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Assessment of oxidative stress tolerance in red bean (*Phaseolus vulgaris* L.) seedling under salinity

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Abstract

In order to evaluate salt tolerance in red bean (*Phaseolus vulgaris* L.) 10 genotypes were exposed to two levels of NaCl i.e., 0 and 400 mM in laboratory conditions. Plant fresh weight, total phenolics, total soluble proteins, hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) in leaves salt-stressed and non-stressed plants were analyzed. Electrophoretic analyses were performed by using 8% slab polyacrylamide gels. Superoxide dismutase (SOD), peroxidase (POX) and catalase (CAT) were stained and for each isozymic band the "density × area" scores onto gels were evaluated by MCID software as enzymatic activity. The salt stress reduced fresh weight of red bean genotypes. MDA, H_2O_2, total soluble proteins and total phenolics were significantly elevated in salinity condition. Salt stress increased activities of SOD, POX_3 and CAT in all red bean genotypes. These results seem to indicate that 31126 genotype of red bean tolerance to salt stress is associated with enhance activity of antioxidant enzymes. Different antioxidant enzymes and other characters analyzed, only SOD_2 isozyme activity was found to be associate with salt tolerance in red bean genotypes examined.

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Introduction

Legumes represent a very significant group of crops in agriculture, and therefore their responses to salt stress are described in several reports (Munns 2002; Lachaal *et al.* 2002). Tolerant varieties and accessions within the legumes have been revealed, such as for soybean and among both cultivated and wild *Phaseolus* species (Bayuelo-Jimenez *et al.* 2002).

Oxidative stress is one of the various influences caused by salt stress (Ashraf 2009). Generally, exposure to salt stress triggers many common reactions in plants that lead to cellular dehydration with concomitant osmotic changes; removal of water from the cytoplasm into the extracellular space results in a decrease of the cytosolic and vacuolar volumes. Another consequence of exposure to this stress is the generation of reactive oxygen species (ROS) such as superoxide (O_2^-) and hydrogen peroxide (H_2O_2), which in turn have a negative oxidative stress effect on cellular structures and metabolism (Wang *et al.* 2009). As salt stress occur frequently and can affect most habitats, plants have developed several strategies to cope with these challenges. One of the stress defense mechanisms is the antioxidant defense system, which includes antioxidant enzymes, such as super dismutase (SOD), peroxidase (POX), catalase (CAT), and low-molecular antioxidants. SOD converts superoxide radicals (O_2^-) into hydrogen peroxide (H_2O_2), POX reduces H_2O_2 to water using various substrates as electron donors, and CAT catalyze H_2O_2 into water and oxygen (Gaber 2010). Malondialdehyde (MDA) a product of lipild peroxidation, showed greater accumulation in plants under stress condition. Cell membrane stability has been widely used to differentiate stress tolerant and susceptible cultivars of some crops, and in some cases, lower MDA content could be correlated with stress tolerance (Wang & Han 2009). Wang *et al.* (2009) studied antioxidant responses of three *Medicago* species including alfalfa to 300 mM NaCl during seed germination stage and found a much weaker glutathione reductase (GR) activity in seeds of alfalfa in the controls and salt treatment and different responses were reported for other species. Similar increases in the activities of SOD and POX have been reported in *Morus alba* (Chinta *et al.* 2001), and tomato (Dogan 2012).

The present study was to evaluate the effect of salinity on fresh weight, MDA, H_2O_2, total poly phenol, and changes in activity profile of some antioxidant enzymes properties of common beans.

Materials and methods

Plant material and Salt stress treatment

The seeds of 10 red bean genotypes (Table 1) were surface sterilized in sodium hypochlorite (2.5% for 30 S) and were washed immediately with large volume of sterile distilled water. Five days old seedlings were then transferred into specially designed dishes containing 1/2 strength sterile Hoagland's nutrient solution with added micronutrients and 0.04 mM ferrous ion as Fe–EDTA, (pH 5.6) as described. The seedlings were grown at 26 °C under 16 h light: 8 h dark photoperiod. Salt stress on white beans was induced by incubating plants in half-strength Hoagland's nutrient solution containing NaCl at a final concentrations of 400 mM for 48 h. Plants grown on half-strength Hoagland's medium without NaCl served as control (Nagesh Babu & Devaraj 2008).

