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POST ANTHESIS RESPONSES OF KODOMILLET (PASPALUM SCROBICULATUM) GENOTYPES AGAINST SALINITY STRESS

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ABSTRACT

Studies on post anthesis salt stress are very important in order to know the growth, enzyme biomarkers against salt stress. A pot culture experiment was conducted to know the responses of different Kodomillet varieties under salt stress. Kodomillet genotypes IPS 145, IPS 351, IPS 583, IPS 610, IC 426676 and IC 382888 were subjected to 0, 200, 400 and 600 mmol L^{-1} NaCl stress at post anthesis for about six days. Growth characters root length, plant fresh and dry weights were inhibited with increased salt stress. But this reduction was low in case of IPS 583 and IC 426676. Antioxidant enzymes such as POX, PPO and SOD were increased under increased salt stress conditions and this increment was best reported in IPS 583 and IC 426676. Catalase activity was decreased in present study with increased NaCl stress. The data obtained from this investigation have revealed that IPS 583 and IC 426676 were better performers under salt stress. Root growth, plant growth along with POX, PPO and SOD could be considered as the suitable biomarkers to identify the Kodomillet genotypes against NaCl stress.

INTRODUCTION

Sodium chloride stress is an uninvited guest to the agriculture and former community especially in arid and semiarid regions, which can severely limit crop growth and yield (Ashraf and Ali, 2008; Borziuei, 2012). Salinity stress at grain filling stage is highly influenced feature resulting in aborted grains and diminished yields (Epstein *et al.,* 1980). Therefore, the attempts to enhance salt tolerance of crop plants hold noteworthy importance for sustainable agriculture. Formation of reactive oxygen species (ROS) is one of the prominent biochemical changes possibly happened during severe salt stress conditions (Dionisio-Sese and Tobita, 1998). The important intracellular generators of these activated oxygen species are nothing but the chloroplast and mitochondria of plant cells (Hu *et al.,* 2012). The important impacts of ROS on plants are oxidative damage of proteins, lipids, nucleic acids and alteration of normal cellular metabolic activities (Tammam *et al.,* 2008). To ameliorate these negative effects of salinity stress, plants developed a plethora of mechanisms at cellular, tissue, organ, or whole plant level (Zhu, 2003). Plants are equipped with a number of antioxidant enzymes to protect themselves against harmful effects of activated oxygen species produced during osmotic/ionic stresses occurred due to salinity stress (Parida and Das, 2005). Of all the antioxidant enzymes superoxide dismutase (SOD: EC 1.15.1.1) is a major scavenger of O_2^-. The enzymatic activity of SOD results in the formation of H_2O_2 and O_2. Hydrogen peroxide produced during this process was scavenged by enzymes like peroxidase (POX: EC 1.11.1.7), polyphenol oxidase (PPO: EC 1.14.18.1) and catalase (CAT: EC 1.11.1.6) (Apel and Hirt, 2004). Various researches reported that increased activities of these enzymes was closely associated with salt tolerance efficiency of many crop plants (Zeng *et al.,* 2003a; Lehner *et al.,* 2008; Liu *et al.,* 2011).

Kodomillet (*Paspalum scrobiculatum*) is a minor millet and one of the important annual grain crop of India usually grown for its grains having a protein content of 11% and has the potency to grow even under drought conditions but the increased drought conditions due to regular dry spells prevail the saline soils resulted in reduced yields. To identify autochthonous varieties of Kodomillet tolerant to saline stress the understanding of biochemical mechanisms and growth traits will useful as suitable tools and it is inevitable. Therefore the present investigation was taken up to better understand the mechanisms relevant in salt tolerance and to find out the salt tolerant Kodomillet genotypes.

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MATERIALS AND METHODS

A pot experiment was conducted to observe the influence of NaCl concentrations on the activity of antioxidant enzymes and crop growth. The experiment was carried out in the Botanical Garden of Acharya Nagarjuna University. The seeds of six Kodomillet genotypes i.e. IPS 145, IPS 351, IPS 583, IPS 610, IC 426676 and IC 382888 were procured from the ICRISAT and NBPGR, Hyderabad. Before sowing, seeds were surface sterilized with 1% mercuric chloride for 3 min, and washed three times with sterilized distilled water. The forest bags of 30 × 20 cm diameter were filled with 5 kg red loamy soil and vermi compost in 4:1 ratio. After seedling establishment, five seedlings were retained. The pots were maintained under natural lighting and day/night temperature of 36/24^{o}C.

