



Changes in activity of Enzymes Involved in Maintaining ROS in ground nut during Salt Stress

Sonawane Amruta, Vadawale Ashutosh, Mihani Ritu, Robin Pushpa*

Department of Biochemistry, Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara-390 002, INDIA

Available online at: www.isca.in, www.isca.me

Received 2nd April 2014, revised 24th April 2014, accepted 7th May 2014

Abstract

Soil salinity is of great concern for agriculturalists as crop production is affected drastically. Understanding the biochemical changes that occur due to salt stress can help scientists decipher approaches to combat it. Ground nut is an important cash crop grown in Gujarat, a state with the longest coast line in India. Increase sea water ingress into land has detrimental effect on groundnut productivity. Salt stress in plants results in various changes, chief among which are a disruption in the oxidant/antioxidant balance. This balance is maintained due to the activity of enzymes like peroxidase, catalase and the extent of damage can be gauged by assessing the levels of hydrogen peroxide. In this study we report the biochemical changes seen in enzymes peroxidase, catalase and also monitor the levels of hydrogen peroxide at different concentrations of salt.

Keywords: Ground nut; salt stress, peroxidase, catalase, hydrogen peroxide, ROS.

Introduction

Salinity is one of the major problems facing agriculture with nearly 800 million hectare of land being rendered fallow due to salt. In India too, salinity is major problem with Gujarat the state with the longest coast line facing the major brunt. Salt stress results in reduced plant growth, altered metabolism, changes in ion concentration etc.¹ have shown that response to salt stress depends on the variety of groundnut. Due to salinity stress the balance between the production of reactive oxygen species (ROS) and the scavenging activity of antioxidants is disturbed resulting in oxidative damage. ROS is an important mediator of stress. Generation of reactive oxygen species (ROS) in plant tissues, such as superoxide anion radicals ($O_2^{\bullet-}$), hydroxyl radicals ($\bullet OH$), singlet oxygen (1O_2) and hydrogen peroxide (H_2O_2), is a common response observed in salt-stressed plants^{2,3}. So, in addition to its known osmotic and ionic effects, salt stress is also manifested as an oxidative stress⁴. In order to prevent oxidative damage, plants have evolved a complex antioxidant system, which includes both enzymatic and non-enzymatic components differentially found in cell compartments⁵. Enzymatic components include superoxide dismutase (SOD), which is a major scavenger of $O_2^{\bullet-}$ converting them into H_2O_2 . The H_2O_2 may be scavenged further by catalase (CAT), glutathione peroxidase (GPX) etc. The capacity to scavenge ROS and to reduce their damaging effects on macromolecules appears to represent an important stress tolerance trait⁶. A close correlation between the antioxidant capacity and NaCl tolerance has been demonstrated in several crops such as rice², tomato⁷ and maize³.

In view of the above discussion, we investigated the effects of salt stress on growth and development of groundnut (one of the

important cash crop in Gujarat) and changes in antioxidative enzyme activity in seedlings in vivo. This will help us understand the mechanism of groundnut's acclimation to salinity conditions.

Material and Methods

Plant material and growth condition: Mature dry seeds of *Arachis hypogea* (variety GG20) were washed under running tap water for 25 min. It was then subjected to, treatment with a solution of detergent Extran for 10 min, treatment of antifungal agent Bavistin for 10 min and finally washed thoroughly in sterile double distilled water and then surface-sterilized in 0.1% aqueous mercuric chloride for 10 min, rinsed 3–4 times with sterile double distilled water. The seeds were left soaked 4–6 hours in sterile double distilled water. The seeds so treated were then grown in vermiculite with distilled watered plants as control. To study the effect of salt stress plants were subjected to NaCl at concentration of 25, 50, 75 and 100 mM. The plants were grown in light/dark for 12/12h at $24 \pm 2^\circ C$. Effect of salt on growth was monitored as mentioned in the Results and Discussion section. To determine the effect of salt stress on height of plants the seeds were planted in separate pots containing mixture of normal soil and Farm yard manure in equal proportion. The pots were kept in dry open space to facilitate normal germination process. Each pot was irrigated separately with the particular NaCl solution twice a day. Control pot was irrigated with normal double distilled water.