Table 1. List of red common beans analyzed in this study.

Code	Genotype	Code	Genotype
1	31109	6	31125
2	31114	7	31126
3	31116	8	31137
4	31120	9	31165
5	31123	10	31167

Determination of Hydrogen peroxide (H_2O_2)

Hydrogen peroxide content in control and stressed seedlings were determined according to Velikova *et al.* (2000). Fresh leaf (500 mg) were homogenized in an ice bath with 5 ml of 0.1% (w/v) trichloroacetic acid. The homogenate was centrifuged at 10,0009g for 15 min and 0.5 ml of the supernatant was added to 0.5 ml of 10 mM potassium phosphate buffer (pH 7.0) and 1 ml of 1 M KI. The absorbance of the supernatant was measured at 390 nm.

Malondialdehyde (MDA)

The level of lipid peroxidation was determined in terms of thiobarbituric acid-reactive substances (TBARS) concentration as described by Noreen & Ashraf (2009). Fresh leaf (1.0 g) was homogenized in 3 mL of 1.0% (w/v) TCA at 4 °C. The homogenate was centrifuged at 20,000g for 15 min and 0.5 mL of the supernatant obtained was added to 3 mL of 0.5% (v/v) thiobarbituric acid (TBA) in 20% TCA. The mixture was incubated at 95 °C in a shaking water bath for 50 min, and the reaction was stopped by cooling the tubes in an ice water bath. Then the samples were centrifuged at 10,000g for 10 min, and the absorbance of the supernatant was read at 532 nm. The value for nonspecific absorption at 600 nm was subtracted. The concentration of TBARS was calculated using the absorption coefficient, 155 $mmol^{-1}\ cm^{-1}$.

Total phenolics

Total phenolics were determined using Folin–Ciocalteau reagent (Noreen & Ashraf, 2009). Fresh leaf tissue (50 mg) was homogenized with 80% acetone and centrifuged at 10,000g for 10 min. One-hundred microlitres of the supernatant were diluted with 2 mL of water and 1 mL of Folin–Ciocalteau's phenol reagent and shaken vigorously. Then 5 mL of 20% sodium carbonate solution was added and the volume was made up to 10 mL with distilled water. The contents were mixed thoroughly and the absorbance was read at 750 nm using a spectrophotometer (IRMECO U2020). The results were expressed as mg/g of fresh leaf.

Enzymes extraction and electrophoresis

The crude extract of fresh and healthy leaves from adult plants were prepared with separate mortar and pestle in a Tris-HCl extraction buffer pH 7.5 (Tris 50 mM, sucrose 5%, ascorbic acid 50 mM, sodium metabisulfite 20 mM, PEG 2% and 2-Mercaptoethanol 0.1% before use) with a ratio of 1 mg μl^{-1} and centrifuged at 4°C and 10,000 rpm for 10 minutes using small Eppendof tubes. Enzyme extracts were immediately absorbed onto 3×5 mm wicks cut from Whatman 3 mm filter paper and loaded onto 8% horizontal slab polyacrylamide gel (0.6×15×12 cm) using TBE (Tris-Borate-EDTA) electrod buffer (pH= 8.8). Electrophoresis was carried out at 4 ^{oC} for 3 h (constant current of 30 mA, and voltage of 180 V) (Valizadeh *et al.* 2013). Total soluble protein content was determined according to the method of Bradford (1976) using BSA as the standard.

Statistical Analysis

A factorial experiment on the basis of completely randomized design was carried out. An image analysis program (MCID[1] software) was used to measure D×A (optical density×area) parameter for each isozymic band to evaluate the activity onto gels. For statistical analysis and relationship estimates between isozyme markers and fresh weight SPSS 16.0 software was used.

