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EVALUATION OF SALT TOLERANT MUNGBEAN (*Vigna radiata* L.) GENOTYPES ON GROWTH THROUGH BIO-MOLECULAR APPROACHES

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Abstract

This study was conducted to obtain saline tolerant mungbean genotypes through evaluating growth, biochemical and molecular parameters, and possible salt tolerant mechanisms were studied in different salt sensitive genotypes. Thirteen prescreened mungbean genotypes were grown on 0, 40, 80 and 120 mM NaCl induced salinity and evaluated by germination percentage, shoot and root length, superoxide ($O_2^{\bullet-}$) generation rate, concentration of H_2O_2, lipid peroxidation (as malondialdehyde, MDA), methylglyoxal (MG), K^+/Na^+ and proline content in leaves. Based on these parameters, genotypes BD-10588, BD-6894 and IR-01 were selected as tolerant genotypes. For studying oxidative stress tolerance mechanism, BD-10588 and IR-01 were used as tolerant and BD-6887 and BD-10741 as susceptible genotypes, and comparative ROS ($O_2^{\bullet-}$ and H_2O_2), and MDA as well as LOX activity between the two groups were determined. Analysis of activities of ROS metabolizing antioxidant enzymes strongly suggested that superoxide dismutase in tolerant genotypes provided first line protection from salt induced $O_2^{\bullet-}$. Higher catalase (CAT) and ascorbate peroxidase (APX) played major role in H_2O_2 metabolism in tolerant genotypes. Both specific and in-gel activities of glutathione peroxidase (GPX) strongly proved the H_2O_2 metabolism for reducing oxidative damage in both tolerant and susceptible genotypes. However, higher peroxidase activity was important for mitigating salt stress in susceptible mungbean genotypes. Therefore, SOD, APX and GPX are very important for protecting salt mediated oxidative damage in mungbean genotype.

Keywords: Salinity, bio-molecular approach, mungbean, oxidative stress

Introduction

The productivity of mungbean has remained low due to susceptibility to various biotic (Mungbean yellow mosaic virus, powdery mildew and Cercospora leaf spot) and abiotic (salinity, drought, temperature, and water-logging) stresses at different growth stages. Salinity stress is a serious problem in arid and semi-arid tropics and in the Indo-Gangetic plains in irrigated areas and also planting areas of mungbean where it is especially grown after Aman rice crop in the coastal

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regions. Mungbean is a short duration moderate saline tolerant crop where 50 mM NaCl can cause yield losses ≥70% (Saha *et al*., 2010; Gosh *et al*., 2015). However, salt tolerant mungbean variety is still limited. Though significant knowledge has been achieved for saline tolerance mechanism, it is not fully understood. Therefore, efficient selection criteria for salt tolerant genotypes of crops including mungbean are essential for obtaining salt tolerant genotypes.

In Bangladesh, salinity is the most important constraints for crop production in coastal zone throughout the year. The coastline of Bangladesh is 710 km long with pH ranges 6.0-8.4 which is composed of the interface of various ecological and economic systems, including mangroves, tidal flat (Ahmad, 2019; Haque, 2006). Salinity problem has been increasing in Bangladesh, and over the last 35 years, salinity has increased around 26 percent in the coastal region of Bangladesh (Mahmuduzzaman *et al*., 2014). Therefore, for sustainable crop production in coastal region, saline tolerant crop species and crop varieties are urgent issues.

Salinity stress is the most devastating stress, and increasing soil salinity in coastal regions has focused attention on the possibility of crop damage in fields located in sea regions worldwide. The problem due to salinity will affect the production of agricultural crops in the upcoming years, particularly in arid and semiarid regions (IPCC, 2014). This situation is a great threat to ensure food security for densely populated countries like Bangladesh where more than one million hectare (ha) of land remain fallow in coastal area, because crops that have a higher yield but have lower adaptability to salinity will need to be replaced by crops that have higher adaptive potential but are likely to have a lower yield. As a result, there is an urgent need to develop highly adaptive crops that also have a higher yield under salinity. Salinity affects plant growth and development in two ways. First, it imposes osmotic stress by reducing the soil water potential, leading to limited water uptake. Second, it causes excessive uptake of ions, particularly Na^+ and Cl^-, that ultimately interfere with various metabolic processes. Plant responses to the osmotic and ionic components of salt stress are complicated and involve many gene networks and metabolic processes (Hasegawa *et al*., 2000; Munns and Tester, 2008). Such responses depend mainly on the inherent salt tolerance of the plant, the severity of salt stress (the concentration of salt in the soil solution), and the duration of the plant roots' exposure to the salt. Salinity tolerance is a complex trait, and plant breeders' efforts to produce crops with higher yields have largely been unsuccessful because of mutagenic adaptive responses to these traits. Moreover, a significant number of physiological activities are involved in the tolerance process.

Salinity causes negative consequences for gas exchange, resulting in low CO_2 assimilation for photosynthesis and consequently a significant reduction in electron transportation. As a result, reactive oxygen species (ROS) are generated, such as singlet oxygen ($^1O_2$), superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2),

perhydroxy radicals ($HO_2^{\bullet}$), and alkoxy radicals ($RO^{\bullet}$) (Gill and Tuteja, 2010; Moller *et al*., 2007). Normally, generation of ROS is balanced with scavenging by various antioxidants (Foyer and Noctor, 2005). This balance between the generation and scavenging of ROS is broken down by various biotic and abiotic stresses, including salinity drought. At higher concentrations, ROS are highly reactive and cause damage to proteins, DNA, lipids, and carbohydrates, resulting in cell death (Rohman *et al*., 2019). As a result, accumulation of ROS under environmental stresses is the foremost reason for reduced productivity of crops (Mittler, 2002; Apel and Hirt, 2004; Mahajan and Tuteja, 2005). On the other hand, methylglyoxal (MG) is a potentially cytotoxic compound, which can react with and modify other molecules, including DNA and proteins (Yadav *et al*., 2005a). Therefore, both ROS and MG must be removed or detoxified or kept below toxic level for cellular survival. Therefore, ROS and MG can be important criteria for selecting saline tolerant genotypes of crops (Yadav *et al*., 2005a, b; Singla-Pareek *et al*., 2008; Azooz *et al*., 2009). ROS cause oxidation of polyunsaturated fatty acid (PUFA) to produce important stress marker melondialdehyde (MDA) for cell wall leakage. Moreover, imbalance between K^+ and Na^+ ions under salinity can be an important indicator. Therefore, these parameters were used as selection criteria for obtaining tolerant mungbean genotypes.

Overproduction of ROS under abiotic stress including salinity causes oxidative damage in plant cell (Gill and Tugeta, 2010; Rohman et al., 2019). Plants possess ROS metabolism system through enzymatic and nonenzymatic antioxidants for cellular protection from oxidative damage. Among the enzymatic antioxidant, superoxide dismutase (SOD), peroxidase (POD) catalase (CAT), ascorbate peroxidase (APX) and glutathione peroxidases (GPX) are major ROS scavengers (Apel and Hirt, 2004; Gill and Tujeta, 2010). SOD provides first line protection from $O_2^{\bullet-}$ and produce H_2O_2 which is further metabolized by POD, CAT, APX and GPX (Gill and Tujeta, 2010). Considering all, we designed the study to obtain salt tolerant genotypes as well as to study oxidative stress tolerance in mungbean seedlings. In this study, we screened some preselected mung bean genotypes at seedling stage by evaluating growth parameters and content of ROS, MDA, MG and K^+/Na^+ as selecting criteria to obtain tolerant mungbean genotypes. Later, the possible oxidative damage in susceptible genotypes were studied.