Catalase Estimation: Catalase activity was estimated as described by Aebi⁸. 0.5 gm of fresh tissue was rinsed with distilled water. The tissue was ground with 5 ml of extraction buffer (K-phosphate buffer pH 7.2 containing 2% PVP) and a

pinch of glass powder in chilled condition with precooled mortal and pestle. The homogenate was centrifuged at 10,000 g for 15 min. at 4°C. The supernatant was used as enzyme extract. 100µl of enzyme extract was added with 875µl of assay buffer (K-Phosphate buffer pH 7.8). The reaction was started with the addition of 25µl of H₂O₂. The absorbance was taken at 240 nm for 1 min. Enzyme activity was computed by calculating the amount of H₂O₂ decomposed.

Peroxidase estimation: Peroxidase activity was estimated by the method Chance and Maehly 1955⁹. 0.5 gm of fresh tissue was rinsed with distilled water. The tissue was ground with 5 ml of extraction buffer (phosphate buffer pH 7.2 containing 2% PVP) and a pinch of glass powder in chilled condition with precooled mortal and pestle. The homogenate was centrifuged at 10,000 g for 15 min. at 4°C. The supernatant was used as enzyme extract. The assay system consisted of 2.4 ml of 0.1M Phosphate buffer (pH 6.0), 0.33 ml of Pyrogallol solution and 0.20 ml of H₂O₂ solution. This mixture was pipetted into a 10 mm cuvette and it was equilibrated in water bath until temperature reaches 20°C. The enzyme was added at zero time and mixed properly. The total reaction volume was made to 3ml and the increase in absorbance at 420 nm was recorded.

H₂O₂ Estimation: H₂O₂ levels were estimated by the method of Patterson et. al 1984¹⁰. 0.5 gm fresh tissue was weighed and rinsed with distilled water. Tissue was then ground under chilled conditions with 5ml 5% TCA and 0.15gm activated charcoal and glass powder in dark condition. The mixture was centrifuged at 10,000g for 20 min at 4°C. Supernatant is adjusted to pH-8.4 with 17M Ammonia solution and filtered. 1ml of colorimetric reagent used for peroxidase estimation was added to 0.5ml sample extract and incubated for 10' at 30°C. The absorbance was read spectrophotometrically at 505nm.

Results and Discussion

Effect of salt stress on seed germination and growth of ground nut: The pot experiment was planned and carried out to find out the level of natural salt tolerance and to study the effect of different concentrations of salt stress on ground nut germination. Salinity is a complex parameter and is contributed by number of salts out of which sodium chloride (NaCl) is dominant salt and is major among all followed by Sodium sulphate which is of secondary importance. To provide salt stress, different concentrations of NaCl solution were used (25 mM, 50 mM 75 mM and 100mM). Various parameters like days required for germination, % germination, shoot height, number of leaves, number of days required for flowering etc. were noted.

As shown in table 1, average 3 days were required for germination of seeds in control as well as low NaCl treatment. While the germination was delayed for the seeds kept on 100 mM NaCl treatment. There was no germination at all for the seeds exposed to 200 mM treatment. All other parameters were more or less similar for initial three NaCl treatments i.e. 10, 25 and 50 mM concentration. Height of plant treated with 25mM was not affected. Whereas in case of plants treated with 50mM, 100mM and 125mM shows significant decrease in plant height (figure 1) with respect to control. It is known that salinity induces a change in the signals of root origin, which changes the hormonal balance of the plant, and this affects root and shoot growth. Another reason for that is at higher salt concentration especially Na⁺ becomes toxic to plant by inactivating the enzymes like rubisco and disturbs the ion homeostasis that leads to cytotoxic effect in cell in turn leading to death of plant cells and thus decreases in shoot length.