Result and discussion

The analysis of variance of data to assess the effect of salinity on red bean and related attributes showed a highly significant difference for genotype and salinity but no significant difference for genotype × salinity interaction (Table 2). According to table 2, there were significant differences among genotypes for fresh weight and MDA. The effect of salinity was significant for fresh weight, MDA, H_2O_2, total phenolics and total soluble proteins (P< 0.01).

Table 2. Analysis of variance of MDA, H_2O_2, Total phenol and Total protein.

		Mean Square			
Source	**df**	**MDA**	**H_2O_2**	**Total phenol**	**Total protein**
Genotype	9	32.264**	1.742^{ns}	0.410^{ns}	2.80^{ns}
Salinity	1	6.480**	113.906**	0.077**	4.80**
G × S	9	1.260^{ns}	1.253^{ns}	0.115^{ns}	1.792^{ns}
Error	20	32.264	1.944	0.091	0.82

** and ^{ns} : significant at 0.01 levels and non-significant.

Salinity was decreased fresh weight of all red bean genotypes. 31126 genotype of red bean was the higher fresh weight than other red bean genotypes. MDA in

[1] www.mcid.co.uk

leaves of all genotypes had different response (Fig. 1). Salt stress was increased the H_2O_2, total soluble protein, and total phenolics contents (Table 3).

Table 3. Mean(±SE) of H_2O_2, total phenolics and total protein.

Treatment	H_2O_2(µmol/g fr. wt.)	Total phenolics (mg/g fr. wt.)	Total protein (µgr/fr. wt.)
0 mM NaCl	12.07±0.24[b]	3.48±0.11[b]	8.59±0.32[b]
400 mM NaCl	15.45±0.33[a]	3.55±0.10[a]	9.28±0.21[a]

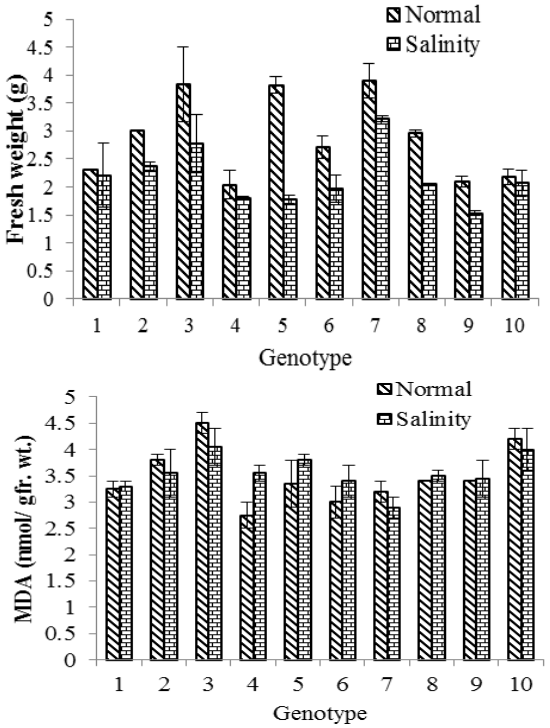

Fig. 1. Fresh weight and MDA of 10 common beans grown on five old seedlings to salt stress for 48 h.

Salinity is a major environmental factor that limits plant growth and crop productivity, and different crops may have varying salt-tolerant mechanisms (Asish *et al.* 2004).

MDA is the decomposition product of polyunsaturated fatty acids of membranes under stress. The rate of lipid peroxidation level in terms of MDA can therefore be used as an indication to evaluate the tolerance of plants to oxidative stress as well as sensitivity of plants to salt stress. It is also known that the formation of ROS enhances peroxidation at the cellular level and that the rate of such enhancement relates to plant species and the severity of stress (Wang *et al.* 2009). Variation in MDA contents were found in rice (Tijen & Ismail 2005) and cotton (Diego *et al.* 2003) cultivars differing in salt tolerance. Our research found significantly increased MDA contents in red beans under salt stress and result showed that the 31126 genotype had a lower MDA content than other genotypes. This indicates that 31126 genotype may be better able to adopt improved antioxidant mechanisms under salt stress than other genotypes of red beans.