Treatment

At 50 DAS, plants were treated with different concentrations (200, 400 and 600 mmol L^{-1}) of NaCl for a period of 6 days and plants were harvested on the final day of the treatment and preserved further to conduct biochemical parameters and morphological characters.

Growth parameters

On the final day of experiment, three plants from each treatment were randomly selected. Root length and shoot length were measured traditionally. Fresh weights (FW) of roots and shoots were measured. To measure the dry weights of root and shoot samples were oven dried at 80 ^{o}C for about 72 h and then weighed.

Antioxidant Enzyme Assays

Healthy leaves were collected washed with double distilled water 2-3 times and the moisture was blotted gently.

Superoxide dismutase

Enzyme extraction: Leaf samples of 0.5 g were homogenized in ice cold 50 mM potassium phosphate buffer (pH 7.8) with pre-chilled pestle and mortar. Each homogenate was transferred to centrifuge tubes and centrifuged at 4 °C in cooling micro centrifuge (Eppendorf – 5415 R) at 10, 000 rpm. The supernatant was used for enzyme activity assay (Esfandiari *et al.,* 2007) within 12 h of extraction. The SOD activity was estimated by recording the decrease in absorbance of superoxide nitro blue tetrazolium complex by the enzyme (Sen Gupta *et al.,* 1993).

Estimation: A reaction cocktail of 33 ml was prepared by mixing the reagents in the following ratio (60 µl 50 mM Phosphate buffer; 390 µl 13 mM Methionine; 0.6 µl 02 µM Riboflavin; 60 µl 0.1 mM EDTA; 300 µl 75 mM NBT and 50 µl Enzyme extract). A blank was set without enzyme and NBT to calibrate the spectrophotometer. Another control was set having NBT but no enzyme as reference control. All the tubes were exposed to 400 W bulbs (4×100 W bulbs) for 15 min. The absorbance was measured at 560 nm immediately and calculated the percentage inhibition of the reaction between riboflavin and NBT in the presence of methionine which is taken as 1 unit of SOD activity. The enzyme activity was expressed as units/mg of protein. To estimate the activity of catalase, peroxidise and polyphenol oxidase frozen leaf samples were ground to a fine powder with liquid nitrogen and extracted with ice-cold 50 mM phosphate buffer (pH 7.0). The extracts were centrifuged at 4 °C for 30 min at 20000 × g and the supernatants were used as the crude extracts (Manoranjan Kar and Mishra, 1976).

Catalase activity (CAT)

One gram of leaf material was macerated into thin paste using pH 7 Phosphate buffer and the enzyme extract was filtered through muslin cloth. Two milliliter of the enzyme extract taken into 50 ml clear conical flask and to this 1ml of 0.45 molar H_2O_2 was added and the set up was kept for 5 min incubation and enzyme activity was stopped by adding 1ml of 12% H_2SO_4. This extract was titrated against 0.05 N of $KMnO_4$ taken in a burette, appearance of pink color and remains constant for about 30 seconds considered as the end point. The enzyme activity was expressed as enzyme units per gram leaf material. One unit of catalase is defined as that amount of enzyme, which breaks down /µmol/ of H_2O_2 / min.

$$\text{Catalase activity} = \frac{25 \times 0.85}{2} \times \frac{V}{W}$$

Where W= Wt. of material used
V= Vol. of $KMNO_4$ utilized (Blank sample value)

Peroxidase activity (POD)

Reaction mixture was prepared by adding 3 ml of pyrogallol phosphate buffer and 0.1 ml of enzyme extract into a cuvette. To the reaction mixture 0.5 ml of H_2O_2 was added and gently shaken. The absorbance was measured after 3 min at 420 nm. The same procedure was continued to know the control value by using boiled enzyme extract. The enzyme activity was measured by subtracting the absorbance value of the blank from the sample and expressed the enzyme activity as absorbing units per 1 g fresh weight per 3 minutes.

Polyphenol Oxidase (PPO)

The reaction mix was prepared by adding 2 ml of buffer and 1 ml of enzyme extract. The mixture was incubated for 5 min and the reaction was stopped by adding 1 ml of 2.5 N H_2SO_4. Optical density was measured at 420 nm against a blank containing 1 ml of H_2SO_4, 2 ml of buffer 1 ml of pyrogallol and 1 ml of boiled enzyme extract. Enzyme activity was calculated by subtracting the absorbance value of blank from the sample and expresses the enzyme activity as absorbing units per 1 gram fresh weight per 5 min.

Statistical analysis

Analysis of variance was performed using AGRISTAT appropriate for randomized complete block design. The data were presented as the means for each treatment. The simple correlation analysis was measured using MINITAB 14 statistical software.

