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Research Article
Sodium Chloride Primed Seeds Modulate Glutathione Metabolism in Legume Cultivars under NaCl Stress

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Abstract

Background and Objective: Soil salinity causes major inhibition in growth and productivity of legumes cultivars. Thus, it is necessary to protect legume crops grown in saline soil by cost effective, eco-friendly and farmer friendly methods. Present study investigated the degree of damage caused by salinity stress on ascorbate-glutathione cycle of some legumes and its possible reversal by priming of seeds with mild dose of NaCl. **Materials and Methods:** Experiments were carried out with the seedlings germinated from non pretreated and pretreated seeds (halo primed with 50 mM NaCl) of arhar (*Cajanus cajan* L. Mill sp. cv T120), maskalai (*Vigna mungo* L. Hepper cv. WBU109) and khesari (*Lathyrus sativus* L. cv. Nirmal) to characterize the toxic effects of sodium chloride on oxidative stress markers and glutathione metabolism of test seedlings and its possible reversal by NaCl pretreatment. Statistical analyses were performed by two-sided Student's t-test (considered statistically significant at $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$) and regression analyses were done using Minitab 17 software. **Results:** Salinity resulted in increased amount of total glutathione contents (136% in root and 39% in shoot of arhar, 103% in root and 67% in shoot of khesari and 86% in root and 81% in shoot of maskalai) and reduction in ascorbate contents (reduced by 15% in root and 18% in shoot of arhar, 13% in root and 20% in shoot of khesari and 14% in root and 21% in shoot of maskalai) in all the three tested legume cultivars. The variations in the above contents were due to differential activities of antioxidant and glutathione metabolizing enzymes as well as altered levels of oxidative stress markers under NaCl stress. However, by adopting pretreatment technology, such damage was found to be altered in pretreated legume seedlings variably promoting better growth. **Conclusion:** NaCl primed seeds exhibited significant alterations of all the tested parameters in legume seedlings that might promote better agricultural productivity in saline prone soils.

Key words: Soil salinity, legumes, NaCl, glutathione metabolism, priming

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

NaCl stress adversely affects growth and metabolism of plants. Salt exposure causes ion cytotoxicity resulting in generation of osmotic stress imparting metabolic imbalances. Salt stress enhances generation of Reactive Oxygen Species (ROS) viz., superoxide, hydrogen peroxide, hydroxyl radical and singlet oxygen leading to oxidative stress, disintegration of membranes and decreased photosynthetic activity[1]. All these factors attribute to cellular toxicity and nutritional deficiency in plants resulting in their retarded growth. Plants have developed an efficient antioxidant defense system bearing enzymatic and non-enzymatic components to combat such unavoidable circumstances[2].

To overcome such effects, plant produces compatible solutes as osmolytes which serve as protectant and maintain the osmotic balance within cell via continuous water influx. It has been reported that proline improves salt tolerance in olive (*Olea europaea*) and tobacco (*Nicotiana tabacum*) by increasing the activity of enzymes involved in antioxidant defense system[3-5]. Antioxidant metabolism, including antioxidant enzymes and non-enzymatic compounds, play critical parts in detoxifying ROS induced by salinity stress. Salinity tolerance is positively correlated with the activity of antioxidant enzymes and with the accumulation of nonenzymatic antioxidant compounds[6].

Glutathione is a metabolite, has many diverse metabolic functions in plants viz., protecting membranes by ROS detoxification and preventing oxidative denaturation of proteins under stress conditions[7].

Glutathione exists in two forms: Oxidized (GSSG) and reduced (GSH). GSH has often been considered to play an important role in the defense of plants against oxidative stress[8,9]. Glutathione reductase (GR) plays a crucial role in maintaining GSSG/GSH levels under stressed conditions[10,11]. In metabolically active tissues, GSH acts as a key redox buffer forming a barrier between cysteine groups of proteins and ROS[12]. In order to prevent the decrement in the total ascorbate pool during oxidation of ascorbate, dehydroascorbate (DHA) must be re-reduced. In plants, the reduction of DHA to ascorbate by GSH is a non-enzymatic reaction catalyzed by dehydroascorbate reductase[13]. During oxidative stress, the constituents of ascorbate-glutathione cycle regulate themselves to eliminate ROS efficiently. The enzymes of GSH synthesis and breakdown are induced during stress[14]. Some of the component enzymes like glutathione-S-transferase (GST) and glutathione peroxidase (GPx) show strong response towards ROS detoxification, thus preventing lipid peroxidation[15].

