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# EFFECT OF SALINITY STRESS ON PHYSIOLOGICAL, BIOCHEMICAL AND ANTIOXIDANT DEFENSE SYSTEMS OF HIGH YEILDING CULTIVARS OF SOYABEAN

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## ABSTRACT

Salinity stress is a major adverse factor that limits agricultural productivity and this is one of the major abiotic stresses faced by plants. Salinity affects plant growth, physiological activities and developmental processes. Soya bean is one of the proteinaceous commercial crops in India, where salt stress is the limiting factor. In the present work, six cultivars of beans (ADB-22, DSB-20, JS-93.05, JS-93.37, JS-335, and LSB-18) were tested under differing NaCl concentrations to assess their performance in salt conditions. The aim was to select salt tolerant bean cultivars. In order to investigate the effect of salt stress on plant height, fresh and dry weight, chlorophyll levels, total protein content, SOD, Catalase, Lipid peroxidase (LPX), Glutathione reductase (GR) and Ascorbate peroxidase (APX) of Soya plants of all the six cultivars subjected to salinity levels at varying concentrations of NaCl (control, 50, 100, 150, 200mM) and collected after 10days of NaCl treatment. The obtained results showed that higher levels of NaCl concentrations, reduced plant height, fresh weight, dry weight, chlorophyll levels and total protein content. Under stress conditions a decline in SOD and Catalase activity were observed in all six cultivars but the percentage of decrease was more significant in DSB-20. Meanwhile there was an increase in APX, LPX and GR activities in all the cultivars of soyabean. Among all the cultivars studied under salt conditions, changes were drastic and more in ADB-22, where as changes were less significant in JS-93.37 and DSB-20. Thus this paves a way to state that ADB-22 may be a salt susceptible variety while JS-93.37 and DSB-20 may be salt tolerant varieties. The present findings indicate that salinity (NaCl) triggered an antioxidant response in *Glycine max* L.

***KEY WORDS: Glycine max L., Cultivars, Antioxidant enzymes, Salinity stress and NaCl.***

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# INTRODUCTION

Abiotic stresses such as high salinity, drought, heavy metal etc can impose limitations on crop productivity and limit land availability for agriculture. Consequently, there is a greater need for understanding and choosing cultivars that can respond to adverse conditions with the hope of improving tolerance of plants to environmental stresses [1]. Among various abiotic stress factors, soil salinization is the biggest threat to agricultural productivity. As per a study on global land use pattern, it is clear that 7% of the world's land area, amounting to 1000 million hectares, has become saline. Salinity stress affects plant growth, as well as development processes such as seed germination, seedling growth and vigour, vegetative growth, flowering and fruit set [2]. In saline conditions, the plants are subjected to primarily an ionic imbalance and hyperosmotic stress. The effect of this imbalance or disruption in homeostasis occurs at the cell levels as well as the whole plant level. Massive changes in ionic and water balance cause molecular damage and growth arrest. Finally in severe salt conditions, this leads to tissue death and ultimately plant dies [3]. A high concentration of salt affects the cellular membrane activities of biochemical enzymes and the functioning of plant photosynthetic apparatus. Production of Reactive Oxygen Species (ROS) is an important cause of this damage. ROS is routinely generated during the normal plant metabolic processes. It has been reported that free radical $O^{.2-}$ and $H_2O_2$ plays an important role in plant damage and injury from NaCl stress in *Vigna catjand*, *Vigna unguiculata* and *oryza sativa* [4, 5]. It is also established that there is a link between increased antioxidant capacity and salt tolerance in different plants like pea, tomato and citrus [6-8]. With all these studies it is confirmed that in plants subjected to NaCl stress, the balance between the production of ROS and quenching activity of antioxidants is upset, resulting in oxidative stress damage. In the absence of a protective mechanism in plants the active oxygen species which is highly reactive can cause serious damage to different aspects of cell structure and function. To keep the levels of active oxygen species under control, plants have non-enzymatic and enzymatic antioxidant systems to protect cells from oxidative damage. Non-enzymatic antioxidants including β-carotenes, ascorbic acid (AA), α-tocopherol (α-toc), reduced glutathione (GSH) and enzymes including: superoxide dismutase (SOD), guaiacol peroxidase (POD), ascorbate peroxidase (APX), catalase (CAT), polyphenol oxidase (PPO) and glutathione reductase (GR). Superoxide dismutases (SODs), a group of metalloenzymes, are considered as the first defence against ROS, being responsible for the dismutation of $O^{2-}$ to $H_2O_2$ and $O_2$. CAT, APX, POD are enzymes that catalyze the conversion of $H_2O_2$ to water and $O_2$. There are many reports in the literature that underline the intimate relationship between enhanced or constitutive antioxidant enzyme activities and increased resistance to environmental stresses in several plant species, such as rice [9], foxtail millet [10], wheat [11] and barley [12].

In order to economically exploit the salinized areas, it is important to search for crop plants that thrive in such soil and produce reasonable yields. Soya bean (*Glycine ma* L.) is one of the important and valuable leguminous crops of India. Apart from providing quality protein for human consumption, *Glycine max* L. also contributes to the profitable agricultural yield. Therefore its culture has been increasing in India in recent years according to the fact that the seed yield of *Glycine max* L. was decreased due to salinity stress, the understanding of the physiological and biochemical mechanisms conferring salinity tolerance of these species is very important in terms of developing selection and breeding strategies.

The present study aims to examine the effect of various concentrations of NaCl (salinity) on physiological activities and antioxidant enzymes in this plant, by comparing six high yielding cultivars of soya bean in A.P, India. The results will definitely provide documentation for breeding/selection of higher salt resistant soya bean and acquisition of good information for future aspect of molecular research.

## MATERIALS AND METHODS

This study was examined by pot culture. The influence of salinity (NaCl) on six different cultivars of *Glycine max* L. (ADB-22, DSB-20, JS-93.05, JS-93.37, JS-335, and LSB-18) were estimated by physical, biochemical parameters, viz., plant length, plant width, fresh weight, dry weight and chlorophyll content, oxidative stress marker enzymes.