RESULTS AND DISCUSSION

Analysis of variance revealed significant differences among plant species. The characteristic growth features like root length, fresh and dry weights of plants were found to be varied significantly. The activity of antioxidant enzymes was also varied to a large extent with increased NaCl stress.

Table 1. Reduced growth characters under salinity stress in Kodomillet

NaCl concentration (mmol L^{-1}) →Variety ↓	Root length (g)				Whole plant fresh weight (g)				Whole plant dry weight (g)			
	0	200	400	600	0	200	400	600	0	200	400	600
IPS 145	13.60	9.80	7.40	7.30	6.965	4.860	2.640	1.240	4.060	2.046	1.024	0.500
IPS 351	15.53	15.20	11.27	10.40	6.424	5.322	3.254	2.212	4.077	3.044	1.037	0.622
IPS 583	17.33	17.00*	14.67*	12.33*	6.419	6.409*	5.346*	4.265*	4.139*	4.106*	3.054*	2.051*
IPS 610	14.63	11.37	10.33	7.60	6.423	4.396	2.242	1.185	4.063	2.033	1.025	0.611
IC 426676	21.83*	21.77*	19.27*	16.23*	6.571	6.500*	5.413*	4.339*	4.153*	4.116*	3.075*	2.073*
IC 38288	16.37	15.83	12.30	11.47	6.431	5.364	3.256	2.134	4.125*	3.053	1.046	0.642
Grand Mean	16.54	15.16	12.54	10.88	6.530	5.450	3.690	2.590	4.100	3.060	1.710	1.080
SEm±	1.386	0.941	1.481	0.889	0.678	0.293	0.471	0.239	0.014	0.030	0.005	0.001
CD	4.368	2.964	4.667	2.799	NS	0.922	1.483	0.752	0.044	0.094	0.017	0.003
CV%	14.5	10.8	20.5	14.1	18.0	8.9	22.1	16.1	0.6	1.7	0.5	0.1

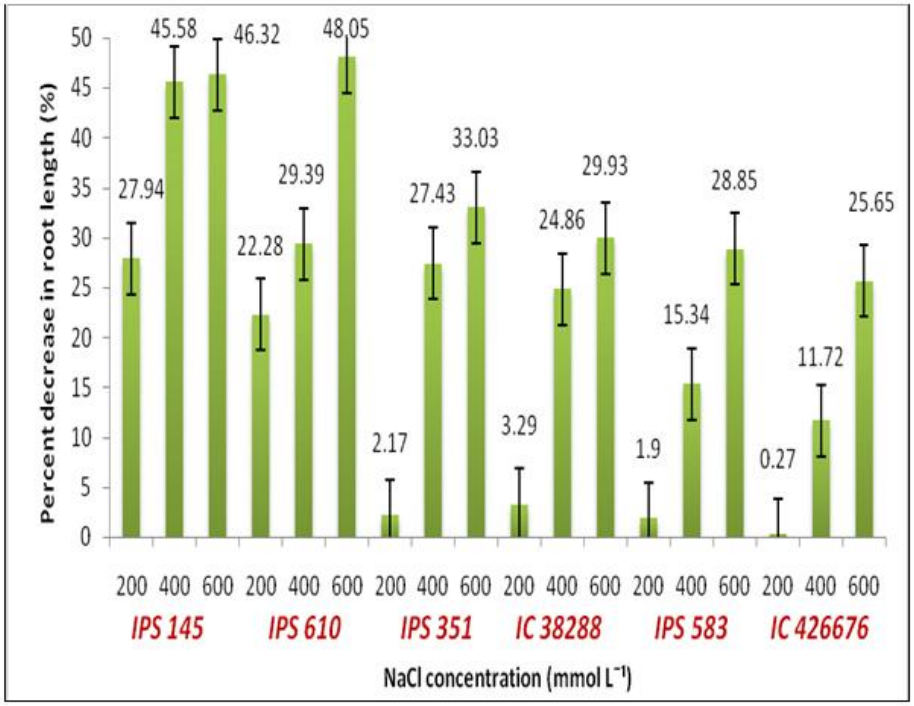

Fig. 1. Percentage decrease in root length against different NaCl concentrations

Root length

Root length varied significantly among all the genotypes both in controls and treatments (Table 1). Among all the treatments i.e. C, 200, 400 and 600 mmol L^{-1} NaCl concentrations high root length was observed in IC 76 (21.83 cm, 21.77 cm, 19.27 cm and 16.23 cm) with less percent reduction in root length 0.27%, 11.72% and 25.65% at 200, 400 and 600 mmol L^{-1} respectively (Fig. 1). Root growth inhibition is a very common phenomenon in response to salt stress and it is considered as the most important agricultural indices of salt stress (Koca *et al.,* 2007).