Table-1
Effect of NaCl on germination and growth parameters of groundnut seeds

Observations	Control	10 mM	25 mM	50 mM	100 mM	200 mM
Pretreatment	4 hrs presoaking in 10 ppm GA3 with respective salt concentration					
Number of replicates	10	10	10	10	10	10
Average number of Days required for germination	3	3	4	6	8	Germination not seen
Avg. 'no. of seeds germinated	10	9	9	7	4	0
% seed germination	100.00	90.00	90.00	70.00	40.00	0.00
Average Number of compound leaves after 10 days	3-4	3-4	2-3	2	2	0
Average number of Days required for 1 st flowering	19	20	21	21	60	-
Average Number of flowers after 3 weeks	8	6	4	1	0	0
Average number of compound leaves after 21 days	9	9	6	5	3	0

Abscisic acid in plant plays major role in germination. It is known that at high salt concentration there will be increase in abscisic acid level that will lead to decrease in activity of α - amylase. Thus inhibiting the germination of seedlings.

A significant decrease in leaf area was observed in plants under high salt stress. Higher concentration of salt leads to accumulation of Na^+ in the cells. This leads to toxicity and in turn reduction in leaf area exposed for photosynthesis. With the increase of NaCl concentration there was reduction in the number of leaves and yellowing of leaves (figure 1 and 2). These parameters were also more or less same for the treatment up to 50 mM NaCl treatment compared to control however germination, number of leaves, morphological growth and days required to flower were severely affected at the 100 mM treatment. Hardly one flower was observed in one plant that to after 60 days of germination which took 3 times more duration than the normal control plants.

Based on these observations it was concluded that ground nut can naturally withstand salinity up to 50 mM without any aberration.

Levles of H_2O_2 during salt stress: H_2O_2 , in addition to being a toxicant, has been regarded as a signaling molecule and a regulator of the expression of some genes in cells. The levels of H_2O_2 could be used as a determinant of stress. H_2O_2 level was measured by Zhou et al.¹¹. The results of hydrogen peroxide estimation showed an increase in H_2O_2 level under high salt stress. At the increasing concentration of salt many reactive oxygen species are produced including hydrogen peroxide as result of free radical chain process in plant.

Effect of salt stress on catalase activity: The activities of catalase increased with the increase in the concentration of NaCl (Figure 4). The increase in the activities of POD and CAT with the increase of NaCl concentration has been shown in S. sieb by Yan Li¹².



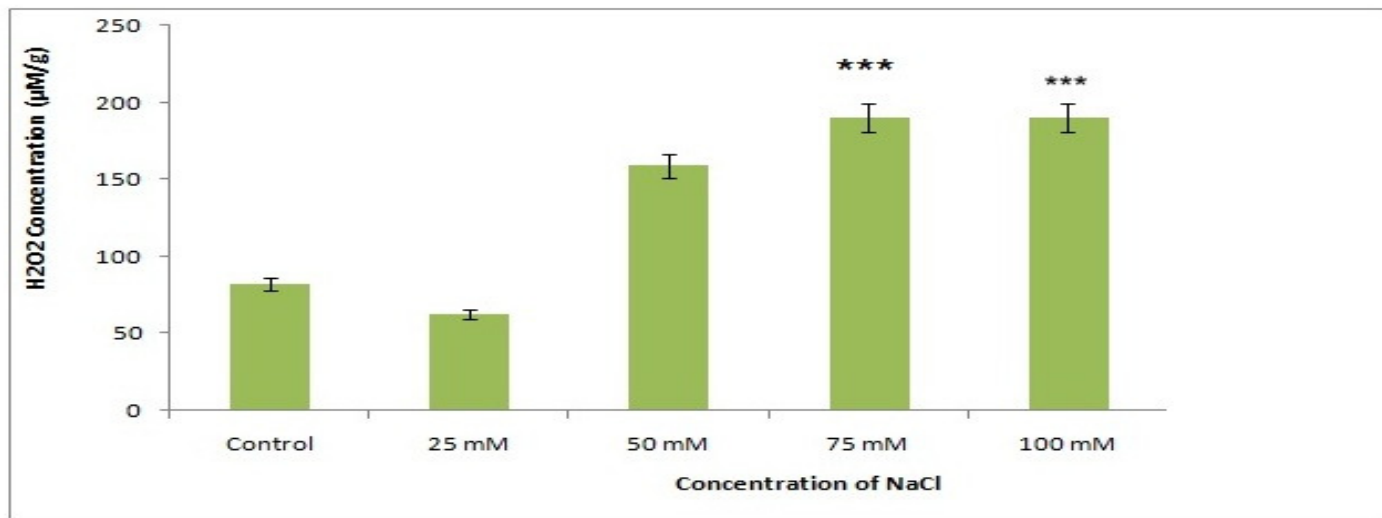
Figure-1
 Effect of different concentration of NaCl on plant growth and development after 21 days