### *Growth Conditions*

The soya bean seeds were surface sterilized with commercial bleach sodium hypochlorite. The plastic tubs were taken and filled with black soil and vermicompost with 70:30 ratios respectively. Seeds of all the 6 bean cultivars were sown in this soil medium and were maintained at 20 to 25˚C. Germination was observed on 3rd day from the day of sowing of seeds. The plants were grown normally by supplying water regularly for 21 days. The 21day old plants were subjected to salinity stress with various concentrations of salinity i,e. 50mM, 100mM, 150mM and 200mM of NaCl for 10 days. Simultaneously, controls and replicates were maintained for all the experimental sets and for all the 6 cultivars of *Glycine max* L. On 31th day the leaf samples of all the 6 cultivars of *Glycine max* L. were collected and stored at 80˚C.

### *Plant Length, Fresh & Dry Weight*

The plants were removed carefully with root system and washed thoroughly. The length of shoot and root were measured by meter scale. Immediately the plants were weighed for fresh weight, Plants were dried in oven by setting at low heat (100°C) over night. Then plants were cooled in a dry environment. After the complication of cooling, the plants were weighed for dry weight.

### *Estimation of chlorophyll content*

The Chlorophyll content was estimated according to the method of Arnon (1949). About 1 gm of leaf sample was cut into small pieces and homogenized in a pre-cooled mortar and pestle using 80-1 (v/v) acetone. The extract was centrifuged at 3000 rmp for 15 min and made upto 25 ml with 80% (v/v) acetone. The clear solution was transferred to a colorimeter and the optical density was measured at 645 nm and 663 nm against blank in Shimadzu double beam spectrophotometer (UV -240).

Total chlorophyll (µg/ml) = (20.2xOD at 645 nm) + (8.02xOD at 663 nm).

### *Determination of total protein content*

Protein content in the extracts was determined according to Lowry *et al*. (1951). The plant tissue was weighed and homogenized in pestle and motor with extraction buffer then centrifuged at 12000 rpm for 10 min at room temperature. The supernatant was used for protein estimation. Its 100µl, and 200µl of the aliquots were taken in triplicate for test and maintain to 500µl by water, followed by the addition of 5ml of reagent-C, (reagent-C: 95 ml of reagent-A mixed with 5 ml of reagent-B,

Reagent-A: 2% sodium carbonate in 0.1 M NaOH, Reagent-B: 1% copper sulphate (CuSO4.5H2O) and 2% potassium-sodium tartarate in ratio of 1:1) mixed properly and incubated for 10 min at room temperature. 500 µl of 1N Folin-Ciocalteu's phenol reagent was mixed and vortexed quickly. This reaction mixture was incubated for 30 minutes at 37$^{o}$C and its absorbance was recorded at λmax 660 nm. The amount of protein was calculated by comparison with standard curve drawn under identical experimental conditions.

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***Enzyme extraction***

For SOD, CAT and GR extraction, leaf samples (0.5g) were homogenized in ice cold 0.1 M phosphate buffer (pH=7.5) containing 0.5 mM EDTA with pre-chilled pestle and mortar. Each homogenate was transferred to centrifuge tubes and was centrifuged at 4°C in refrigerated centrifuge for 15 min at 15000×g. The supernatant was used for enzyme activity assay.

***Enzyme activity assay***

SOD activity was estimated by recording the decrease in absorbance of superoxide nitro blue tetrazolium complex by the enzyme. About 3 ml of reaction mixture, containing 0.1ml of 200mM methionine, 0.1ml of 2.25mM nitro-blue tetrazolium (NBT), 0.1 ml of 3 mM EDTA, 1.5 ml of 100 mM potassium phosphate buffer, 1ml distilled water and 0.05 ml of enzyme extraction, were taken in test tubes in duplicate from each enzyme sample. Two tubes without enzyme extract were taken as control. The reaction was started by adding 0.1 ml riboflavin (60 μM) and placing the tubes below a light source of two 15 watts florescent lamps for 15 min. Reaction was stopped by switching off the light and covering the tubes with black cloth. Tubes without enzyme developed maximal colour. A non-irradiated complete reaction mixture which did not develop colour served as blank. Absorbance was recorded at 560 nm and one unit of enzyme activity was taken as the quantity of enzyme which reduced the absorbance reading of samples to 50% in comparison with tubes lacking enzymes.

CAT activity was measured according to Aebi (1984). About 3 ml reaction mixture containing 1.5 ml of 100 mM potassium phosphate buffer (pH=7), 0.5 ml of 75 mM $H_2O_2$, 0.05 ml enzyme extraction and distilled water to make up the volume to 3 ml. Reaction started by adding $H_2O_2$ and decrease in absorbance recorded at 240 nm for 1 min. Enzyme activity was computed by calculating the amount of $H_2O_2$ decomposed.

APX activity was measured according to Yoshimura *et al*. (2000) by monitoring the rate of ascorbate oxidation at 290 nm (E=2.8mM-1cm-1). The reaction mixture contained 25 mM phosphate buffer (pH=7), 0.1 mM EDTA, 1 mM $H_2O_2$, 0.25 mM AsA and the enzyme sample. No change in absorption found in the absence of AsA in the test medium.

GR activity was assayed by recording the increase in absorbance in the presence of oxidized glutathione (GSSG) and 5,5-dithiobis-2-nitrbenzoic acid (DTNB) according to Sairam *et al*., (2002). The reaction mixture contained 1 ml of 0.2 M potassium phosphate buffer (pH=7.5) containing 0.1 mM EDTA, 0.5 ml of 3 mM DTNB in 0.01 M potassium phosphate buffer (pH=7.5), 0.1 ml of 2 mM NADPH, 0.1 ml enzyme extract and distilled water to make up a final volume of 2.9 ml. Reaction initiated by adding 0.1 ml of 2 mM GSSG. The increase in absorbance at 412 nm recorded at 25°C over a period of 5 min on a spectrophotometer.