This decrease in root length is may be due to the altered external water potential, which increased ion toxicity and causing an ion imbalance (Jaleel *et al.,* 2007a). Further this situation brings the biochemical restraints on cell wall expansion, which in turn can inhibit the root growth (Hernandez and Almansa, 2002). But the varieties IC 76 and IPS 583 performed well and showed less decrease in root growth even under increased salinity levels, this peculiarity is may be due to their inherent genetic potentiality in having a mechanism of stopping the excess input of ions into their cells.

Whole plant fresh and dry weights

Crop growth inhibition is a general response to salinity. In the present investigation the reduction in fresh and dry weights was observed (Table 1) among all the millet varieties. Maximum reduction in fresh and dry weights was observed at 600 mmol L^{-1} NaCl concentrations (Fig 2). At this stress level less crop growth reduction was noticed in IPS 583 (2.051 g) and IC 76 (2.073 g) less percent decrease at 600 mmol L^{-1}. This reduction in biomass may be due to toxic effects of high NaCl concentrations and unbalanced nutrient uptake by roots (Hajibagheri *et al.,* 1989; Werener and Finkelstein, 1995; Glyn Bengough *et al.,* 2011).

Catalase activity

The catalase activity was found to be decreased with increased NaCl concentration (Table 2).

Table 2. Responses of different ROS enzymes under increased NaCl stress in Kodomillet

NaCl concentration (mmol L^{-1}) → Variety ↓	Catalase (ug^{-1})				Peroxidase (ug^{-1})				Polyphenol oxidase (ug^{-1})				Superoxide dismutase(umg^{-1})			
	0	200	400	600	0	200	400	600	0	200	400	600	0	200	400	600
IPS 145	5.24	4.22	3.21	1.14	0.17	0.24	0.32	0.64	0.03	0.11	0.22	0.38	14.10	16.25	17.10	18.40
IPS 351	9.58	7.43	5.26	3.28	0.16	0.31	0.46	0.69	0.08	0.30	0.32	0.48	14.25	16.90	21.90*	23.80*
IPS 583	12.31*	8.47*	7.34*	5.29*	0.29*	0.44*	0.56*	0.85*	0.09	0.31	0.47*	0.77*	15.00*	18.10*	22.90*	25.10*
IPS 610	8.49	7.42	4.63	3.21	0.14	0.26	0.46	0.66	0.07	0.13	0.31	0.38	14.10	16.42	19.60	22.10
IC 426676	13.74*	9.59*	8.38*	5.42*	0.32*	0.44*	0.72*	0.85*	0.26	0.46*	0.47*	0.77*	15.30*	18.90*	23.60*	26.25*
IC 38288	11.10*	7.44	6.04	4.19*	0.16	0.41*	0.55*	0.69	0.08	0.30	0.47*	0.74*	14.25	17.20	22.30*	24.0*
Mean	10.07	7.42	5.81	3.75	0.20	0.35	0.51	0.73	0.10	0.26	0.37	0.58	14.50	17.29	21.23	23.27
SEm±	0.024	0.193	0.464	0.009	0.011	0.007	0.009	0.013	0.14	0.011	0.014	0.016	0.069	0.108	0.082	0.270
CD	0.076	0.608	1.462	0.030	0.036	0.022	0.028	0.041	NS	0.036	0.045	0.050	0.219	0.342	0.257	0.851
CV%	0.4	4.5	13.8	0.4	21.2	4.6	71.15	3.8	92.8	5.6	4.9	3.7	0.8	1.1	0.7	2.0

* Significant at 5% level

Table 3. Association analysis between different parameters against various NaCl concentrations in Kodomillet