Plants can modulate their physiological ability to adapt in different environmental stresses. This phenomenon is known as acclimation. There are several evidences on acclimation of seedling growth to cold, drought, salinity and other environmental stresses[16,17]. Present study has been conducted with three legumes. Since the productivity of legumes are hampered under saline soils, cost effective, eco friendly and farmer friendly seed priming technique has been adopted which might generate seedlings in saline soils exhibiting better growth, metabolism and productivity. The objective of this study was to investigate the degree of damage in glutathione metabolism imposed by salt stress and its amelioration by priming of seeds with NaCl. The study also intended to find out the oxidative stress induced metabolic status of antioxidant defense system constituted by ascorbate-glutathione cycle in arhar (*Cajanus cajan* L.), maskalai (*Vigna mungo* L.) and khesari (*Lathyrus sativus* L.) seedlings under salt toxicity.

MATERIALS AND METHODS

Plant materials and experimental design: Experiments were carried out in three legume cultivars under controlled conditions (27-30°C under the influence of 16 h photo period at 200 μmol m^{-2} sec^{-1} photon irradiance) in the Plant Physiology and Biochemistry Laboratory, Department of Botany, University of Calcutta during 2014-17.

From Pulse and Oil Seed Research Institute Berhampore, West Bengal, India the seeds of arhar (*Cajanus cajan* cv. T120) maskalai (*Vigna mungo* cv. WBU109) and khesari (*Lathyrus sativus* cv. Nirmal) were collected. Surface sterilized seeds were placed on sterilized glass plate lined by blotting papers with 3 replicates containing suitable concentration of NaCl added in required proportion in hydroponic solution[18] (where hydroponic solution was treated as control) to prepare the salt solutions of 50, 100 and 150 mM concentrations. The plates containing seeds were kept under controlled laboratory conditions for 3 weeks. The possible alteration of NaCl toxicity was determined by pretreating the seeds with 50mM NaCl for 2 h prior to their exposure in higher NaCl concentrations[19]. During every year, seedlings were raised from non-pretreated and NaCl pretreated seeds between first week of November to first week of March. After stipulated period of growth, samples were harvested and stored at -80°C for further biochemical studies. All biochemical assays were performed during rest of the period of every year.

Chemicals: All the chemicals used for biochemical assays were of analytical grade. Sigma Aldrich (St. Louis, MO) supplied

Ninhydrin reagent, Tris-HCl buffer, bovine serum albumin (BSA), riboflavin, glutathione reductase (GR), 5,5'-Dithiobis [2-nitrobenzoic acid] (DTNB), L-methionine, L-glutathione-oxidized (GSSG), guiacol, L-glutathione-reduced (GSH) and 1-Chloro-2,4-dinitrobenzene (CDNB). Sulphosalicylic acid (SSA), toluene, potassium iodide (KI), trichloroacetic acid (TCA), glacial acetic acid, nitroblue tetrazolium (NBT), Nicotinamide Adenine Dinucleotide Phosphate (NADPH), L-ascorbic acid, Ethylenediaminetetraacetic acid (EDTA), Folin Ciocalteu reagent were supplied by Sisco Research Laboratories Pvt. Ltd. (SRL). Hydrogen peroxide (H_2O_2) was supplied by Merck.

Estimation of oxidative stress markers: Proline contents were estimated following the method of Huang *et al.*[20]. Plant tissues extracted with 5 mL of 0.1 M SSA was centrifuged at 5000 rpm for 30 min and supernatant was collected. Reaction mixture (having 2 mL of supernatant, 5 mL glacial acetic acid and 5 mL of 140 mM acid ninhydrin) after heated was cooled, followed by extraction in 10 mL of toluene. Absorbances of mixtures were measured at 520 nm and proline content was calculated from standard curve. Then, H_2O_2 contents was estimated according to the method followed by Gondim *et al.*[21]. Plant tissues extracted in 5 mL of TCA (0.1%, w/v) was centrifuged at 12,000 rpm for 15 min to obtain supernatant. Absorption of the reaction mixtures [0.5 mL supernatant, 0.5 mL of 0.05 M sodium phosphate buffer (pH 7.0) and 1 mL of 1 M KI solution] were measured at 390 nm. Then, H_2O_2 contents were determined using extinction coefficient (ε) (0.28 μM^{-1} cm^{-1}).