Materials and Methods

Evaluation of mungbean genotypes through germination and growth parameters

For screening of mungbean accessions, the laboratory experiment was conducted in the Molecular Biology lab, PGRC, BARI, Gazipur. In this study 13 germplasm preselected from 91 were used. The laboratory experiment was made to screen

best performing accessions against salinity tolerance of mungbean at germination and seedling growth stage under room temperature using procedures followed by Taffouo *et al*. (2009). In order to investigate the response of mungbean accessions to different concentrations of NaCl solution (0, 50, 80 and 120 mM NaCl) were used. Glass petridishes (10 cm diameter) were thoroughly washed and sterilized in hot air oven. After sterilization, petridishes were lined with Whatman No.1 filter paper. Ten ml of distilled water as a control and salt solutions [50, 80 and 120 mM NaCl (Laboratory grade)] were added in to separate petridishes. Ten uniform seeds of each mungbean accession were placed in each Petri dish (five petridishes per genotypes per treatment) at uniform distances. Each petridish was filled with 10 ml of the respective treatment solution of NaCl on alternate days. The petridishes were put in a hood to avoid the loss of moisture through evaporation. Germination was started after three days of sowing, and a seed was considered to have germinated when the lengths of the emerging plumule and radicle were about 0.5 cm. Seeds were checked for germination every other day and the germination count was continued for 10 days. After 10 days, growth parameters like germination percentage, seedling shoot and root length were measured.

Determination of proline

Proline was measured according to Bates *et al.* (1973) based on proline's reaction with ninhydrin.

Evaluation of mungbean genotypes through biochemical parameters

Different biochemical parameters as selection criteria were measured at Molecular Breeding Lab, Plant Breeding Division, BARI. Thirteen accessions screened from germination test were grown in petridishes and one-week old seedlings were subjected to NaCl induced salinity of 0, 40 and 80 mM for one week and data were taken on different parameters from leaves. For comparative oxidative stress, two relative tolerant genotypes (BD-10588 and IR-01) and two susceptible genotypes (BD-6887 and BD-10741) were grown again and after germination, salinity of 0 and 80 mM were imposed for one week. After one week, data were taken from leaves.

Measurement of the $O_2^{\bullet-}$ generation rate

Superoxide radical was determined in 3rd leaf according to the method described in Rohman *et al*. (2016a). Leaves (0.3 g) were homogenized in 3 ml of 65 mmol phosphate buffer (pH 7.8) on an ice bath and were then centrifuged at 4°C and 5,000 × g for 10 min. The supernatants (0.75 ml) were mixed with 0.675 ml of 65 mM K-phosphate buffer (pH 7.8) and 0.07 ml of 10 mM hydroxylamine chlorhydrate and were placed at 25°C. After 20 min, 0.375 ml of 17 mM

sulfanilamide and 0.375 ml of 7 mM α-naphthylamine were added, and the mixture was placed at 25°C for another 20 min before it was mixed with 2.25 ml of ether. The absorbance was measured at 530 nm and the $O_2^{\bullet-}$ concentration was calculated from a standard curve of $NaNO_2$.

Measurement of H_2O_2

H_2O_2 was assayed according to the method described by Yu *et al*. (2003). Third leaf tissue (0.5 g) was homogenized in 3 ml of 50 mM K-P buffer (pH 6.5) at 4°C. The homogenate was centrifuged at 11,500×g for 15 min. The supernatant (3 ml) was mixed with 1 ml of 0.1% $TiCl_4$ in 20% H_2SO_4 (v/v), and the mixture was then centrifuged at 11,500×*g* for 15 min at room temperature. The optical absorption of the supernatant was measured spectrophotometrically at 410 nm to determine the H_2O_2 content (ϵ= 0.28 μM^{-1} cm^{-1}) and expressed as micromoles per gram FW.

Measurement of MDA

The level of lipid peroxidation was measured by estimating melondialdehyde (MDA), a decomposition product of the peroxidized polyunsaturated fatty acid component of the membrane lipid, using thiobarbituric acid (TBA) as the reactive material following the method of Heath and Packer (1968). Briefly, leaf tissue (0.5 g) was homogenized in 3 ml 5% (w/v) trichloroacetic acid (TCA), and the homogenate was centrifuged at 11,500×g for 10 min. The supernatant (1 ml) was mixed with 4 ml of TBA reagent (0.5% of TBA in 20% TCA). The reaction mixture was heated at 95°C for 30 min in a water bath and then quickly cooled in an ice bath and centrifuged at 11,500×*g* for 15 min. The absorbance of the colored supernatant was measured at 532 nm and was corrected for non-specific absorbance at 600 nm. The concentration of MDA was calculated by using the extinction coefficient of 155 mM^{-1} cm^{-1} and expressed as nanomole of MDA per gram FW.

Measurement of K^+/Na^+: The sap was extracted from leaves and was put on compact Na^+ ion meter (Horiba-731, Japan) and compact K^+ ion meter (Horiba-722, Japan) to estimate the Na^+ and K^+ ions in leaves. The K^+/Na^+ ratio was measured from the estimated values.

Extraction of soluble protein for activity assay

Fresh leaf (0.5g) was homogenized in 1 ml of 50 mM ice-cold K-P buffer (pH 7.0) by mortar and pestle containing 100 mM KCl, 1 mM ascorbate, 5 mM β-mercaptoethanol and 10 % (w/v) glycerol. The homogenates were centrifuged at 11,500×*g* for 10 min and the supernatants were used for determination of enzyme activities. All procedures were performed below 4 °C.

Determination of protein

The protein concentration in the leaf extracts was determined according to the method of Bradford (1976) using BSA as a protein standard.

Assay activities of enzymes

Lipoxygenase (LOX, EC: 1.13.11.12): LOX activity was measured following Doderer *et al.* (1992). For the measurement of activity, the substrate solution was prepared by adding 35µl linoleic acid to 5 ml distilled water containing 50 µl Tween-20. LOX activity was determined spectrophotometrically by adding 10 µl of sample to 590 µl substrate solution. The increase in absorbance at 234 nm was measured for 1 min at 25°C. The activity was expressed as µM hydroperoxide formed min^{-1} mg^{-1} protein using a molar extinction coefficient of 25,000 M^{-1} cm^{-1}.

SOD (EC: 1.15.1.1): To determine SOD activity of whole cell homogenate, the reaction was prepared on ice in 50 mM potassium phosphate buffer (p^H 7.8, with 1.34 mM diethylenetriaminepentaacetic acid) and an indirect competitive inhibition assay was used for the measurement (Spitz, and Oberley, 1989). This assay is based on the competition between SOD and an indicator molecule nitroblue tetrazolium (NBT), for superoxide production from xanthine and xanthine oxidase. One unit of activity was defined as the amount of protein required to inhibit NBT reduction by 50%.

POD (EC: 1.11.1.7): POD activity was estimated according to Hemeda and Klein (1990). The reaction mixture contained 25 mM K-P buffer (pH 7.0), 0.05% guaiacol, 10 mM H_2O_2 and the protein solution). Activity was determined by the increase in absorbance at 470 nm due to guaiacol oxidation for 1 min using extinction coefficient of 26.6 mM^{-1} cm^{-1}.

APX (EC: 1.11.1.11): APX activity was assayed following the method of Nakano and Asada (1981). The reaction solution contained 50 mM K-P buffer (pH 7.0), 0.50 mM ascorbic acid (ASA), 0.10 mM H_2O_2, 0.1 mM EDTA, and enzyme extract in a final volume of 0.7 ml. The reaction was started by the addition of H_2O_2, and the activity was measured by observing the decrease in absorbance at 290 nm for 1 min using an extinction coefficient of 2.8 mM^{-1} cm^{-1}.

GPX (EC: 1.11.1.9): GPX activity was measured as described by Elia *et al.* (2003) using H_2O_2 as a substrate. The reaction mixture consisted of 100 mM sodium-phosphate buffer (pH 7.5), 1 mM EDTA, 1 mM NaN_3, 0.12 mM NADPH, 2 mM GSH, 1 unit glutathione reductase (GR), 0.6 mM H_2O_2, and 20 µl of sample solution. The reaction was stated by the addition of H_2O_2. The oxidation of NADPH was recorded at 340 nm for 1 min, and the activity was calculated using the extinction coefficient of 6.62 mM^{-1} cm^{-1}.