Lipid peroxidation was measured as described by Hedges *et al*. (1999). Approximately 0.5 g plant tissues was homogenized in 80% ethanol and centrifuged at 3000 rpm. Afterwards, the extract obtained was analyzed in two steps. At the first step, 1 volume of 20% (w/v) (TCA) and 1 volume of 0.01% Buthylated hydroxyl toluene (BHT) (an antioxidant used to block lipid peroxidation during the assay) were added to 1 volume of supernatant. At the second step, 1 volume of 20% TCA that contained 1 volume of 0.65% TBA and 1 volume 0.01% BHT were added to 1 volume extract taken from the supernatant. After vortexing the sample for 10 sec, they were incubated in a hot water bath adjusted to 95$^{o}$C for 25 min followed immediately by a shock treatment in an ice bath. The cooled samples were centrifuged at 3000 rpm and absorbance values of supernatants were measured in spectrophotometer. First step samples were measured at 532 and 600 nm, whereas second samples at 440, 532 and 600

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nm. Results were obtained using the following formulae (Ab: absorbance, MDA: malondialdehyde).

$[(Ab_{532} + TBA) – (Ab_{600} + TBA) – (Ab_{532} - TBA) – (Ab_{600} - TBA)] = A$

$[(Ab_{440} + TBA – Ab_{600} + TBA) \times 0.0571] = B$

mol MDA/ml = $(A-B/157000) \times 10^6$

## RESULTS

The present study revealed that the application of salt stress of 50, 100, 150 and 200mM concentrations of NaCl for 10 days showed the alterations in morphological studies. Minimum growth occurred in plants in 200mM concentration. The physiological changes under salt stress were evaluated by measuring the length, fresh and dry weight of shoot tissues by comparing the controlled with the Stressed plants.

As the application of salt (NaCl) concentration increases, the chlorophyll content decreased generally in all the cultivars of *Glycine max* L. The percentage of chlorophyll content varied by 44.10% in ADB-22, 60.84% in DSB-20, 84.05% in JS-93.05, 63.86% in JS-93.37, 63.96% in JS-335 and 52.86% in LSB-18 when compared to the control ones. There was high significant percentage of decrease in JS-93.05. In the present study minimum protein content was observed in plants at the highest concentration of 200mM NaCl. The high salt concentration showed a significant decrease in total protein content of all the cultivars of *Glycine max* L. There was a decrease of 18.32% to 70.52% of protein content in ADB-22, 15.85% to 55.94% in DSB-20, 18.60% to 65.49% in JS-93.05, 21.1% to 64.99% in JS-93.37, 11.13% to 56.74% in JS-335 and 11.92% to 64.52% in LSB-18 from 50mM to 200mM NaCl in leaves of *Glycine max* L. Among all the cultivars of *Glycine max* L., ADB-22 had a greater significant decrease in total protein content when compared to all varieties.

As NaCl concentration increased from 50mM to 200mM, the leaves showed decreasing trend of SOD activity (Fig-6). In leaves of ADB-22 the percentage change in superoxide dismutase (SOD) activity was 89.33%, 68.02% in DSB-20, 62.83% in JS-93.05, 61.84% in JS-93.37, 66.06% in JS-335 and 71.02% in LSB-18. Highest significant decrease was found in ADB-22 and increase was found in JS-93.37. In 10 days treated 31 days old plants, with increasing concentration of NaCl, the activity of catalase in leaves showed decreasing trend (Fig-7). At 200mM concentration of NaCl, the percentage decrease observed in catalase enzyme activity was 76.99% in ADB-22, 64.50% in DSB-20, 44.07% in JS-93.05, 60.80% in JS-93.37, 70.23% in JS-335 and 44.52% in LSB-18 in leaves of *Glycine max* L. The highest percentage decrease was observed in ADB-22 and lowest percentage decrease in JS-93.05.

As the concentration of NaCl increases, glutathione reductase increases in leaves of *Glycine max* L. (Fig-8). In leaves of ADB-22 cultivar, the percentage decrease in glutathione reductase activity was 30.33%, 16.35% in DSB-20, 26.17% in JS-93.05, 31.97% in JS-93.37, 21.56% in JS-335 and 21.28% in LSB-18. The highest percentage increase was observed in JS-93.37 and lowest percentage increase in DSB-20. In all the cultivars, the ascorbate peroxidase activity was significantly increased with the increase in concentration of NaCl from 50mM to 200mM compared to control plants (Fig-9). In leaves of ADB-22 cultivar, the percentage change in ascorbate peroxidase activity was 53.54%, 50.37% in DSB-20, 68.70% in JS-93.05, 52.52% in JS-93.37, 47.06% in JS-335 and 83.94% in LSB-18 under 200mM NaCl. The highest percentage increase was observed in LSB-18 and lowest percentage increase in JS-335.

As the concentration of salinity increases, lipid peroxidation increases in leaves of *Glycine max* L. (Fig-10). In leaves, the

percentage change in lipid peroxidation activity was 31.44% in ADB-22, 80.94% in DSB-20, 50.55% in JS-93.05, 43.82% in JS-93.37, 30.69% in JS-335 and 32.29% in LSB-18 in leaves of *Glycine max* L. The highest percentage increase of LPX activity was observed in JS-93.05 and lowest percentage increase in JS-93.37.