Control	10 mM	25 mM	50 mM	100 mM	200mM
---------	-------	-------	-------	--------	-------



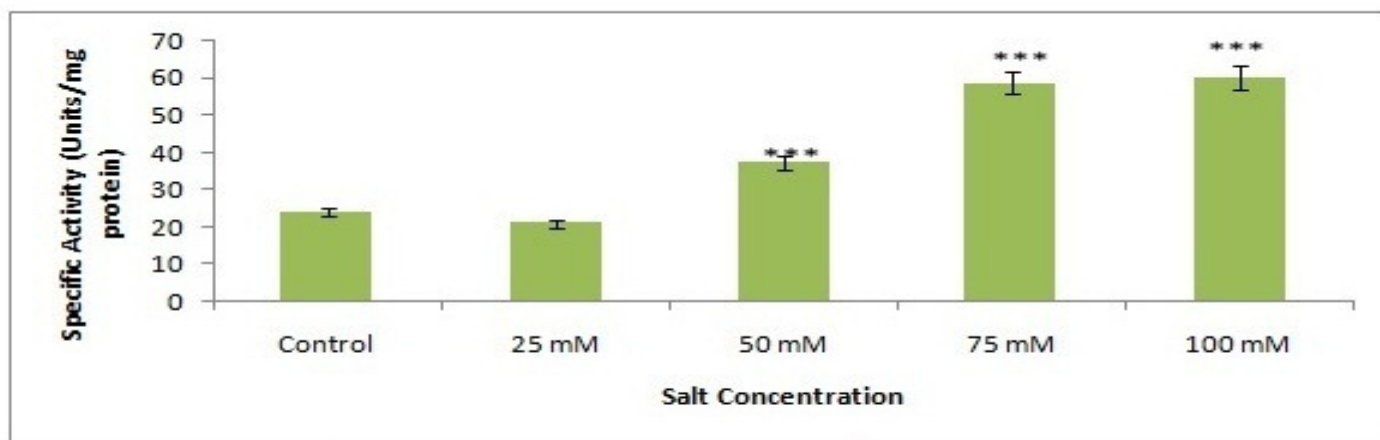
Figure-2
 Effect of NaCl on leaf size on 21 days

100mM	75mM	50mM	25mM	10 mM	Control
-------	------	------	------	-------	---------



Values are reported as Mean + SEM, *, ** and *** indicates significantly difference at $p < 0.05$ and $p < 0.001$ respectively as compared to the corresponding control. N=5

