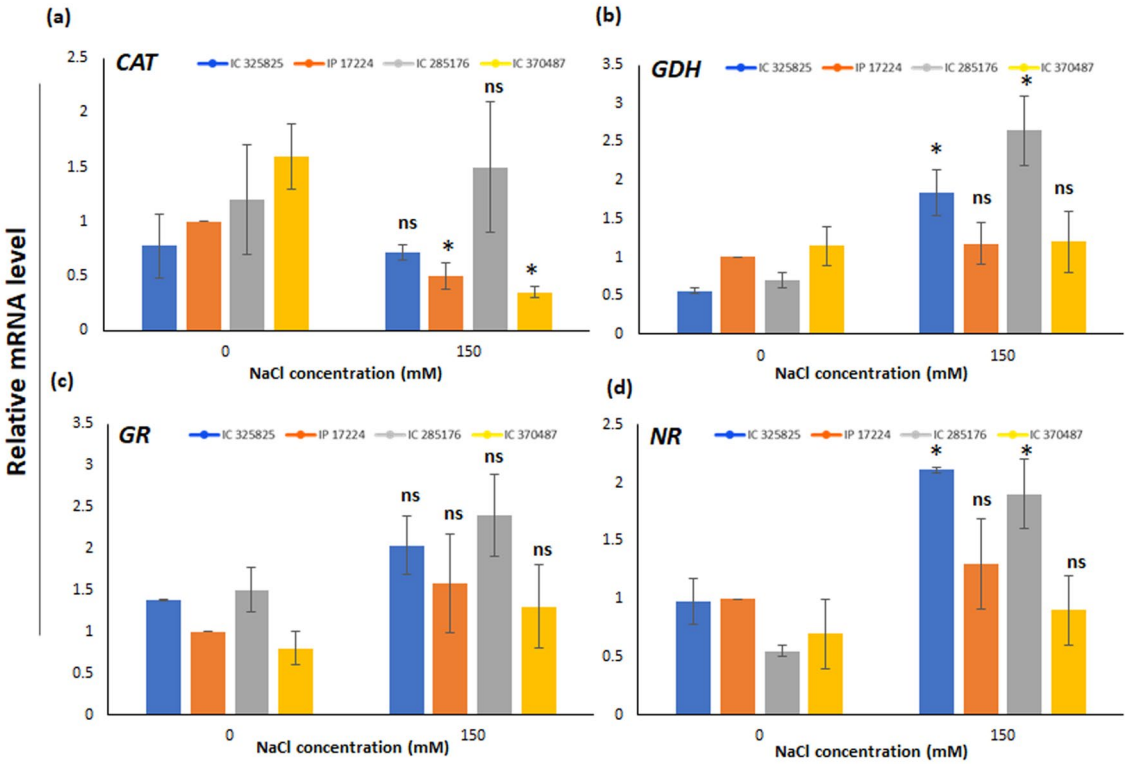

Fig. 5 Expression profiling of the selected stress-responsive genes in both tolerant (IC 325825 and IC 285176) and sensitive (IP 17224 and IC 370487) genotypes of pearl millet by Real-time quantitative PCR. **a** Catalase (*CAT*); **b** glutamate dehydrogenase (*GDH*); **c** glutathione reductase (*GR*); **d** nitrate reductase (*NR*) genes. The data presented here represent mean ± standards errors of three independent biological replicates with three technical replicates for each sample. Values were calculated relative to the unstressed control (0 MM NaCl) of the sensitive genotype IP 17224. Asterisks on the graphs denote significant differences according to student's t-test ($P < 0.05$), ns—non-significant

2005; Rajabi Dehnavi et al. 2020). Abiotic stress during the early vegetative stage is the major cause of grain yield reduction in pearl millet due to the death of the seedlings and poor establishment of the crop (Shivhare and Lata 2017). Therefore, in the present study, 33 pearl millet genotypes were screened for salt stress tolerance during early vegetative growth stage. Although the seedling stage salt stress responses may not correlate well with the adult plant stage, these are highly predictive of adult plant performance under salinity (Uddin et al. 2017). Rice is found to be salt-tolerant at germination and later vegetative stage, but highly sensitive during seedling and reproductive stages, whereas, both vegetative and reproductive stages were affected by salinity in wheat (Maity and Satya 2014). In pearl millet, early and late seedling growth stages and reproductive stages were found to be affected under salt and water-deficit stress (Hussain et al. 2008; Radhouane 2008; Shivhare and Lata 2019).