**Figure. 1**
***Effect of different concentrations of NaCl (salinity) on plant height of different cultivars of Glycine max L.***

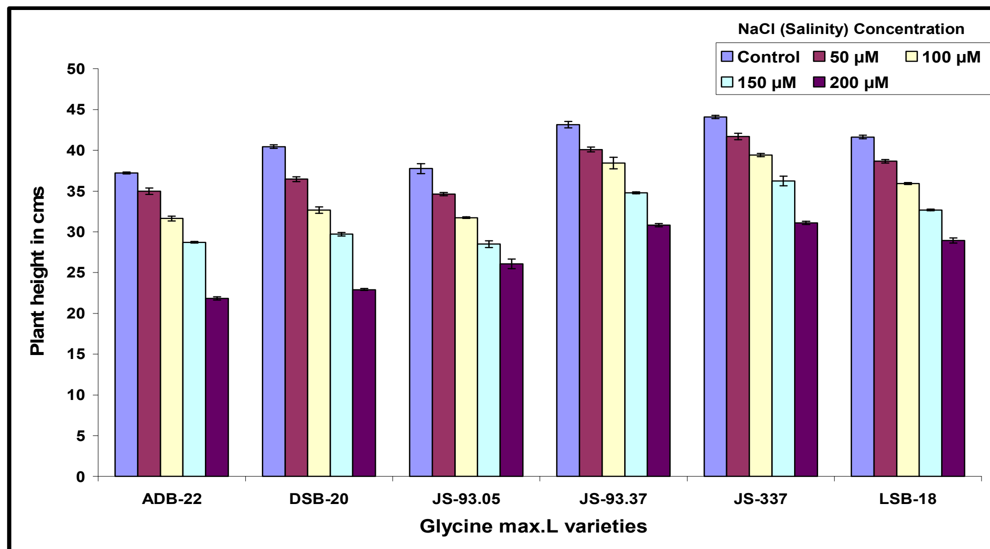


**Figure -2**
***Effect of different concentrations of NaCl (salinity) on plant fresh weight of different cultivars of Glycine max L.***

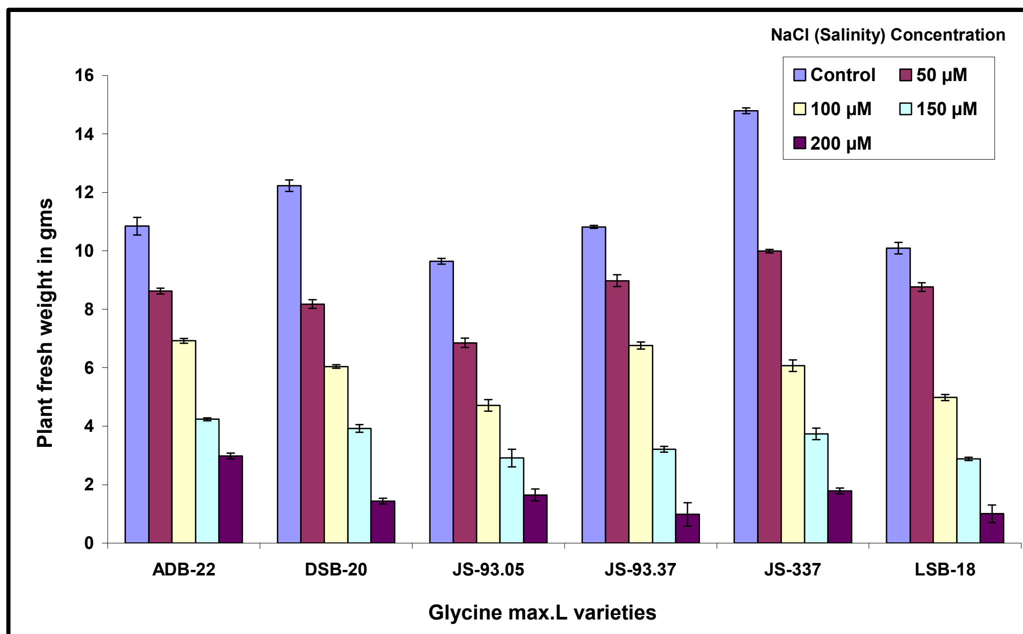

**Figure 3**
***Effect of different concentrations of NaCl (salinity) on plant dry weight of different cultivars of Glycine max L.***

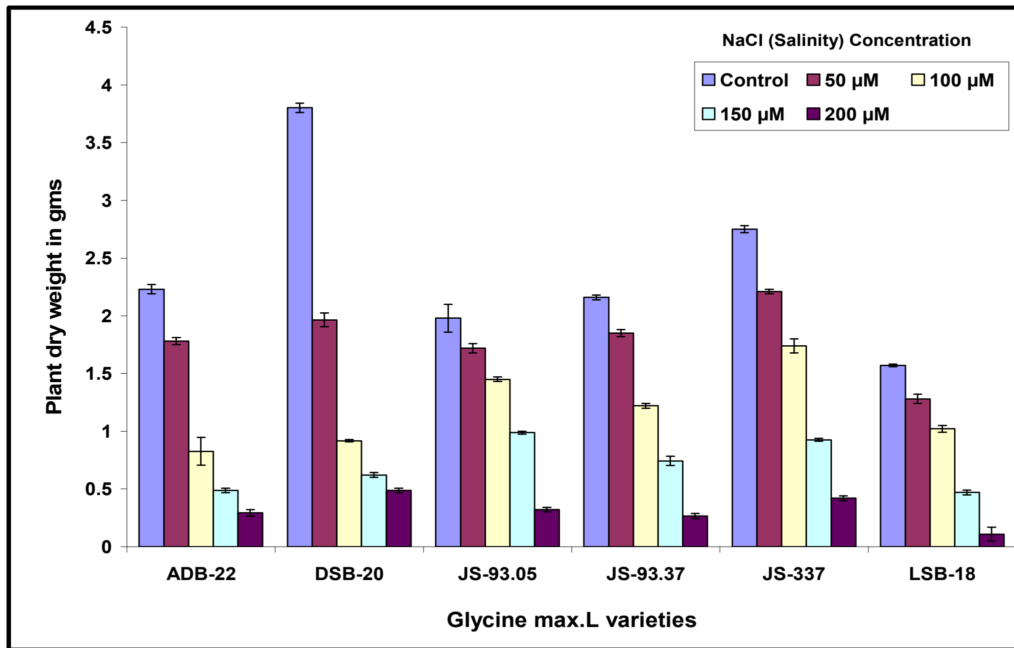


**Figure 4**
***Effect of different concentrations of NaCl (salinity) on Chlorophyll content of different cultivars of Glycine max L.***