CAT (EC: 1.11.1.6): CAT activity was measured according to the method of Csiszár *et al*. (2007) by monitoring the decrease of absorbance at 240 nm for 1 min caused by the decomposition (or degradation) of H_2O_2. The reaction mixture contained 50 mM K-P buffer (pH 7.0), 15 mM H_2O_2, and enzyme solution in a final volume of 0.7 ml. The reaction was initiated with the addition of enzyme extract, and the activity was calculated using the extinction coefficient of 39.4 M^{-1} cm^{-1}.

SDS-PAGE and Native In-gel activity staining

Changes in proteins having isoenzymic activity of the ROS scavenging enzymes were studied using PAGE under non-reduced, non-denatured conditions at 4^0C according to Laemmli (1970). Native PAGE analysis was performed for SOD 10% PAGE for other enzymes 8% were done. SOD gel activity was assayed following Beauchamp and Fridovich (1971). After completion of electrophoresis, the gel was incubated in a solution containing 2.45 mmol NBT for 20 min, followed by incubation in 50 mmol potassium phosphate buffer (pH 7.8) containing 28μmol riboflavin and 28 mmol TEMED under dark condition. SOD expression was observed after light exposure for 10 to 20 min at room temperature.

APX isozymes were separated by native PAGE (gels containing 10% glycerol) for 4.5 h at 4°C at a constant current of 35 mA per gel. Ascorbate (2 mM) was included in the carrier buffer. The gels were pre-run for 30 min to allow ascorbate present in the carrier buffer to enter the gel prior to the application of samples, according to the method of Rao *et al*. (1996). Following electrophoretic separation, gels were equilibrated with 50 mM potassium phosphate buffer (pH 7.0) containing 2 mM ascorbate for 30 min. The gels were incubated in the above buffer containing 4 mM ascorbate and 2 mM H_2O_2, for 20 min. After a brief wash in the buffer for 1 min, the gels were submerged in a solution of 50 mM potassium phosphate buffer (pH 7.8) containing 28 mM TEMED and 2.45 mM nitroblue tetrazolium, and subjected to gentle agitation. The reaction was continued for 10 to 15 min and stopped by a brief wash in water. The gel was exposed to light to visible the APX bands. In-gel activity of GPX were visualized using 2mM o-Dianisidine. The gel washed for 15 min in 2.5% Triton-X-100 following wash in distilled water for 15 minutes. The gel was immersed in 10 mM K-P buffer (pH 7.2) containing 2mM o-Dianisidine for 1 hour following immerse in 10 mM H_2O_2 to appear band.

Statistical Analysis:

All data obtained was analyzed by STATISTIX 10 program following complete randomized design (CRD) with at least three replications. The mean differences among the treatments were compared by least significant (LSD) tests. P value of ≤ 0.05 was considered to be significant.

Results

Effect of salinity on germination percentage of mungbean genotypes

The effects of different concentrations of NaCl solution on germination of mungbean seeds are shown in Table 1. The results showed germination percentage varied among the accessions, and different salinity levels. Increase in NaCl concentration reduced germination percentage. At 120 mM of NaCl only BD-10588, BD-6894, BD-10733, IR-01 showed few germinations. At 80 mM salinity, BD-6894, BD-10588, BD-10733 and IR-01 showed 80% germination; whereas, BD-6887, BD-10589 and BD-10741 had 50% germination.

Table 1. Germination percentage and germination rate of selected mungbean genotypes against different salinity levels. Different letters within a column are significant at P≤0.05. Mean separation was not performed for at 120 mM salinity

Genotypes	Germination percentage			
	Control	40 mM	80 mM	120 mM
BD-6887	80c	70c	50d	0
BD-6894	100a	100a	80a	5
BD-10588	98a	90ab	80a	10
BD-10589	100a	100a	50d	0
BD-10590	100a	100a	70b	0
BD-10733	90b	90ab	80a	2
BD-10740	90b	80bc	52cd	0
BD-10741	100a	80bc	50d	0
BD-10744	100a	100a	70b	0
IR-01	100a	100a	80a	6
IR-03	100a	100a	70b	0
IR-04	100a	100a	60c	0
IR-05	100a	100a	60c	0

Effect of salinity on seedling shoot and root length of mungbean genotypes

All accessions responded differently to salt stress for shoot and root length. Increment of salinity level reduced seedling shoot and root length in all accessions (Table 2). At 80 mM salinity, IR-01 showed the highest shoot length (6.25 cm) followed by BD- 10588 (6.16 cm) and BD-6894 (6.07 cm) while BD-10740 had the lowest shoot length (3.56 cm). In case of root length, under 80 mM salinity, BD-6894 had the highest root length (2.03 cm) followed by BD-

10588 (1.96 cm) and IR-01 (1.51 cm) while BD-10744 had the lowest root length (0.46 cm).

Table 2. Shoot and root length of selected mungbean genotypes against different salinity levels. Different letters within a column are significant at P≤0.05.

Genotypes	Root length (cm)			Shoot length (cm)		
	Control	40 mM	80 mM	Control	40 mM	80 mM
BD-6887	13.97de	9.86de	4.81cd	4.16c	1.83d-f	1.27bc
BD-6894	17.83ab	12.43bc	6.07ab	6.17a	2.33bc	2.03a
BD-10588	15.62b-d	13.05b	6.17ab	2.76e	2.08b-d	1.96a
BD-10589	16.76a-c	15.57a	5.20bc	3.63d	3.16a	0.83de
BD-10590	14.60c-e	12.28bc	5.83ab	2.76e	2.06b-d	1.49b
BD-10733	17.37a-c	13.67b	4.16de	2.96e	2.5b	0.66ef
BD-10740	15.76b-d	7.12g	3.56e	3.40d	1.23g	1.02cd
BD-10741	13.83de	7.83fg	4.17de	2.76e	2.12b-d	1.04cd
BD-10744	17.56ab	9.26d-f	4.83cd	4.6b	1.46e-g	0.46f
IR-01	18.96a	15.22a	6.25a	2.16f	1.93cd	1.51b
IR-03	12.56e	11.13b-d	4.47cde	2.93e	2.50b	1.36b
IR-04	12.36e	8.68e-g	4.67cd	1.66g	1.43fg	0.77d-f
IR-05	13.06de	10.63c-e	4.25c-e	4.42bc	1.90c-e	1.20bc
Mean	15.40	11.29	4.95	3.41	2.04	1.20
CV (%)	5.41	4.04	3.54	3.52	4.91	3.05

The mean seedling root length was 3.41 cm in control, 2.19 cm in 40 mM NaCl and 1.09 cm was in 80 mM NaCl. In this study, with the increment of salt concentration, seedling shoot and root length gradually decreased in all accessions of mungbean, but the effect of salinity varied among genotypes. The present study is in good agreement with Shitole and Dhumal (2012) who reported that shoot and root length in mungbean seedlings reduced with increasing NaCl concentration.

Biochemical changes in leaves of munbean genotypes

Salinity stress causes morphological, physiological, biochemical and molecular changes in cell which causes functional loss of cell organelles and finally cell death (Gill and Tujeta, 2010). Salinity stress increased the $O_2^{\bullet-}$ generation rate in leaves of all the mungbean genotypes (Fig. 1). At 80 mM salinity, BD10741 produced the highest $O_2^{\bullet-}$ followed by BD10590 and IR-04 and being statistically similar to each other. Importantly, BD-10588 produced the lowest $O_2^{\bullet-}$ followed by IR-01 and BD-6894.