Figure-3
 Effect of NaCl on H₂O₂ levels



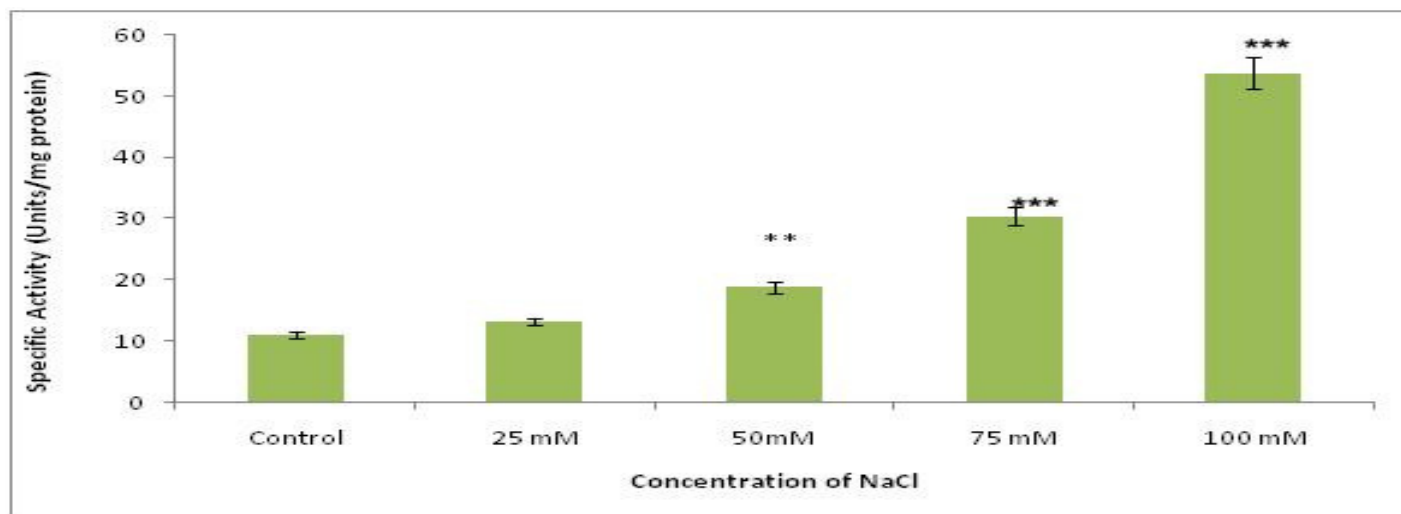
Values are reported as Mean + SEM, *, ** and *** indicates significantly difference at $p < 0.05$ and $p < 0.001$ respectively as compared to the corresponding control. N=5

Figure 4
 Catalase activity at different concentrations of NaCl

Catalase is involved in scavenging of reactive oxygen species. Results show that increasing activity with increased concentration of salt. At 75 and 100 mM NaCl the CAT activity increases 2-3 folds compared to that of control. The plant combat salt stress by increasing catalase activity as this would mean decrease in H₂O₂ levels. But at higher concentration of salt as 75mM and 100mM there was no further increase in activity and it is evident that, CAT activity was not adequate for the complete scavenging of H₂O₂ and thus to combat salt stress at high levels of stress.

Effect of salt stress on Peroxidase release: Peroxidase activity increased with increased exposure to salt indicating that the hydrogen peroxide produced during salt stress could be effectively removed by it. Peroxidase activity increased with respect to salt concentration. Unlike for catalase peroxidase activity was higher at 100 mM than at 75 mM indicating that peroxidase plays a major role in scavenging of H₂O₂ at higher levels of stress (figure5).

Our results showed that enzyme activities of CAT, peroxidase and levels of H₂O₂ increased significantly under salt stress conditions over their controls.