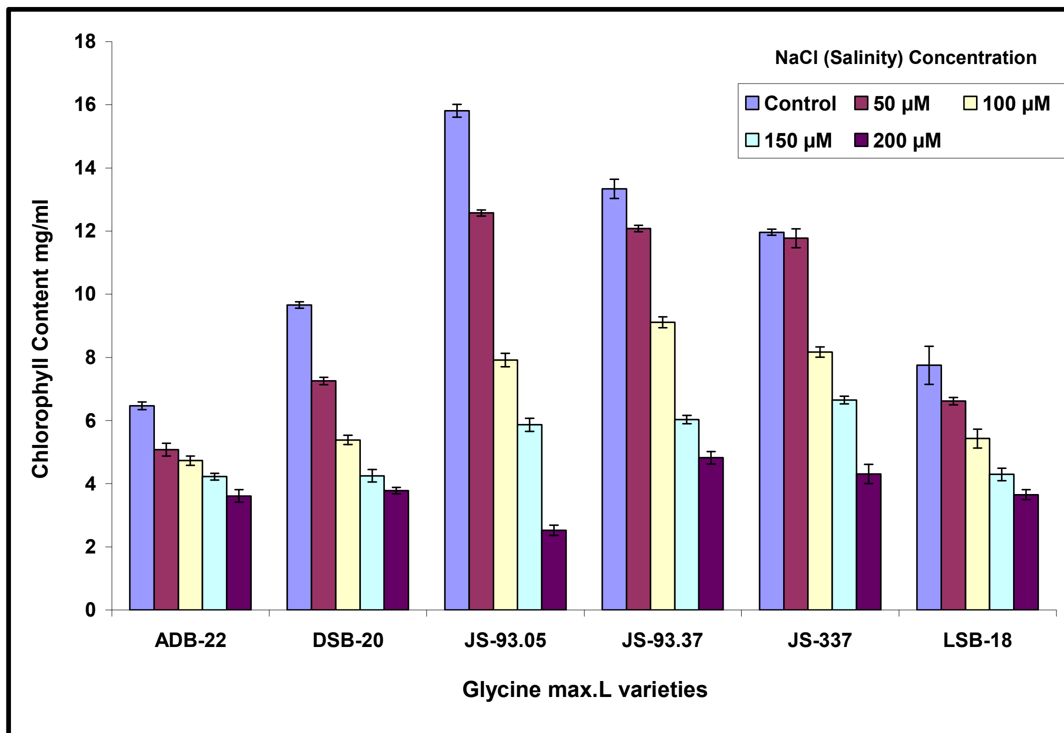

**Figure 5**
***Effect of different concentrations of NaCl (salinity) on total protein content of different cultivars of Glycine max L.***

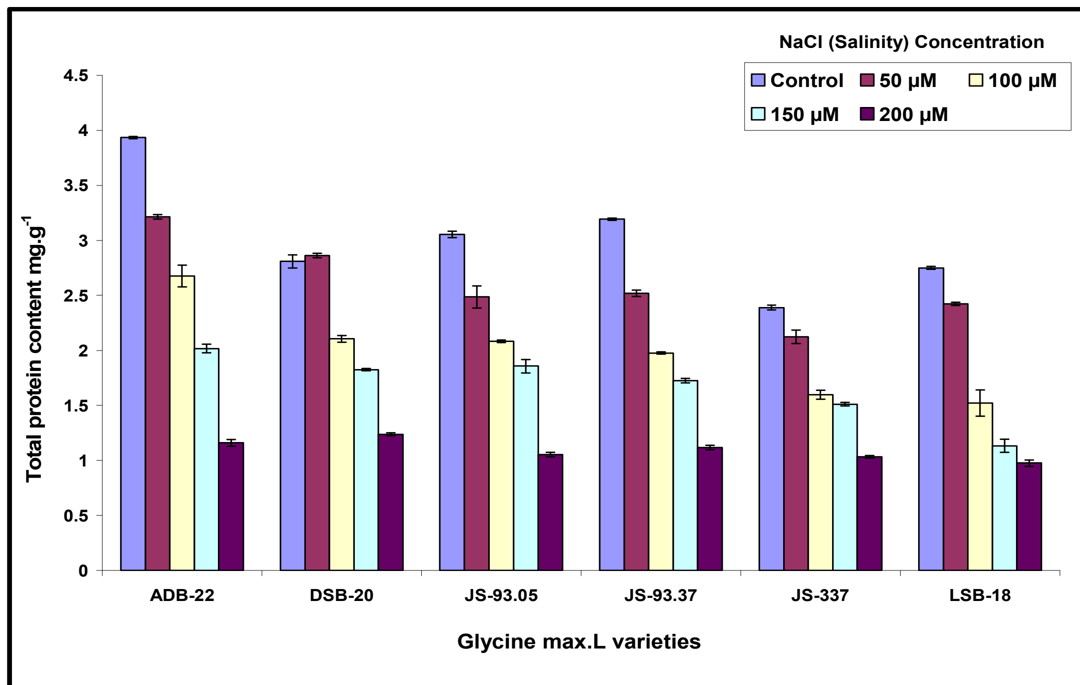


**Figure 6**
***Effect of different concentrations of NaCl (salinity) on Superoxide dismutase activity of different cultivars of Glycine max L.***