In red bean genotypes leaf H_2O_2 remained changed due to salt stress. While, in contrast, it is generally known that salt stress enhances the production of singlet oxygen, superoxide anion, H_2O_2 and hydroxyl radical in plants. However, regulation of these ROS depends on their rates of generation, their rate of reaction with other metabolites such as proteins, lipids and nucleic acids, their rate of degradation and rate of their scavenging/neutralizing by enzymatic and/or non-enzymatic antioxidants. Generally, the dismutation of two superoxide anions, either enzymatically or non-enzymatically, give rise to H_2O_2. H_2O_2 is also produced from the β oxidation of fatty acids and peroxisomal photorespiration reactions (Noreen & Ashraf 2009).

Of various secondary metabolites, terpenes and phenolics are more important to abiotic stress tolerance than the others due to their structural properties. For example, enhanced synthesis of soluble phenolics has been directly correlated with salt and heat tolerance of sugarcane (Wahid & Ghazanfar 2006). In the present study, salt stress increased total phenolics of all red bean genotypes.

Activity analysis of red bean genotypes at both normal and salt stressed conditions stained onto same gels showed a substantial increment of SOD, POX and CAT antioxidant isozymes (Fig. 2). According to statistical analyses of all data obtained from all replications and gels, it has been shown that there

were significant differences in SOD_1, SOD_2, SOD_3, POX_1, POX_2, POX_3 and CAT among red bean genotypes. At the salinity condition, there were significant differences for SOD_1, SOD_2, SOD_3, POX_3 and CAT (Table 4). For genotype × salinity interactions there were no significant differences for their isozymic activities.

Table 4. Analysis of variance of densitometric activities of catalase, peroxidase and superoxide dismutase isozymes.

		Mean Square						
Source	**df**	**SOD_1**	**SOD_2**	**SOD_3**	**POX_1**	**POX_2**	**POX_3**	**CAT_1**
Genotype	9	1.047E-6**	2.729E-7**	1.306E-7**	2.208E-4**	0.957**	1.605**	0.799**
Salinity	1	4.692E-6**	2.116E-6**	3.331E-7**	7.076E-5^{ns}	0.006^{ns}	0.494**	0.276**
G × S	9	3.078E-7^{ns}	7.489E-8^{ns}	9.590E-9^{ns}	4.836E-6^{ns}	0.042^{ns}	0.032^{ns}	0.042^{ns}
Error	20	1.672E-7	4.450E-8	9.437E-9	2.850E-5	0.059	0.014	0.027