Values are reported as Mean + SEM indicates significantly different at $p < 0.05$ and $p < 0.001$ $p < 0.001$ respectively as compared to the corresponding control. N=5, *, ** and ***

Figure-5
Peroxidase activity at different concentrations of NaCl

Conclusion

Sessile plants have over the years learnt to use deleterious molecules like H_2O_2 to combat stress in more ways than one. The role of H_2O_2 as a signaling molecule is being proved without doubt by many scientists. Accumulation of H_2O_2 has a crucial role as a potent inducer of cell death and in cell signaling during stress. It is important to unravel the regulatory processes that govern H_2O_2 metabolism and find a way to prevent excessive H_2O_2 synthesis. In this paper too an attempt is made to understand the regulatory process by which H_2O_2 levels are regulated. From our studies it is clear that increase in salt stress increases H_2O_2 production. And this increase is kept in check by the concerted action of catalase and peroxidase. Our results also show that it is peroxidase that plays a greater role at higher levels of salt stress. Research has been directed to understand our overall knowledge concerning H_2O_2 physiology. It has proved the role of this molecule as a signaling network regulator, however more work in this area would be necessary to understand the entire mechanism. These studies will tell us how RSO is generated, maintained and more so how it could be used to improve crop yield under adverse conditions of stress.

Acknowledgement

The authors would like to acknowledge GSFC-Science Foundation for funding this project.

Reference

1. Nithila S., Durga Devi D., Velu G., Amutha R. and Rangaraju G, Physiological Evaluation of Groundnut (*Arachis hypogaea* L.) Varieties for Salt Tolerance and Amelioration for Salt Stress., *Res. J. Agriculture and Forestry Sci.*, **1(11)**, 1-8 (2013)
2. Vaidyanathan H., Sivakumar P. and Chakrabarty R., Thomas G Scavenging of reactive oxygen species in NaCl-stressed rice (*Oryza sativa* L.) differential response in salt-tolerant and sensitive varieties, *Plant. Sci.*, **165**, 1411-1418 (2003)
3. Azevedo-Neto A.D., Prisco J.T., Enéas-Filho J., Abreu CEB, Gomes-Filho E., Effect of salt stress on antioxidative enzymes and lipid peroxidation in leaves and roots of salt-tolerant and salt-sensitive maize genotypes, *Environ. Exp. Bot.*, **56**, 87-94 (2006)
4. Hernández J.A., Olmos E., Corpas F.J., Sevilla F. and Del Río L.A., Salt-induced oxidative stress in chloroplasts of pea plants, *Plant Sci.*, **105**, 151-167 (1995)
5. Mittler R., Oxidative stress, antioxidants and stress tolerance, *Trends Plant Sci.*, **7**, 405-410 (2002)
6. Møller IM, Jensen PE, Hansson A., Oxidative modifications to cellular components in plants, *Annu. Rev. Plant Biol.*, **58**, 459-481 (2007)
7. Amor N.B., Hamed K.B., Debez A., Grignon C. and Abdelly C., Physiological and antioxidant responses of the perennial halophyte *Crithmum maritimum* to salinity, *Plant Sci.*, **168**, 889-899 (2005)
8. Mittova V., Tal M., Volokita M. and Guy M., Salt stress induces up-regulation of an efficient chloroplast antioxidant system in the salt-tolerant wild tomato species *Lycopersicon pennellii* but not in the cultivated species, *Physiol. Plant.*, **115**, 393-400 (2002)

9. Aebi H., Catalase *in vitro*, *Method of Enzymology.*, **105**, 121-126 (1984)
10. Chance B. and Maehly A.C., Estimation of Peroxidase Activity, *Methods in Enzymology.*, **11**, 773-775 (1955)
11. Patterson B.D., Macrae E.A. and Ferguson I.B., Estimation of hydrogen peroxide in plant extracts using titanium (IV), *Anal. Biochem.*, **139**, 487-492 (1984)
12. Zhou B., Wang J., Guo Z., Tan H. and Zhu X., A simple colorimetric method for determination of hydrogen peroxide in plant tissues, *Plant. Growth. Reg.*, **61**, 121-125 (2006)
13. Yan Li., Effects of NaCl Stress on Antioxidative Enzymes of Glycine *Soja sieb*, *Pakistan. J. Biological Sciences.*, **12**, 510-513 (2009)