Characters → ↓	NaCl Concentration (mmol L^{-1})	Root length (cm)	Whole plant fresh weight (g)	Whole plant dry weight (g)	Catalase (ug^{-1})	Peroxidase (ug^{-1})	Polyphenol oxidase (ug^{-1})
Whole plant fresh weight (g)	0	0.799*					
	200	0.913**					
	400	0.967**					
	600	0.944**					
Whole plant dry weight (g)	0	0.895**	0.934**				
	200	0.898**	0.926**				
	400	0.920**	0.818**				
	600	0.965**	0.780**				
Catalase (ug^{-1})	0	0.230	0.329	0.212			
	200	0.368	0.394	0.365			
	400	0.427	0.401	0.378			
	600	0.453	0.421	0.460			
Peroxidase (ug^{-1})	0	0.851*	0.668*	0.871**	0.264		
	200	0.928**	0.757**	0.883**	0.364		
	400	0.943**	0.913**	0.890**	0.467		
	600	0.983**	0.969**	0.968**	0.980		
Polyphenol oxidase (ug^{-1})	0	0.562*	0.540*	0.543*	0.239	0.718**	
	200	0.619*	0.758**	0.548*	0.319	0.806**	
	400	0.888**	0.833**	0.874**	0.389	0.816**	
	600	0.986**	0.962**	0.962**	0.458	0.914**	
Superoxide dismutase (umg^{-1})	0	0.507*	0.864**	0.634*	0.333	0.486	0.562*
	200	0.826**	0.865**	0.739**	0.436	0.655*	0.681*
	400	0.895**	0.940**	0.787**	0.485	0.844**	0.814**
	600	0.928**	0.962**	0.808**	0.564*	0.871**	0.870**