** and ^{ns} : significant at 0.01 levels and non-significant.

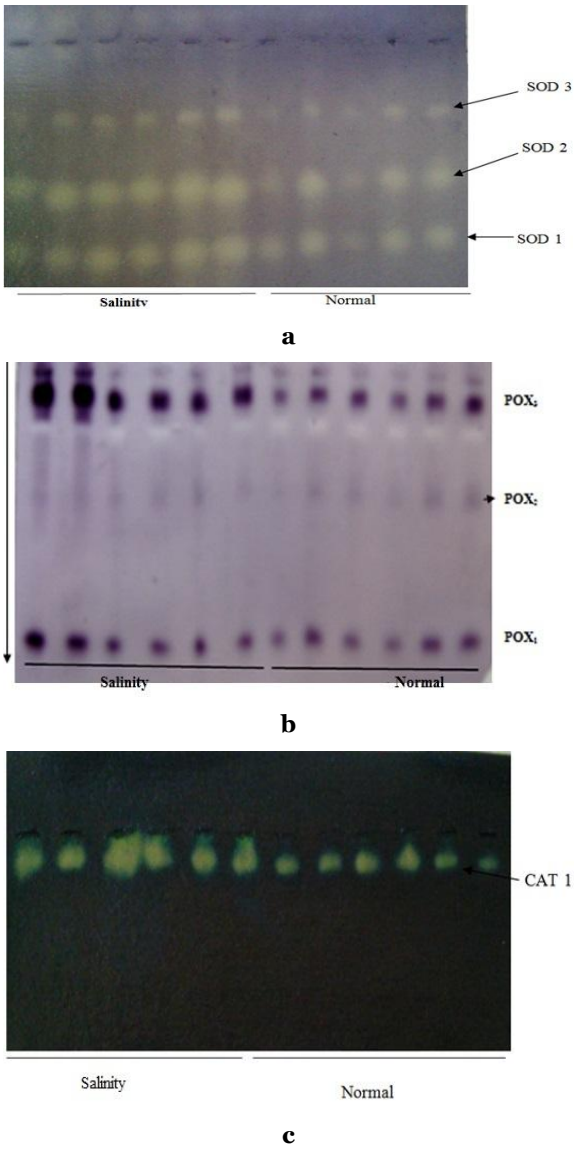

Fig. 2. Example SOD(a), POX(b) and CAT(c) banding pattern for normal and stress conditions.

Antioxidant enzymes such as SOD, POX and CAT are known to substantially reduce the levels of superoxide and hydrogen peroxide in plants. SOD catalyses the dismutation of O_2^- to molecular oxygen and H_2O_2. It is one of the most important enzymes used against oxidative stress in the plant defense system, and it occurs ubiquitously in every cell of all types of plants. Since SODs are mutlimeric metallo proteins they have different isoforms, based on the metal species present at their active sites. The most common isoforms of SOD known in the literature are copper–zinc containing superoxide dismutase (Cu/Zn-SOD), manganese containing (Mn-SOD) and iron containing (Fe-SOD) and nickel containing (Ni-SOD) (Ashraf, 2009). We observed three isoforms for SOD, POX and one isoform for CAT (Fig. 2). The result indicates that in salt stress, mean activities of SOD, POX and CAT isozymes are significantly higher than normal conditions (Fig. 3). Similar increases in the activities of SOD, POX and CAT have been reported in French bean (Nagesh Babu & Devaraj 2008) and alfalfa (Wang *et al.* 2009). Significant roles of POX have been suggested in plant development processes, which was involved in scavenging of H_2O_2 produced in chloroplast (Gaber 2010). Increases in the activities of CAT have been reported in soybean (Comba *et al.* 1998).

Data for fresh weight of 10 red bean genotypes grown under salt stress was subjected to multiple regression analysis. The results obtained from this analysis show that growth inhibition in different red bean genotypes was associated with activity of SOD_2 (Table 5).

Table 5. Multiple regression analysis showing the dependence of fresh weight on different biochemical of 10 red bean genotypes grown under varying levels of salt (R^2=0.80).

Variables	Coefficient
Intercept	0.283**
Total soluble proteins	-0.116ns
MDA	-0.156 ns
H_2O_2	-0.079 ns
Total Phenolics	0.005 ns
SOD_1	-0.172 ns
SOD_2	-0.510**
SOD_3	-0.210 ns
POX_3	0.006 ns
CAT	-0.131 ns

** and ns : significant at 0.01 levels and non-significant.

Diego *et al.* (2003) found that the POX activity significantly increased in salt-tolerant cotton cultivars but was unchanged in salt-sensitive cotton cultivars under salt stress. Mittal & Dubey (1991) compared two sets of rice cultivars differing in salt tolerance to determine a possible correlation between POX activity and salt tolerance of rice cultivars. Most of POX and SOD isozymes showed positive and significant correlations for plant height of alfalfa. This means that plant height was affected by salinity stress as antioxidant enzyme activities. However, dry weight and stem weight some alfalfa cultivars had negative and significant correlations with SOD_3 isozyme activity (Valizadeh *et al.* 2013). Of various antioxidant enzymes and metabolites, activity of CAT as well as the amounts of total soluble proteins and γ-tocopherols were found to be associated with the differential response of nine pea cultivars to salt stress (Noreen & Ashraf 2009).