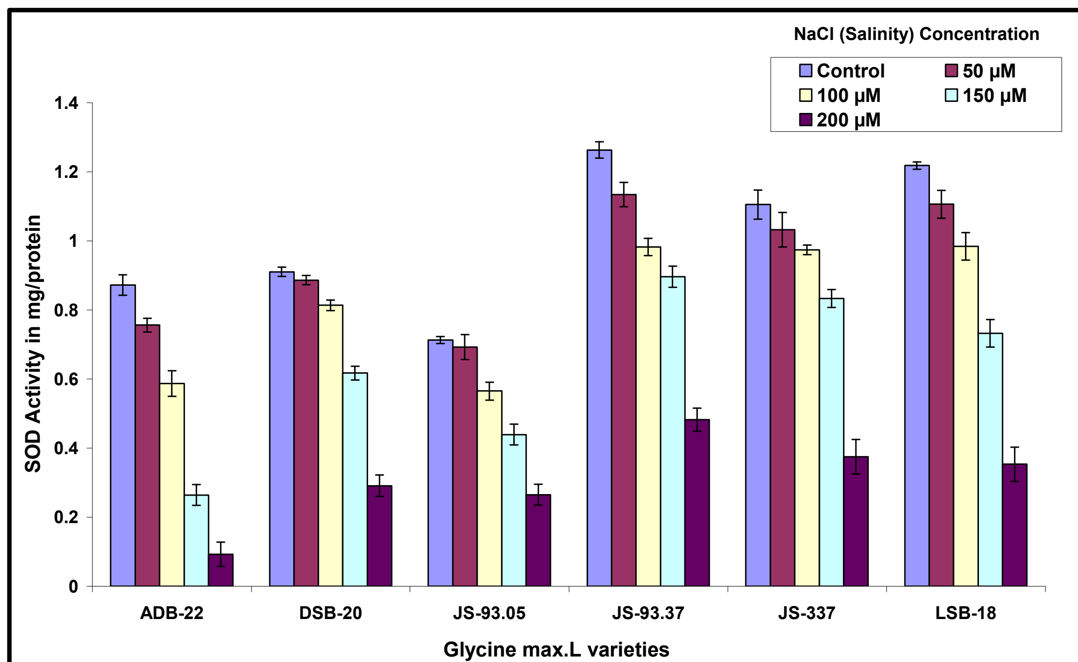

**Figure 7**
***Effect of different concentrations of NaCl (salinity) on catalase activity of different cultivars of Glycine max L.***

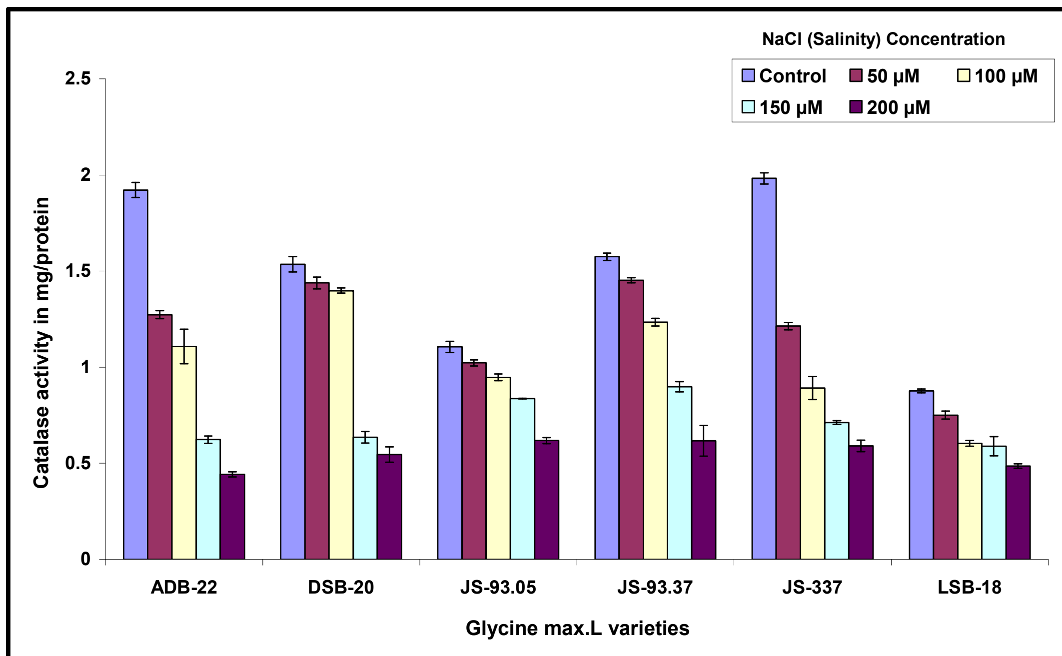


**Figure 8**
***Effect of different concentrations of NaCl (salinity) on Glutathione reductase activity of different cultivars of Glycine max L.***