Assay of antioxidants: Ascorbate contents were recorded at 265 nm spectrophotometrically according to protocol followed by Huang *et al.*[22]. Superoxide dismutase (EC 1.15.1.1) activity was assayed following the method of Esfandiari *et al.*[23]. Reaction mixtures composed of 1.5 mL of 100 mM potassium phosphate buffer, 0.1 mL of 3 mM EDTA, 0.1 mL 2.25 mM NBT, 0.1 mL of 200 mM methionine, 0.1 mL supernatant (obtained from the extraction of 1g plant tissue in 50 mM Tris-HCL buffer) and finally 0.1 mL of 0.6 mM riboflavin was added to start reaction. Absorbance of sets exposed to fluorescent light (40 W) was read at 560 nm. Ascorbate peroxidase (EC 1.11.1.11) activity was measured following protocol of Ghosh *et al.*[10]. Then, APX activity was recorded from reaction mixtures containing 0.1 M sodium phosphate buffer (pH 7.0), 0.5 mM ascorbic acid, 0.1 mM H_2O_2, 0.1 mM EDTA and 0.1 mL supernatant (1 g tissue homogenized in 0.1 M sodium phosphate buffer, pH 7) at 290 nm. Data were calculated using extinction coefficient of 2.8 mM^{-1} cm^{-1}.

Assay of glutathione and glutathione based enzymes: Total glutathione contents were measured following the protocol followed by Sakr and El-Metwally[24]. Spectrophotometric values from reaction mixture composed of 0.1 mL supernatant (obtained from 1 g homogenized plant tissue), 100 mM potassium phosphate buffer (pH 7.5), 1 mM EDTA, 6 U mL^{-1} GR, 10 mM DTNB and 0.16 mg mL^{-1} NADPH was read at 412 nm and plotted in standard curve to determine GSH content. Glutathione reductase (EC.1.6.4.2) activity was done according to Sairam *et al.*[25]. Spectrophotometric readings from assay mixture containing 0.2 M potassium phosphate buffer (pH 7.5), 0.1 mM EDTA, 0.5 mL of 3 mM DTNB, 0.1 mL of 2 mM NADPH, 0.1 mL of 2 mM GSSG and 0.1 mL supernatant [extracted in 100 mM potassium phosphate buffer (pH 7.5)] at 412 nm was used to calculate GR activity using extinction coefficient of 6.22 mM^{-1} cm^{-1}. Glutathione peroxidase (EC 1.11.1.9) activity was measured according to Scebba *et al.*[26]. GPx activity was assayed in 65 mM phosphate buffer (pH 5.0) containing 11 mM H_2O_2 and 2.25 mM guaiacol. Activity of glutathione peroxidase enzyme was recorded at 470 nm and calculated using the extinction coefficient of 26.6 mM^{-1} cm^{-1}. Glutathione-S-transferase (EC 2.5.1.18) activity was measured according to Zhao and Zhang[27]. Enzyme activity assay was performed in 100 mM potassium phosphate buffer (pH 6.5) containing 5 mM GSH and 1 mM CDNB. Then, GST activity was measured at 340 nm and calculated using extinction coefficient of 9.6 mM^{-1} cm^{-1}.

Estimation of protein content for enzymatic assays: For all the enzyme preparations, protein contents were estimated according to Lowry *et al.*[28], using bovine serum albumin (BSA, Sigma) as standard and Folin Ciocalteu reagent. All sets were spectrophotometrically read at 660 nm and values were determined from standard curve.

Calculation of promotion and inhibition: Results for all the tested parameters have been expressed as average percentage values of promotion or inhibition. Promotion or inhibition percentage for a single parameter has been calculated.

The comparison between non-pretreated control sets and other directly salt treated sets is calculated by:

$$\text{Promotion or inhibition percentage} = \left[\frac{\text{Mean value of treatment}\{50/100/150\ (\text{mM})\} - \text{Mean value of non pretreated control}}{\text{Mean value of non pretreated control}}\right] \times 100$$

The comparison between non-pretreated control sets and other pretreated salt treated sets is calculated by:

$$\text{Promotion or inhibition percentage} = \left[\frac{\text{Mean of pretreated sets\{pretreated control 50/100/150 (mM)\}-Mean of nonpretreated control}}{\text{Mean value of nonpretreated control}}\right]\times 100$$

Thereafter, mean of all individual promotion/inhibition was calculated for every single parameter tested which has been used to express results.

Statistical analysis: Experimental data were means from three independent series, each done with two replicates and the results presented as mean±standard error (SE) were based on three repeats. Differences in significance of mean values of non-pretreated control and non pretreated/pretreated grown samples were statistically evaluated by two-sided Student's t-test. Regression analysis was performed using 'Minitab 17' software. For all tested parameters, different NaCl treated sets (X1) and NaCl pretreated sets (X2) were considered in generating the regression equations. The effects of stress on various parameters were considered statistically significant at $p \leq 0.001$ (denoted by 'a' indicating extremely significant), $p \leq 0.01$ ('b' indicating very statistically significant) and $p \leq 0.05$ ('c' indicating statistically significant).