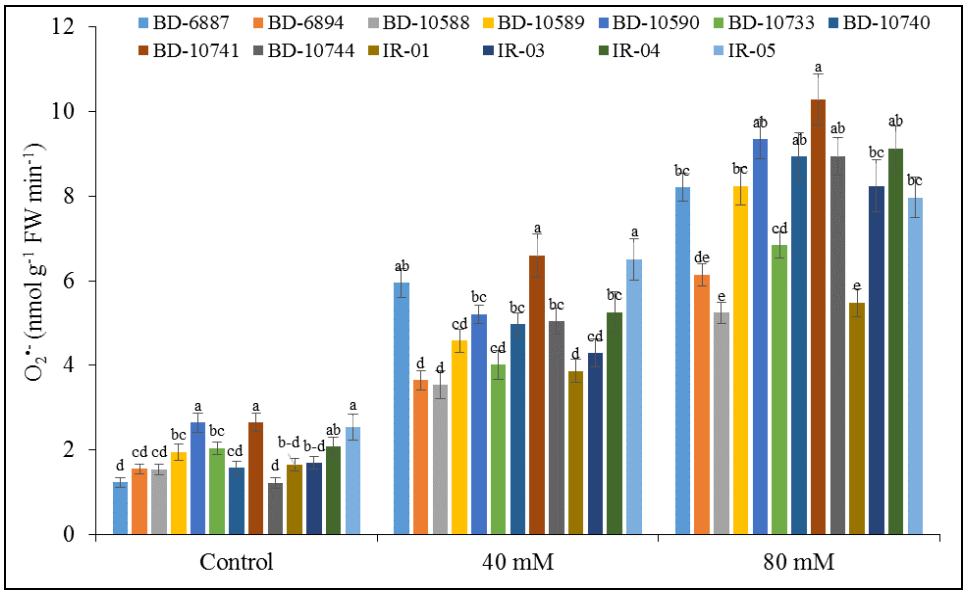

Fig.1. Comparative $O_2^{\bullet-}$ content in leaves of different among mungbean genotypes under salinity. Different letters above the bars within a treatment are significant at P≤0.05. Vertical bar represents Mean ± SE.

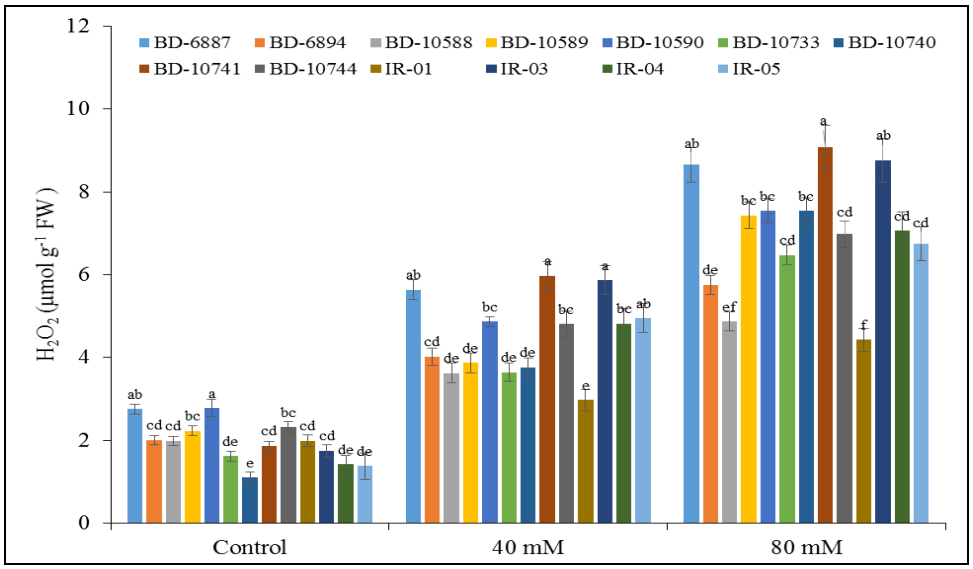

Fig. 2. Comparative H_2O_2 content in leaves of different among mungbean genotypes under salinity. Different letters above the bars within a treatment are significant at P≤0.05. Vertical bar represents Mean ± SE.

Like $O_2^{\bullet-}$ generation, H_2O_2 was also enormously increased with increasing NaCl concentration (Fig. 2). Here, also significant variation was observed among

different genotypes. At 80 mM salinity, BD10741 produced the highest H_2O_2 followed by BD-6887 and IR-03. Importantly, IR-01 produced the lowest H_2O_2 followed by BD-10588 and BD-6894.

Malondialdehyde is a lipid peroxidation product of PUFA of cell wall. In this study, MDA content increased significantly with increasing salinity, and the content varied among different genotypes (Fig. 3). Higher content of MDA in BD-10741, BD-6887 and IR-03 at 80 mM salinity indicated their susceptibility to salinity. On the other hand, lower MDA in BD-10588, BD-6894 and IR-01 suggested their better cell membrane integrity.

Methyl glyoxal (MG) level significantly increased in leaves of all mungbean genotypes under salinity stress (Fig. 4). At 80 mM salinity, IR-03 produced the highest MG followed by BD-10741, IR-05 and BD-6887. Importantly, BD-10588, BD-6894, BD-10589 and IR01 produced lower MG.

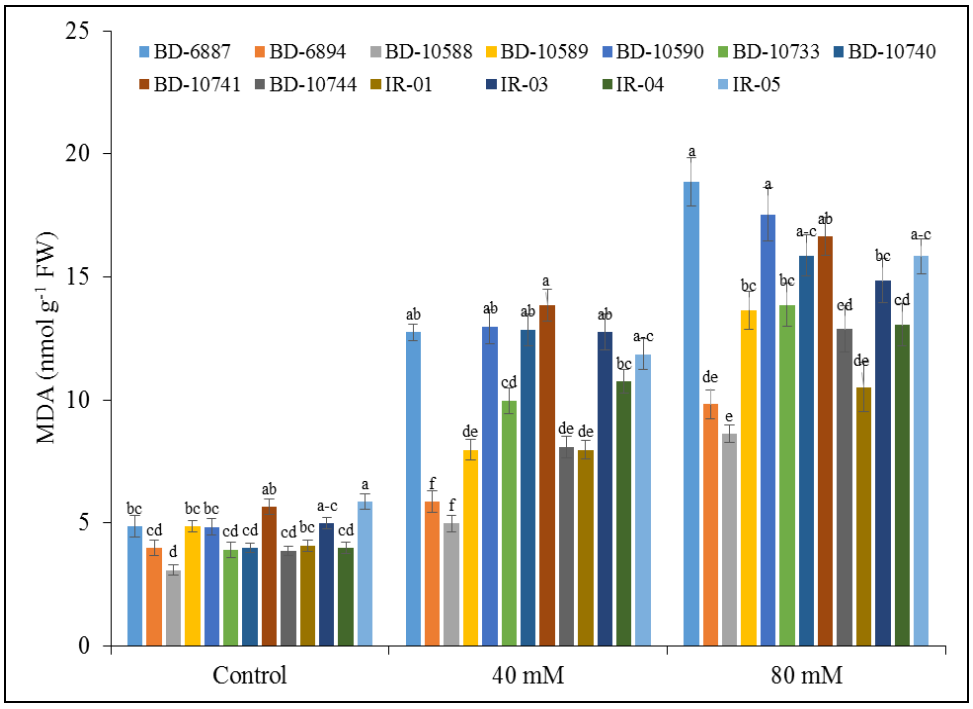

Fig. 3. Comparative MDA content in leaves of different among mungbean genotypes under salinity. Different letters above the bars within a treatment are significant at P≤0.05. Vertical bar represents Mean ± SE.