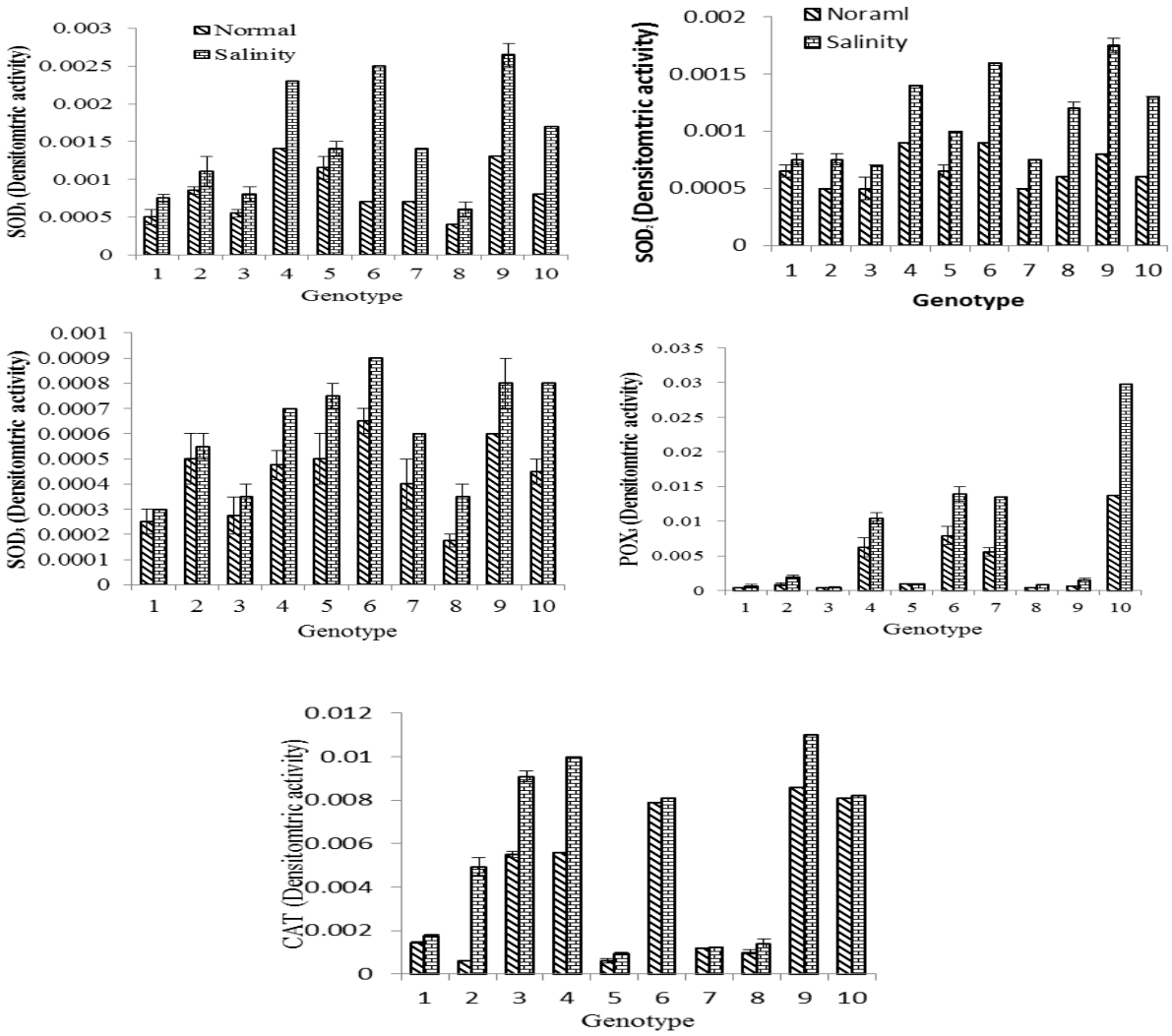

Fig. 3. SOD, POX and CAT isozymes of 10 genotypes of red bean grown on five old seedlings to salt stress for 48 h.

Conclusion

Evaluation of effect of relatively high salinity (400 mM NaCl) on some red bean genotypes fresh weight related traits along with analysis of antioxidant and non-antioxidant enzymes by native polyacrylamide gel electrophoresis showed that significant associated exist only between changes on SOD_2 activity and fresh weight, which are consistent with previous findings in several plants. These results seem to indicate that 31126 genotype of red bean tolerance to salt stress is associated with enhance activity of antioxidant enzymes.