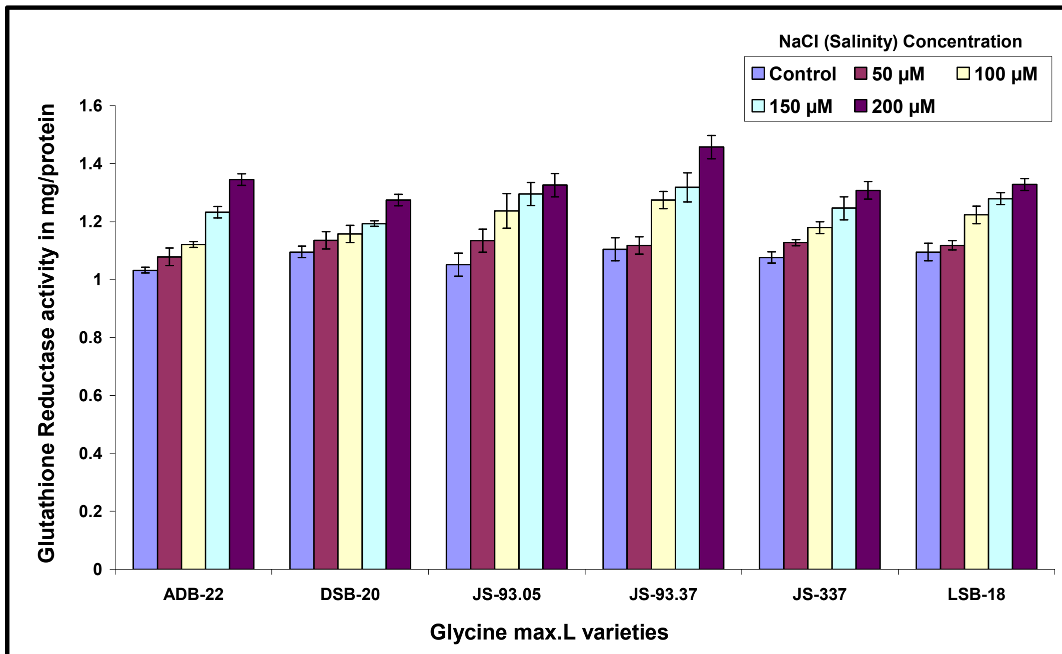


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**Figure 9**
***Effect of different concentrations of NaCl (salinity) on Ascorbate peroxidase activity of different cultivars of Glycine max L.***

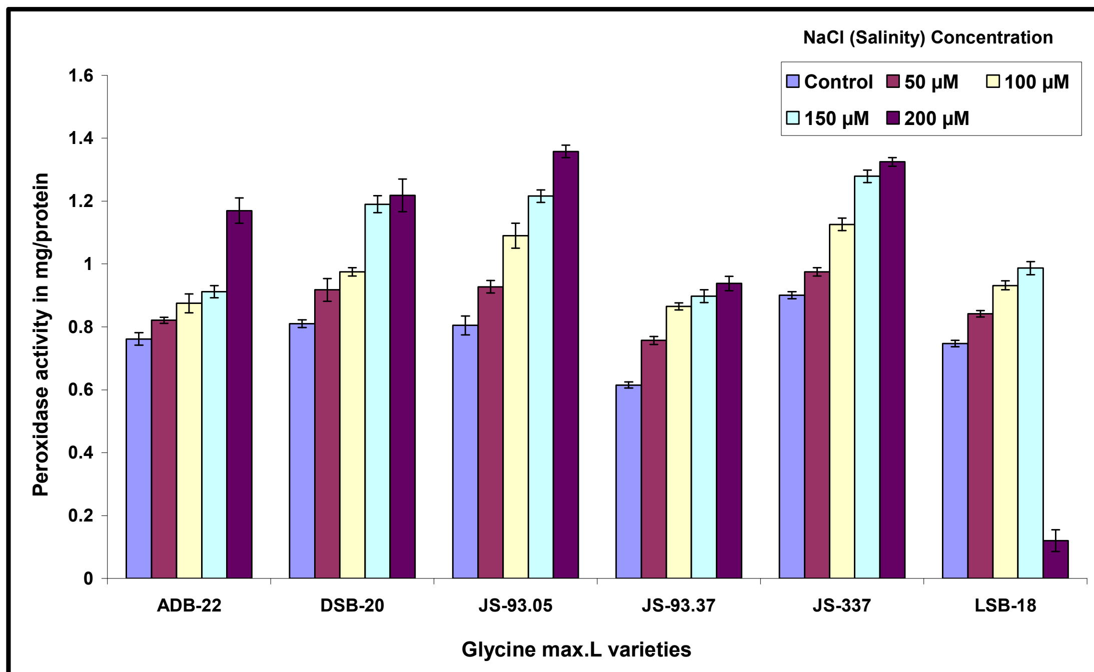
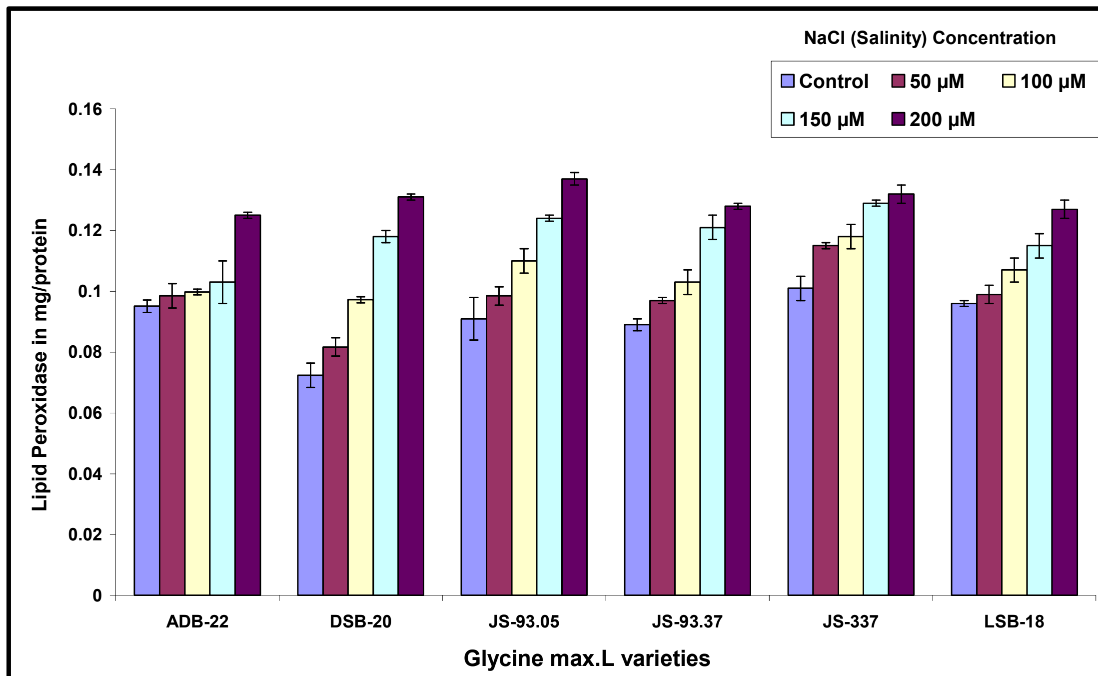