* Significant at 5% level; ** Significant at 1% level

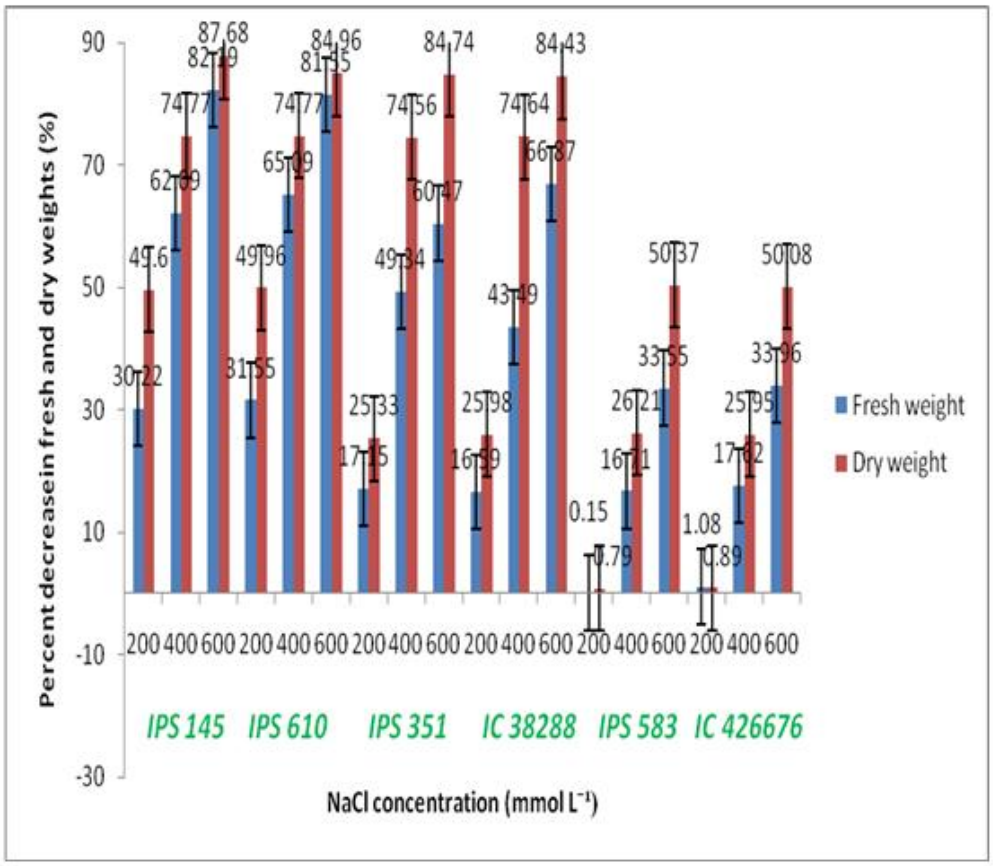

Fig. 2. Variations in fresh and dry weights at different salt treatments

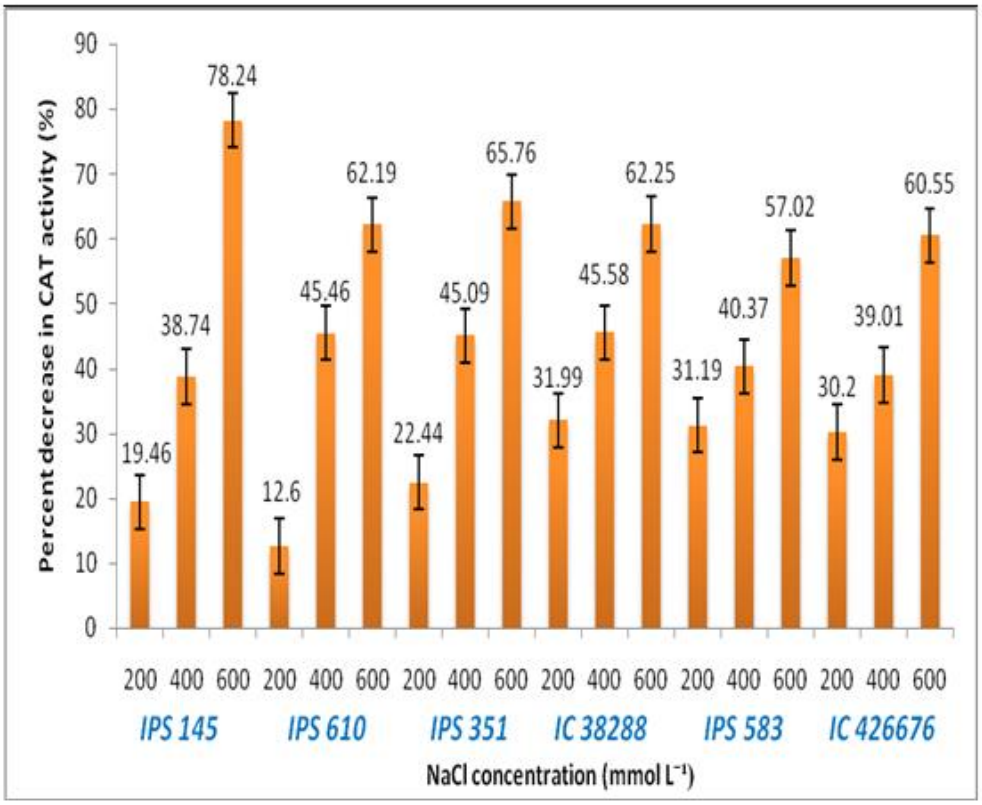

Fig. 3. Percent decrease in CAT activity with increased NaCl stress

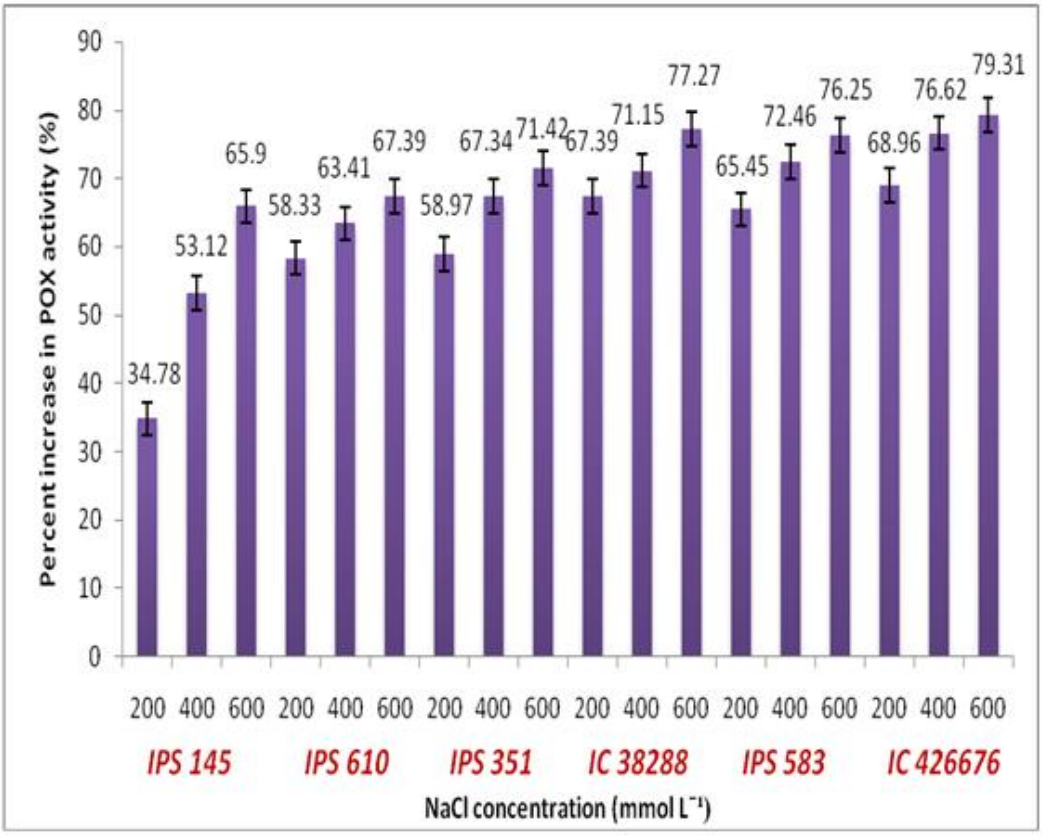

Fig. 4. Percent hike in POX activity at 200 > 400 > 600 NaCl stress

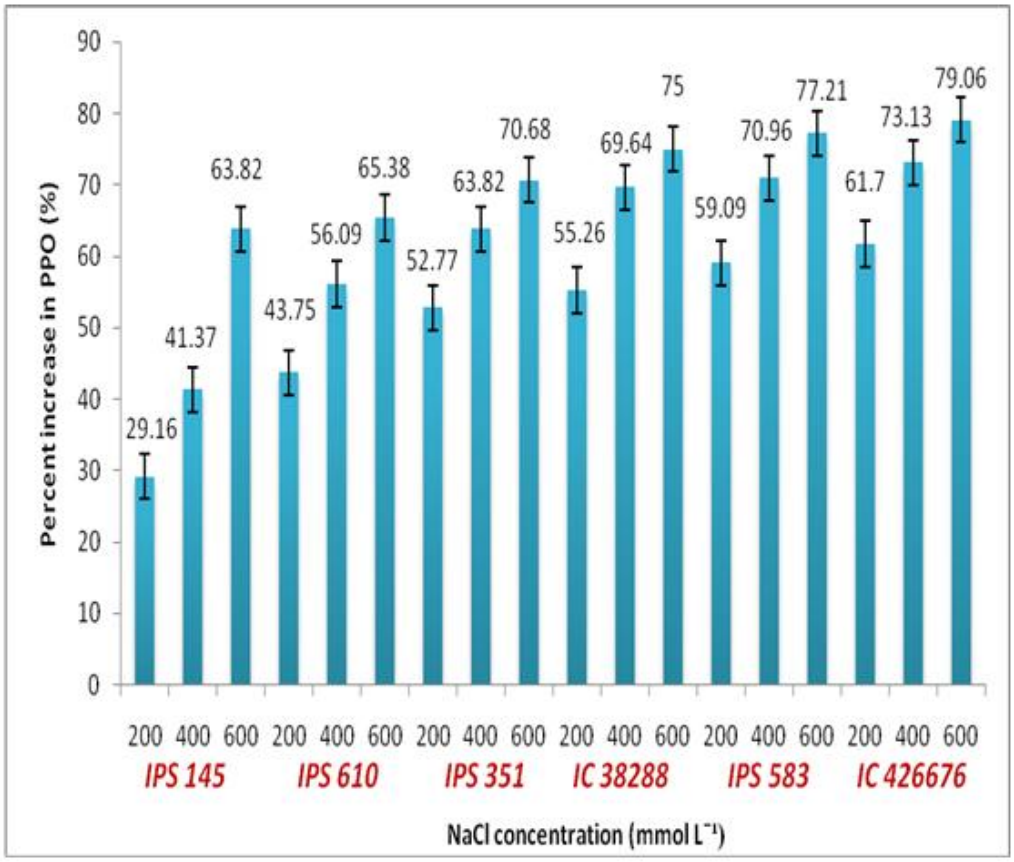

Fig. 5. Percent increase in PPO activity under different salt stress treatment

IC 76 was recorded maximum activity of CAT and less percent decrease even under increased salt stress (5.42 at 600 mmol L^{-1}) (Table 2; Fig. 3). The decreased CAT activity is may be due to increased peroxidases activity which has a protective role in cell membrane strengthening (Velikova *et al.,* 2000). The decrease in catalase activity with increase in salt stress was noticed in all the cultivars and in all treatment levels especially in the varieties IPS 145 and IPS 610. Similar observations were also made by Jaleel *et al.* (2007a) in *rosy periwinkle* and Anitha and Usha (2012) in soyabean.

Peroxidase activity

The increased peroxidase activity in response to NaCl stress has been reported in all the genotypes (Table 2). Increased peroxidase activity will change the mechanical properties of cell wall, which in turn could be related to the salt adaptation process (Sancho *et al.,* 1996; Harinasut *et al.*, 2003). In present investigation also increased activity of peroxidase was reported in IC 76 in all the treatments i.e. 0 mmol L^{-1} – 0.26; 200 mmol L^{-1} – 0.46; 400 mmol L^{-1} – 0.47; 600 mmol L^{-1} – 0.77 and percent increase in POX activity (Fig. 4).

substances like phenolic compounds which are accumulated during NaCl stress (Weisany *et al.,* 2012).