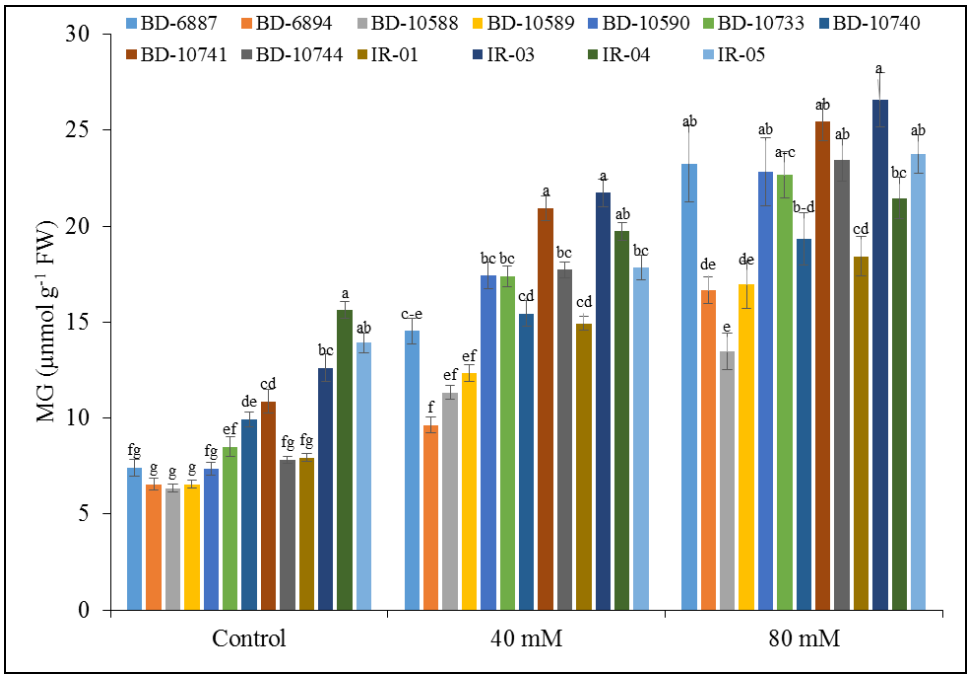

Fig. 4. Comparative MG content in leaves of different among mungbean genotypes under salinity. Different letters above the bars within a treatment are significant at P≤0.05. Vertical bar represents Mean ± SE.

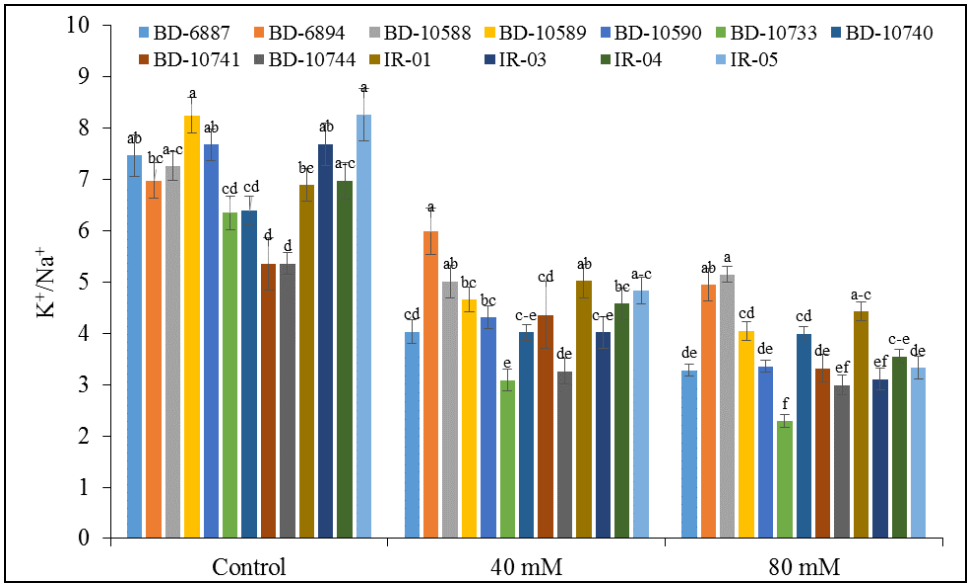

Fig. 5. Comparative K^+/Na^+ in leaves of different among mungbean genotypes under salinity. Different letters above the bars within a treatment are significant at P≤0.05. Vertical bar represents Mean ± SE.

Comparative K^+/Na^+ in leaves and roots of mungbean genotypes

Salinity stress increases Na^+ uptake which severely reduces K^+ content in both leaves and roots of plants. However, reduction of K^+ uptake varies among genotypes. In this experiment, generally the genotypes which had lower ROS and MG showed higher K^+/Na^+ than the other genotypes (Fig. 5) depicting their higher tolerance to salinity.

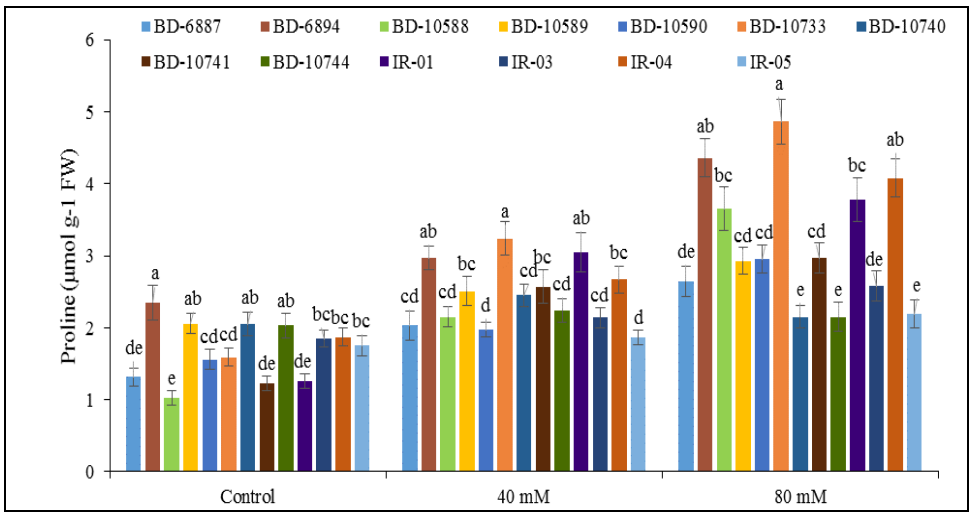

Fig. 6. Comparative proline content in leaves of different among mungbean genotypes under salinity. Different letters above the bars within a treatment are significant at P≤0.05. Vertical bar represents Mean ± SE.

Comparative proline content in leaves of mungbean genotypes

Proline, a free amino acid with adaptive role, accumulated differently in leaves of mungbean genotypes (Fig. 6). Among the genotypes, BD-10733 showed the highest concentration (4.86 µmol g^{-1} FW) followed by BD-6894 (4.36 µmol g^{-1} FW) while BD-10740 and BD-10744 had the least concentration (2.15 µmol g^{-1} FW) at 80 mM salinity. The genotypes BD-10588 and IR-01 which had lower ROS, MDA and MG showed moderate concentration of proline (3.65 and 3.78 4.86 µmol g^{-1} FW, respectively). Contrarily, BD-6887 and BD-10741 showing higher ROS, MDA and MG contained comparatively lower proline content.

Selection of genotypes based on physiological and biochemical parameters for studying ROS regulation mechanism

Based on germination, root-shoot length, ROS, MDA, MG, K^+/Na^+ and proline content, BD-10588, BD-6894 and IR-01 seemed to be capable to tolerance salinity. Contrary, BD-6887 and BD-10741 were not germinated under 120 mM salinity. To study the ROS regulation mechanism, genotypes BD-10588 and IR-01 were selected as relative tolerant genotypes and BD-6887 and BD-10741 as

susceptible genotypes. It should be mentioned that phenotypically differently saline sensitive genotypes are required for such type of research.