The relative decrease in shoot growth, fresh weight or total biomass are strong indicators of salt stress response in plants (Negrão et al. 2017). Therefore, we have selected these two traits for the screening of pearl millet genotypes under salinity stress. The genotypic variability and genotype x treatment interactions of these traits were highly significant, as evident from ANOVA. These results were in agreement with the earlier studies in the contrasting genotypes of rice, wheat, pearl millet, foxtail millet and other plants under drought or salinity stress (Krishnamurthy et al. 2016; Lapuimakuni et al. 2018; Singh et al. 2015; 2018; Vaezi et al. 2020). In the present study, 33 genotypes of pearl millet were ranked according to salt tolerance potential based of visual symptoms of salt injury as per the standard evaluation system (Negrão et al. 2017). In addition, multiple stress indices viz. STI, SSI, ST, and TOL were employed for determining the stress tolerance potential of pearl millet genotypes. The STI and ST are commonly used indices that have been reported previously to select the superior genotypes (Fernandez 1992; Krishnamurthy et al. 2016; Singh et al. 2015). The TOL index measures the differences in biomass production or yield under stressed and control conditions (Rosielle and Hamblin 1981), whereas the SSI identifies genotypes exhibiting a minimum reduction in growth parameters under a stressed environment (Fischer and Mourer 1978). In our study, the lower value of SSI and TOL and higher value of STI and ST indicate the superiority of the genotypes having enough plasticity to respond to extreme conditions. These observations are in accordance with the earlier studies in rice, wheat, and many other crops (Singh et al. 2015; Krishnamurthy et al. 2016). Besides this, the correlation among various stress indices and the observed traits is a valuable parameter for the identification of superior genotypes (Negrão et al. 2017). The current study demonstrated a negative correlation of Ys (biomass production under stress) with TOL and SSI, while it was positively correlated with STI and ST, further confirming the direct association between stress tolerance and multiple stress indices. Based on multiple stress indices, all the 33 pearl millet genotypes were classified into sensitive, moderately tolerant, and highly tolerant groups, depending on their relative potential to sustain good growth under high salinity. These results were in agreement with the previous reports describing multivariate cluster analysis for salt and drought stress screening of genotypes of wheat, rice, barley etc. (Ahmad et al. 2008; Singh et al. 2015; Zeng 2005). Moreover, PCA analysis was successfully used in the present study for identification of the key attributes contributing to stress tolerance, as reported earlier (Lapuimakuni et al. 2018; Vaezi et al. 2020).

In the present study, we have selected some salt-tolerant and sensitive genotypes for further physio-biochemical, and gene expression analysis. Our results showed that NaCl treatment resulted in a significant reduction in seed germination percentage in the sensitive genotypes of pearl millet, due to sodium ion toxicity or osmotic stress (Singh et al. 2018). Furthermore, free proline levels were found to be increased in all the selected pearl millet genotypes under salt stress, however, the increase was more profound in the tolerant genotypes, as reported earlier (Mukhopadhyay et al. 2007). Proline is an important amino acid, which act as an osmoprotectant, ROS scavenger, metal chelator, membrane protein stabilizer, and signaling molecule (Haya et al. 2012). It provides salt tolerance via regulating solute potential (osmotic adjustment), thereby enhancing water uptake from the soil. Moreover, soluble sugars also act as an osmoprotectant and play a critical role in plant defense against abiotic stress (Zulfiqar et al. 2020). In our study, the total soluble sugar content was increased in all the four genotypes of pearl millet with higher basal levels in the tolerant genotype, which was in agreement with the previous studies (Shinde et al. 2018). A strong reactive oxygen species (ROS) scavenging antioxidant system dictates the salt tolerance potential of plants. In our study, a significant increase in the activity of catalase (CAT) enzyme was evident in the tolerant genotypes under stress condition, as observed earlier (Jogeswar et al. 2006; Mukhopadhyay et al. 2007). This finding confirms that the tolerant genotypes utilize catalase as a potent antioxidant enzyme for improved scavenging of H_2O_2. Peroxidases (POX) are other enzymes involved in the decomposition of peroxide radicals. Its activity was significantly enhanced under salt stress in the sensitive genotypes, but it was not adequate to overcome the detrimental effect of a large number of ROS generated under high salinity. On the other hand, a non-significant change in POX activity in the tolerant genotypes indicates that this enzyme is not directly involved in protection against oxidative stress, and there might be activated coordination among other antioxidant enzymes for establishing proper ROS homeostasis.