**Figure 10**
***Effect of different concentrations of NaCl (salinity) on Lipid peroxidation activity of different cultivars of Glycine max L.***



# DISCUSSION

The increase in concentration of salt in soil is causing decrease in agricultural yields in a wide variety of crop all over the world. Salinity causes many morphological, physiological and biochemical changes in growing plants. The biomass of *Glycine max* L. gradually reduced under saline conditions is an indication of severe growth limitations. The increasing concentration of salinity stress ie, NaCl concentration showed adverse not only the biomass, but also on plant height and its dry weight. In several legumes, such as faba bean [13], soybean (*Glycine max*) [14], and bean (*Phaseolus vulgaris*) [15], salinity was reportedly found to reduce shoot and root weights. At 200mM concentration of NaCl, more growth retardation was observed in the plants of *Glycine max* L. Decrease in chlorophyll content is the primary bioindicator of salt stress. Gama et al., 2007 reported that chlorophyll content, photosynthesis, transpiration rate and stomatal conductance were adversely affected in cultures of common bean during salinity stress. Other researchers suggested that more accumulation of sodium ($Na^+$) ions in shoots of salt sensitive genotypes (compared to the salt tolerant ones), is one of the most important factors affecting chlorophyll losses [16]. The total protein content in leaves showed decrease in all the six cultivars of *Glycine max* L. with the increasing concentration of NaCl.

The present study reveals that, as the concentration of salt increases, SOD and CAT activity decreases. Superoxide dismutase and catalase have been identified as enzymatic protectors against peroxidation reactions. Superoxide dismutase is an essential component of antioxidative defense system in plants; results showed decreased activity of superoxide dismutase in *Glycine max* L. plants growing under stress levels of NaCl. Superoxide dismutase activity in response to stress appears to be probably due to denovo synthesis of the enzymatic proteins. Catalase is universally present oxidoreductase that decomposes $H_2O_2$ to water and molecular oxygen and it is one of the key enzymes involved in removal of toxic peroxides [17]. The ability of plants to overcome oxidative stress is dependent partially on the induction of SOD activity and subsequently on the up regulation of other downstream oxidative enzymes. A decline in Catalase activity under stress conditions were observed in the present study which suggests a possible delay in removal of $H_2O_2$ and toxic peroxides mediated by catalases and inturn as enhancement in the free radical mediated lipid peroxidation under abiotic stress conditions. CAT activity decreased in all experimental plant cultivars. The decline in CAT activity is regarded as a general response to many stresses [8,18&19]. The reduction of CAT activity is supposedly due to the inhibition of enzyme synthesis or change in the assembly of enzyme subunits under stress conditions. It may also be associated with degradation caused by induced peroxisomal proteases or may be due to the photo-inactivation of the enzyme. Salinity stress is inevitably associated with increased oxidative stress due to enhanced accumulation of ROS, particularly O2- and $H_2O_2$ in chloroplasts, mitochondria and peroxisomes. As a result, the induction of antioxidant enzyme activities is a general adaptation strategy which plants use to overcome oxidative stress [20].

The present study indicates a significant increase in GR, APX and LPX activities induced due to the salinity stress in all the cultivars of *Glycine max* L. GR is found in chloroplast, mitochondria and cytoplasm and it catalyzes the rate limiting step of the ascorbate Glutathione pathway. GR activity increased with the rise of salt stress in all the cultivars tested. Elevated levels of GR activity could increase the ratio of $NADP^+$/ NADPH thereby ensuring the availability $NADP^+$ to accept electrons from photosynthetic electron

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transport chain, and minimizing the reduction of oxygen and formation of superoxide radicals. Effect of salt stress on GR activity has been studied in several plants. In rice [21], soybean [22] and mulberry [23] increased activity of GR was indicated under salt stress. Also GR activity was found to be higher in salt tolerant cultivars of mulberry, tomato and soybean [24]. In our study it was observed that in leaf tissues of all the six cultivars, there was a significant increase in APX activity under salt stress. APX activity has been studied in many other plants. It is indicated that APX seems to be a key enzyme in determining salt tolerance in citrus as its constitutive activity much higher in salt tolerant cultivar. APX activity has been shown to be higher in tolerant cultivars of pea [25], mulberry [23], and tomato [26] under salt stress suggesting its role in salt tolerance mechanism.

As the concentration of salinity (NaCl) increases, LPX increased in all the varieties of *Glycine max* L. The adverse effects of salt on membranes are results of the accumulating toxic ions and ROS. This ROS, especially the hydrogen peroxide and hydroxyl radicals, damage the membrane lipid peroxidation, damaging the membrane structure and integrity. Membrane lipid peroxidation is also mechanically important from the perspective of production of oxygen free radicals like OH and $H_2O_2$ that further causes enhanced oxidative injury [16].

## CONCLUSION

Salinity stress, which is an environmental problem has become a major constraint for crop production, because salt stress (NaCl) has both osmotic (cell dehydration) and toxic (ion accumulation) effects on plants. In the present study, after evaluating, the physiological, biochemical and antioxidant enzyme activities, it is clear that JS-93.37 may be the resistant variety followed by DSB-20 and ADB-22 may be the susceptible cultivar among the six high yielding cultivars of *Glycine max* L. examined. So from these findings it is clear that salinity (NaCl) triggered an antioxidant response in the plants. It is possible that induction of antioxidants and osmolytes is a part of an integrated strategy for stress defense mechanism. These antioxidants and osmolytes may serve as biochemical markers for salinity tolerance traits in plants. Moreover the recent molecular tools would eventually help in understanding and discovering the key enzymes involved in this stress tolerance. So exemplification by the identification and validation of severe key genes that improve stress tolerance of crops in field should be improved, which would increase the crop yield and there by agricultural economy.

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