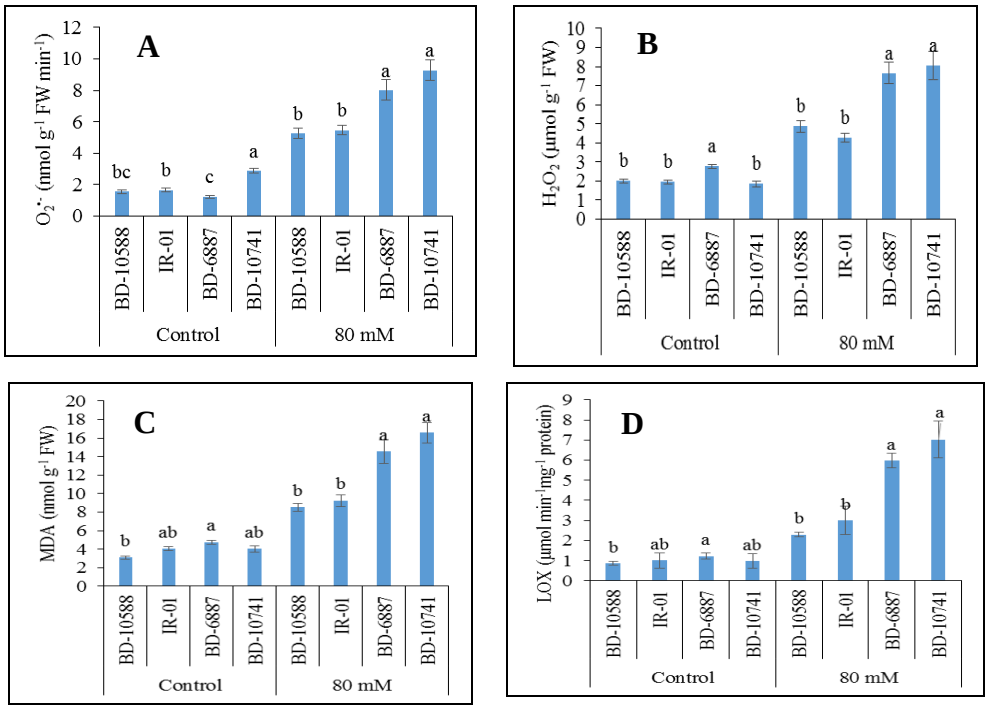

Fig. 7. Comparative $O_2^{\bullet-}$ (A), H_2O_2 (B), MDA (C) contents and LOX activity (D) in leaves of selected mungbean genotypes. Different letters above the bars within a treatment are significant at P≤0.05. Vertical bar represents mean ± SE.

Comparative ROS, MDA content and LOX activity in leaves of selected mungbean genotypes

The content of ROS, MDA and LOX activity in tolerance and susceptible genotypes were found to be increased at 80 mM salinity as compared to the control treatment (Fig. 7). Importantly, $O_2^{\bullet-}$ generation rate, concentrations of H_2O_2, MDA as well as LOX activity in tolerant genotypes were significantly lower than those in susceptible genotypes under saline condition.

Regulation of ROS metabolizing antioxidant enzymes in leaves of selected mungbean genotypes

Superoxide dismutase (SOD) activity:

Activity of SOD varied significantly among tolerant and susceptible genotypes under stress although in control condition significant variation was not observed (Fig. 8). As compare to control treatment, SOD activity at 80 mM was increased

by 36% and 29% in BD-10588 and IR-01, respectively. On the other hand, the activity was increased by 27% and 16% in Bd-6887 and BD-10741, respectively (Fig. 8).

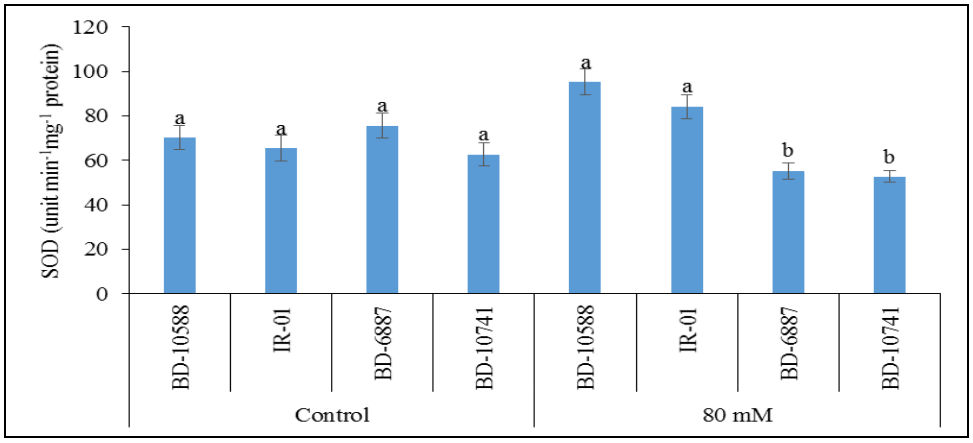

Fig. 8. Activity of SOD in leaves of selected mungbean genotypes. Different letters above the bars within a treatment are significant at P≤0.05. Vertical bar represents mean ± SE.

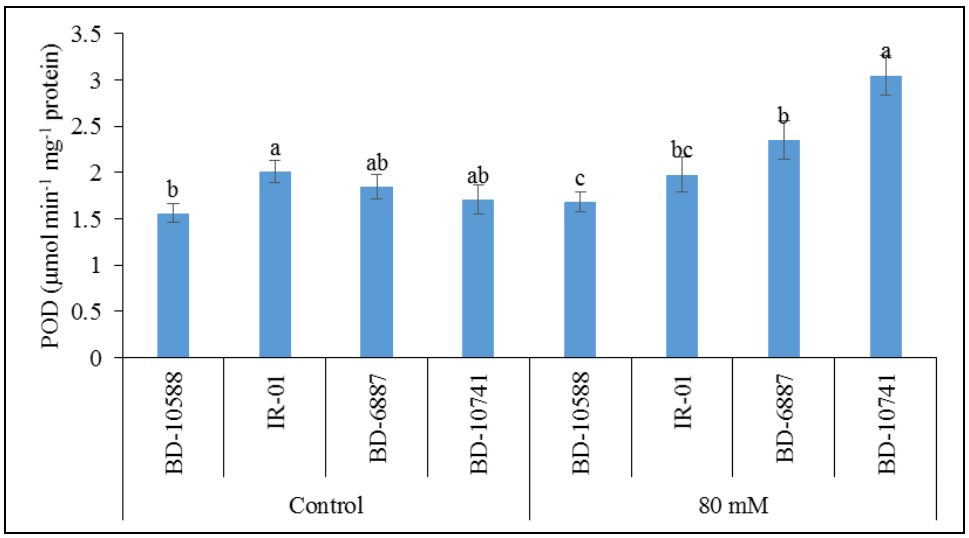

Fig. 9. Activity of POD in leaves of selected mungbean genotypes. Different letters above the bars within a treatment are significant at P≤0.05. Vertical bar represents mean ± SE.

Peroxidase (POD) activity:

The peroxidase (guaiacol) activity varied significantly among the genotypes under both control and salinity (Fig. 9). The POD activity in tolerant genotypes

remained almost similar at 80 mM salinity as compare to control. On the other hand, the activity increased in susceptible genotypes under salinity by 27% and 78% in BD-6887 and BD-10741, respectively, as compare to control treatment.

Catalase (CAT) activity:

The catalase activity among the genotypes were almost similar in control condition (Fig. 10). At 80 mM salinity, significant variation was not observed in tolerant genotypes. However, the activity in susceptible genotypes decreased by 35% and 34% in BD-6887 and BD-10741 as compared to respective control. Importantly, the activity was significantly lower than the tolerant genotypes.