Superoxide dismutase

The genotype IC 76 recorded a little bit more increased activity i.e. 15.30, 18.90, 23.60, 26.25 at 0, 200, 400 and 600 mmol L^{-1} stress level respectively (Table 2) and also noticed maximum percent increase in SOD activity in all the stress levels (Fig. 6). Superoxide dismutase has the ability to repair oxidation damage caused due to the ROS by regulating O_2- and H_2O_2 and decreases the formation of $OH^{\bullet}$ radicle and hence it can reduce the alarming risk to the cells and can be considered as key enzyme to combat salt stress (Agarwal *et al.,* 2005; Ashraf and Ali, 2008).

Correlation studies

Correlation studies were performed to know the association between different parameters under different NaCl stress levels and represented in(Table 3). The results of association analysis have revealed that increased NaCl stress promotes the

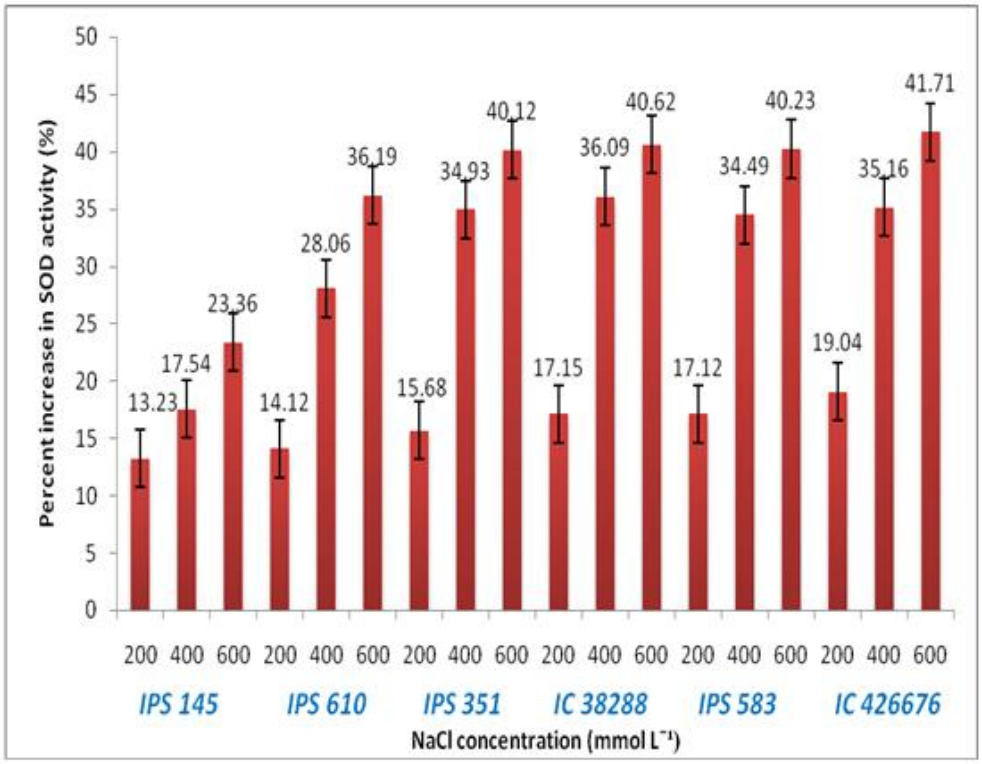

Fig. 6. Percent enhance in SOD at different salt stress levels

Polyphenol oxidase

The polyphenol oxidase activity was also increased in leaf tissues with increased salinity stress up to 600 mmol L^{-1} (Table 2). The varieties IPS 583 and IC 76 showed their inherent potentiality of having high PPO activity even under non-stress conditions (0.29, 0.32) and the same tendency was maintained further in 200 mmol L^{-1} (0.44,0.44); 400 mmol L^{-1} (0.56, 0.72), 600 mmol L^{-1} (0.85, 0.85) high percent increase in PPO activity (Fig 5). Increased PPO activity under saline conditions shows the ability to oxidize and degrade the toxic

more positive association between antioxidant enzymes and growth. Enhanced ROS enzymes had a positive association with root length, plant fresh and dry weights in the present study which is in agreement with Esfandiari *et al.* (2007) in wheat; Noreen and Ashraf, (2009) in pea.

Conclusion

Considering results obtained on activity of ROS enzymes and growth characters under salinity stress, it is clear that the varieties IPS 583 and IC 426676 are more tolerant to NaCl

stress. On the other hand root length, fresh weight, dry weight and ROS enzymes could be use as good biomarkers in screening of salt tolerant varieties.

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