The differential expression of antioxidant genes has been reported in the contrasting genotypes of rice and pearl millet under abiotic stress (Shivhare and Lata 2019; Singh et al. 2018). Interestingly, we did not find any correlation between *CAT* transcript level and enzyme activity. This discrepancy could be explained by complex multi-level regulation of *CAT* gene expression at post-transcriptional, translational, and post-translational levels (Ara et al. 2013; Luna et al. 2005; Ni and Trelease 1991; Palma et al. 2020). For example, the enhanced catalase activity in rice and wheat under salt and drought stress was negatively correlated with its transcript level (Luna et al. 2005; Rossatto et al. 2017). Similar results were reported by Zhang et al. (2014) in seedlings of *Limonium sinense* Kuntze, where catalase enzyme activity decreased after 4 days of salt stress, whereas mRNA level of *LsCAT* showed a significant increase throughout the treatment period. Recently, catalase activity is shown to be modulated by post-translational modifications (Palma et al. 2020). Glutathione reductase (GR) is one of the important enzymes of the ascorbate–glutathione pathway that detoxify the ROS by catalyzing NADPH-dependent reduction of oxidized glutathione. Our results showed no significant change in the *GR* transcript level in all the genotypes under salinity, in contrast to the previous reports (Jogeswar et al. 2006; Mukhopadhyay et al. 2007). This indicates that the impact of salinity on antioxidant system is much complicated and influenced by the salt concentration, treatment time, and genotype. However, we found a profound increase in the transcript levels of two antioxidant enzymes namely, *NR* and *GDH* in the tolerant genotypes, which suggests their major role as ROS scavengers under salt stress. Nitrate reductase catalyzes the reduction of nitrate to nitrite, a rate-limiting step in plant development, whereas GDH is a major component of the ammonium assimilation pathway. Their levels were reported to be regulated in tomato, rice and wheat under salinity (Fariduddin et al. 2013; Guellim et al. 2019; Nguyen et al. 2005). Therefore, it is suggested that these free radical detoxifying enzymes may function in providing tolerance to salinity stress in pearl millet genotypes.

Conclusion

The present study demonstrated the presence of substantial genetic diversity among the 33 genotypes of pearl millet for salinity stress tolerance at the early vegetative stage. Our observations were supported by extensive statistical analyses of morphological parameters such as multivariate cluster analysis, correlation, and PCA. This study favors the use of multiple stress indices to determine the salt tolerance potential of pearl millet genotypes. We have identified two highly tolerant, 18 moderately tolerant, and 13 salt-sensitive genotypes from this study. The selected contrasting genotypes of pearl millet exhibited considerable differences for salinity stress tolerance, as evident from the morphological, physio-biochemical, and gene expression analysis. Our results indicated that the higher content of osmolytes plays a major role in ameliorating the harmful effects of salinity stress in the tolerant genotypes. Thus, osmotic adjustment and efficient scavenging of free radicals can be considered as key mechanisms controlling salt tolerance among pearl millet genotypes, although there is a possibility of involvement of multiple mechanisms, to be investigated in the future. These contrasting genotypes of pearl millet could be utilized for mining novel candidate genes imparting salt tolerance, aiming toward crop improvement through genomics and molecular breeding approaches.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00344-021-10424-0.

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Authors' contribution SJ conceptualized and designed the experiments. JS performed the screening of genotypes, CC performed physio-biochemical analysis and SJ carried out the molecular analysis. SJ analyzed and interpreted the data, and wrote the entire manuscript. OS and RKS critically reviewed and helped in the revision of the manuscript.

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Data Availability Available to scientific community.

Code Availability NA

Declarations

Conflict of interest The authors declare no financial or commercial conflict of interest.

Ethics Approval NA

Consent to Participate NA

Consent for Publication NA

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