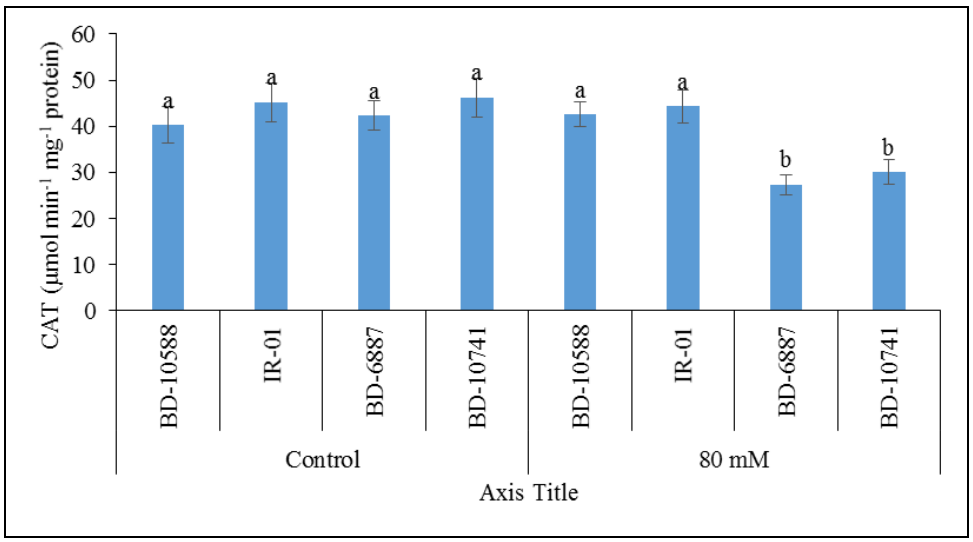

Fig. 10. Activity of CAT in leaves of selected mungbean genotypes. Different letters above the bars within a treatment are significant at P≤0.05. Vertical bar represents mean ± SE.

Ascorbate peroxidase (APX) activity:

The APX activity varied among different genotypes under both control and stress condition (Fig. 11A). Remarkably, tolerant genotypes, BD-10588 and IR-01 maintained higher activity under salinity as compare to susceptible ones. The in-gel activity also showed the similar result (Fig. 11B).

Glutathione peroxidase (GPX) activity:

As compared to control, GPX activity increased under salinity stress in all the genotypes (Fig. 12A). As compare to respective control, 86%, 21%, 55% 80% higher activity under salinity were observed in BD-10588, IR-01, BD-6887 and BD-10588, respectively. In in-gel analysis of GPX activity, two isozymes were found (Fig. 12B). Here increased activity was also observed in these genotypes.

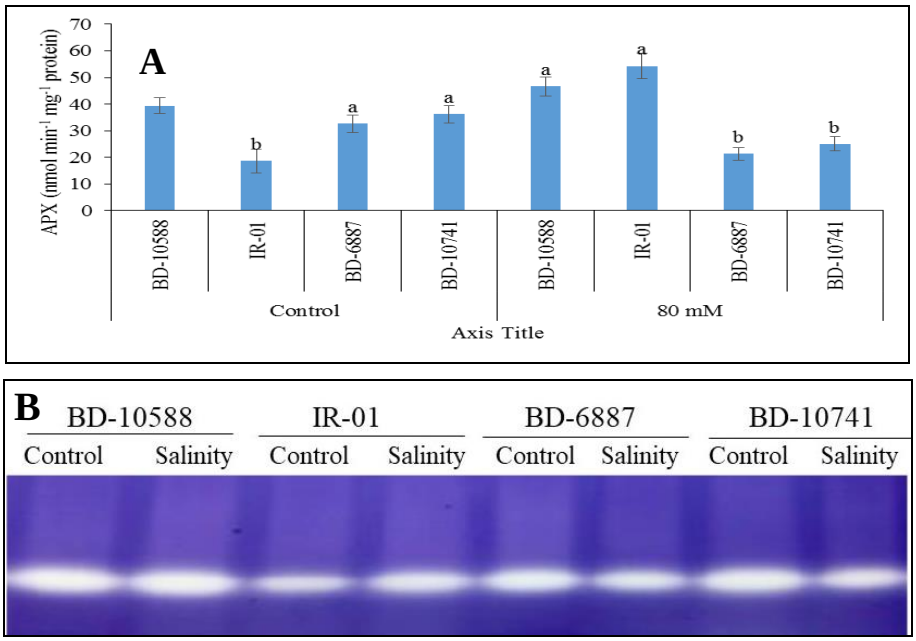

Fig. 11. Specific activity (A) and in-gel activity (B) of APX in leaves of selected mungbean genotypes. Different letters above the bars within a treatment are significant at P≤0.05. Vertical bar represents mean ± SE.

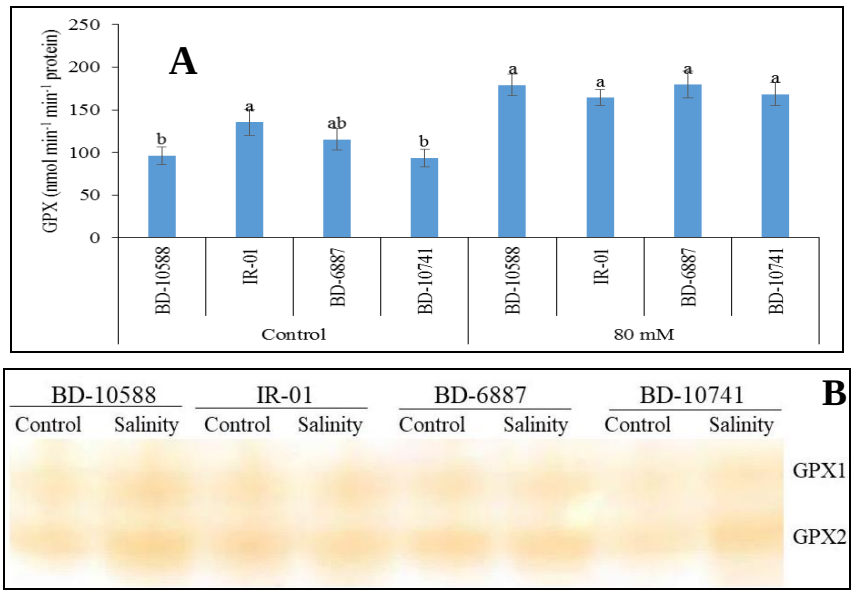

Fig. 12. Specific activity (A) and in-gel activity (B) of GPX in leaves of selected mungbean genotypes. Different letters above the bars within a treatment are significant at P≤0.05. Vertical bar represents mean ± SE.

Discussion

Selection of salt tolerant genotypes by growth and biochemical parameters

As salinity level increased, germination percentage, root and shoot length decreased (Table 1 and 2). Genotypes with higher germination percentage along with higher root and shoot length suggested their saline tolerance ability. At higher salinity (120 mM), only BD-6894, BD-10588, BD-10733 and IR-01 showed few germinations. Hence, for growth and biochemical parameters, 0, 40 and 80 mM concentrations were considered. The length of root and shoot also decreased with increasing salinity level (Table 2). Genotypes like BD-10588, BD-6894 and IR-01 had higher shoot length at 80 mM concentration. On the other hand, roots of BD-10744, IR-04 and BD-10733 were reduced remarkably at 80 mM salinity. Naseer *et al*. (2001) reported that the reduction in shoot length occured due to excessive accumulation of salt in the cell wall, which modifies the metabolic activities and limits the cell wall elasticity. The possible reason for the reduced shoot development could also be due to the toxic effects of the NaCl as well as the unbalanced nutrient uptake by the seedlings. These results can further be explained by biochemical parameters like ROS, MDA, MG, proline contents and LOX activity. Under abiotic stress like, salinity causes over production of ROS which interact with cellular organelles like protein, DNA, lipids and pigments to oxidation and functional loss, finally to cause cell death (Rohman *et al*., 2019). In this study, Genotypes like BD-10588, BD-6894 and IR-01 produced lower ROS like $O_2^{\bullet-}$ and H_2O_2 (Fig. 1 and 2). On the other hand, other genotypes exhibited higher $O_2^{\bullet-}$ and H_2O_2. It can be critical factor to cell wall damage, because these genotypes had higher MDA with higher LOX activity (Fig. 7A, B). It is established that in presence of LOX, higher ROS oxidize the PUFA of cell wall to MDA causing electroleakage (Doderer *et al*., 1992; Gill and Tujeta, 2010). Therefore, higher shoot and root growth of BD-10588, BD-6894 and IR-01 were associated with lower ROS, MDA and LOX activity. Moreover, lower MG content can least damage of root and shoot (Saxena *et al*., 2011). Higher MG with poor growth in mungbean also reported under drought stress (Nahar *et al*., 2015). The lower ration of K^+/Na^+ in these three genotypes also suggested lower Na^+ mediated injury along with higher K^+. Since the remaining genotypes had comparatively higher ROS, MDA, MG and K^+/Na^+, cell death and ionic toxicity would be higher. Thus, these genotypes were sensitive to salinity stress. Among the sensitive genotypes, BD-6887 and BD-10741 were most sensitive due to containing higher ROS, MDA, MG as well as K^+/Na^+ contents with very poor germination and shoot and root length. Previously, susceptible genotypes with higher ROS, MDA, MG as well as K^+/Na^+ were reported in maize (Rohman *et al*., 2016a, b; 2018), and rice (Hasanuzzaman *et al*., 2014). Generally, plants accumulate compatible solutes like proline under abiotic stress including salinity stress. It maintains cellular water status and membrane stability, inhibits protein oxidation and scavenges free radicals through antioxidative action under osmotic stress (Ashraf and Foolad, 2007). Therefore,