Reference

Ashraf M. 2009. Biotechnological approach of improving plant salt tolerance using antioxidants as markers. Biotechnology Advances **27**, 84–93.

Asish KP, Anath BD, Prasanna M. 2004. Defense potentials to NaCl in mangrove, *Bruguiera parviflora*: Differential changes of isoforms of some antioxidant enzymes. Journal of Plant Physiology **161**, 531-542.

Bayuelo-Jimenez JS, Debouck DG, Lynch JP. 2002. Salinity tolerance in *Phaseolus* species during early vegetative growth. Crop Science **42**, 2184-2192.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of micro gram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry **72**, 248–54.

Chinta S, Lakshmi A, Giridarakumar S. 2001. Change in the antioxidant enzyme efficacy in two high yielding genotypes of mulberry (*Morus alba* L.) under NaCl salinity. Plant Science **161**, 613-619.

Diego AM, Marco AO, Carlos AM, José C. 2003. Photosynthesis and activity of superoxide dismutase peroxidase and glutathione reductase in cotton under salt stress. Environmental and Experimental Botany **49**, 69-76.

Dogan M. 2012. Investigation of the effect of salt stress on the antioxidant enzyme activities on the young and old leaves of salsola (*Stenopera*) and tomato (*Lycopersicon esculentum* L.). African Journal of Plant Science **6**, 62-72.

Comba ME, Benavides MP, Gallego SM, Tomaro ML. 1998. Relationship between nitrogen fixation and oxidative stress induction in nodules of salt-treated soybean plants. Environmental and Experimental Botany **60**,155-126.

Gaber MA. 2010. Antioxidative defense under salt stress. Plant Signaling and Behavior **5**, 369-374.

Lachaal M, Grignon C, Hajji M. 2002. Growth rate affects salt sensitivity in two lentil populations. Journal of Plant Nutrition. **25**, 2613-2625.

Mittal R, Dubey RS. 1991. Behaviour of peroxidases in rice: changes in enzyme activity and isoforms in relation to salt tolerance. Plant Physiology and Biochemistry **29**, 31-40.

Munns R. 2002. Comparative physiology of salt and water stress. Plant Cell and Environment **25**, 239–250.

Nagesh Babu R, Devaraj VR. 2008. High temperature and salt stress response in French bean (*Phaseolus vulgaris*). Australian Journal of Crop Science **2**, 40-48.

Noreen Z, Ashraf M. 2009. Assessment of variation in antioxidative defense system in salt-treated pea (*Pisum sativum*) cultivars and its putative use as salinity tolerance markers. Journal of Plant Physiology **166**, 1764-1774.

Tijen D, Ismail T. 2005. Comparative lipid peroxidation, antioxidant defense systems and praline content in roots of two rice cultivar differing in salt tolerance. . Environmental and Experimental Botany **53**, 143-151.

Valizadeh M, Moharamnejad S, Ahmadi M, Mohammadzadeh Jalaly H. 2013. Changes in activity profile of some antioxidant enzymes in alfalfa half-sib families under salt stress Journal of Agricultural Science and Technology **15**, 801-809.

Velikova V, Yordanov I, Adreva A. 2000. Oxidative stress and some antioxidant systems in acid rain treated bean plants; protective role of exogenous polyamines. Plant Science **151**, 59–66.

Wahid A, Ghazanfar A. 2006. Possible involvement of some secondary metabolites in salt tolerance of sugarcane. Journal of Plant Physiology **163**, 723–730.

Wang X, Han J. 2009. Changes of proline content, activity, and active isoforms of antioxidative enzymes in two alfalfa cultivars under salt stress. Agricultural Sciences in China **8**, 431-440.

Wang X, Zhao G, Gu H. 2009. Physiological and antioxidant responses of three *Leguminous* species to saline Environment during seed germination stage. African Journal of Biotechnology **8**, 5773-5779.

Wang W, Kim Y, Lee H, Kim K, Deng X, Kwak S. 2009. Analysis of antioxidant enzyme activity during germination of alfalfa under salt and drought stresses. Plant Physiology and Biochemistry **47**, 570–577.