genotypes with higher proline content are considered as tolerant to saline and other abiotic stresses (Gilll and Tujeta, 2010). In this study, considerable proline accumulation under salinity increased in BD-10588 and IR-01, although BD-10733 and BD-6894 showed the highest accumulation (Fig. 6). Conversely, BD-6887 and BD-10741 with higher ROS, MDA, LOX and MG showed comparatively lower proline accumulation under salinity. Therefore, salt tolerance could be partly associated with proline content in mungbean. Similar results were also found in maize (Moussa and Abdel-Aziz, 2008; Rohman *et al*., 2016a).

Comparative oxidative stress tolerance mechanism in mungbean genotypes

Based on germination percentage, length of shoot and root, contents of ROS, MDA, MG and K^+/Na^+, BD-10588 and IR-01 were considered as most tolerant and BD-6887 and Bd-10741 as most susceptible genotypes to study oxidative stress tolerance mechanism. ROS generation is a common phenomenon in crop under abiotic stress including salinity (Bartoli *et al*., 1999; Noctor *et al*., 2012). In this study, leaves of BD-10588 and IR-01 had higher $O_2^{\bullet-}$ and H_2O_2 concentrations under 80 mM salinity stress suggesting more oxidative damage in cell. It might be due to limited capacity of scavenging of $O_2^{\bullet-}$ and H_2O_2. This capacity is explained by the activities of different antioxidant enzymes. SOD is considered as the first line defense against ROS, being responsible for the dismutation of $O_2^{\bullet-}$ to H_2O_2 (Apel and Hirt, 2004; Gill and Tujeta, 2010). On the other hand, enzymes like CAT, POD, GPX and APX catalyze the conversion of H_2O_2 to water and O_2 (Gratao *et al*., 2005). The balance between ROS production and activities of antioxidative enzymes determines whether oxidative signaling and/or damage will occur (Moller *et al*., 2007). Tolerance of a plant to salinity depends on the capability of scavenging ROS and reducing their damaging effects as well as Na^+ and Cl^- uptake (Farooq *et al*., 2015). Tolerance level also depends upon intensity and duration of stress as well as plant species and its developmental stage (Chaves *et al*., 2003; Jung, 2004). Like ROS, MDA is regarded as a marker for evaluation of lipid peroxidation or damage to plasmalemma and organelle membranes that increases with increasing ROS accumulation under environmental stresses (Garg and Manchanda, 2009). In this study, $O_2^{\bullet-}$ generation rate and H_2O_2 and MDA contents were significantly lower in tolerant genotypes (Fig. 7A, B, C). Previously, Rohman *et al*. (2019) also reported comparatively higher ROS and MDA levels in a number of saline susceptible maize. However, such types of results were not reported in mungbean previously.

LOX enzyme catalyzes the peroxidation of polyunsaturated fatty acids to their corresponding hydroperoxides (Doderer *et al*., 1992). The increased LOX activity was assumed as a reason for increased lipid peroxidation of polyunsaturated fatty acids as reported in many plants (Demiral and Türkan, 2004; Azooz *et al*., 2009; Sánchez-Rodríguez *et al*., 2012; Rohman *et al*., 2016a,

b). However, LOX activity was significantly higher in susceptible genotypes under saline stress. LOX activity was also correlated with increased MDA content in susceptible genotypes (Fig. 7C, D). Previously, Hossain *et al*. (2010) and Nahar *et al*. (2015) reported higher MDA with higher LOX activity. In this report, we found that susceptible genotypes were associated with higher LOX and MDA.

The SOD activity was higher in both tolerant genotypes, BD-10588 and IR-01 while susceptible genotypes BD6887 and Bd-10741 had comparatively lower activity under salinity stress (Fig. 8). Previously, we reported higher SOD activity in tolerant maize genotype (Rohman *et al*., 2016a). Higher SOD activity under salinity in tolerant genotypes may convert cytotoxic $O_2^{\bullet-}$ to H_2O_2, and thus, lower $O_2^{\bullet-}$ mediated cytotoxicity. Previously, Nahar et al. (2015) reported increased SOD activity in mungbean under salinity stress. In that study, they did not use any susceptible genotypes. Therefore, in susceptible mungbean genotypes, SOD activity can decrease.

POD catalyze the decomposition of H_2O_2 to water, thus alleviating the damaging effects (Jin *et al*., 2011). In this study, POD activity increased only in susceptible genotypes (Fig. 9). Therefore, increment of POD activity can be important strategy of salt sensitive mungbean genotypes. Besides, CAT, GPX and APX are associated with oxidative tolerance by scavenging of H_2O_2 (Gill and Tujeta, 2010; Miller *et al*., 2010). However, CAT, as compared to APX, GPX and POD, shows low affinity to H_2O_2 with a high processing rate (Scandalios, 2005). This is essential because, unlike other H_2O_2 scavenging enzymes (APX, GPX and POD), CAT is not substrate dependent with low affinity to of peroxide (Scandalios, 2005). In this study, CAT activity remained almost similar in saline condition, but it was reduced in susceptible genotypes. (Fig. 10). Therefore, higher CAT activity can reduce H_2O_2 mediated oxidative damage in tolerant genotypes.

APX, the most important antioxidant enzyme in plants which reduces H_2O_2 mediated oxidative damage by using ascorbic acid as electron donor (Gill and Tujeta, 2010; Rohman *et al*., 2019). The spectrometric and in-gel activity results strongly proved that APX activity increased in tolerant mungbean genotypes (Fig. 11A, B). The decreased activity in susceptible genotypes may spell them cellular damage. Comparatively lower APX activity was also reported in susceptible genotypes of maize and rice (Hasanuzzaman *et al*., 2014; Rohaman *et al*., 2016a).

In glutathione (GSH) dependent metabolism, H_2O_2 is removed by GPX where GSH is converted to oxidized glutathione (GSSG). In this study, the increased GPX activities in both types of mungbean genotypes suggested a role of this enzyme in H_2O_2 metabolism under salinity (Fig. 12A). This result is supported by Nahar *et al*. (2015). Additionally, the GPX isozymes provided strong proof of increased GPX activity in mungbean genotypes under saline stress (Fig. 12B).

Conclusion

Considering growth, biochemical and molecular parameters, genotypes, BD-10588, BD-6894 and IR-01 were selected as tolerant genotypes. Higher SOD, CAT and APX activities played major role in $O_2^{\bullet-}$ and H_2O_2 metabolism as well as lower MDA production in tolerant genotypes. On the other hand, GPX was equally important for both tolerant and susceptible genotypes while POD played better role in susceptible genotypes. Through in-gel activity analysis, we provide strong evidence of salt mediated oxidative stress mitigation through in-gel activity of enzymes. As GPX had equal role in both tolerant and susceptible genotypes, both GPX1 and GPX2 can further be investigated through biotechnological and molecular approaches to obtain detailed roles in mungbean.

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