RESULTS

Effect of NaCl on proline contents: In both root and shoot of arhar, maskalai and khesari seedlings, the amount of proline contents found to be increased by NaCl treatment than that of non-pretreated control (Fig. 1). NaCl treatment showed an increase in proline contents on an average, in arhar seedlings, to about 144% in root and 180% in shoot; in maskalai seedlings increased by about 25% in root and 22% in shoot; while in khesari seedlings increased by about, 28 and 30% in root and shoot, respectively over non-pretreated control. This effect was narrowed down with the application of seeds pretreated with 50 mM NaCl to about 123% in root and 152% in shoot of arhar seedlings, 17% in root and 16% in shoot of maskalai seedlings and 14% in root and 8% in shoot of khesari seedlings compared to non-pretreated control. Regression analysis showed that in non-pretreated sets,

For arhar:

- $Y = 44.32+(0.879)X1+(-0.225)X2$ (in root)

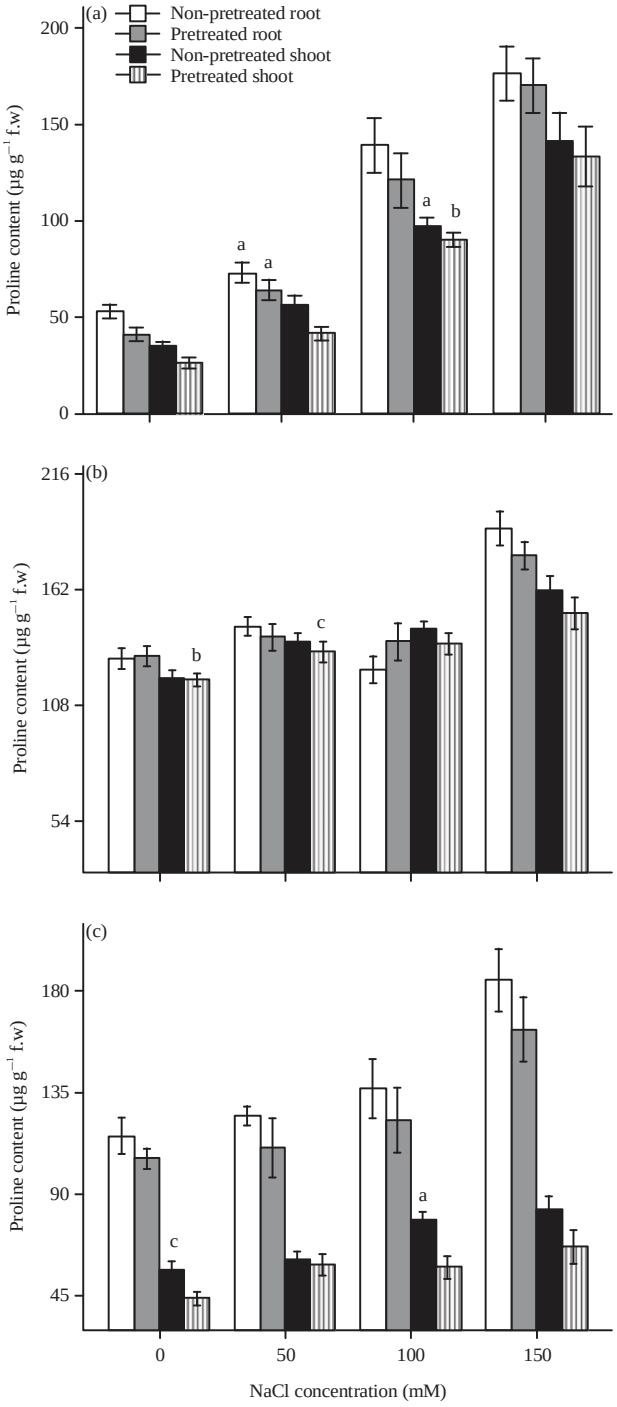

Fig.1(a-c): Effect of NaCl on proline content in root and shoot of 21 days old non-pretreated and pretreated (with 50 mM NaCl) test seedlings, (a) Arhar, (b) Maskalai and (c) Khesari

Data values are expressed as means of three experiments conducted with two replicates in each treatment±SE. a, b, c indicates statistically significant at $p \leq 0.001$, $p \leq 0.01$, $p \leq 0.05$, respectively over non-pretreated control

- Y = 27.76+(0.7275)X1+(-0.1935)X2 (in shoot)

For khesari:

- Y = 110.6+(0.402)X1+(-0.3)X2 (in root)
- Y = 57.7+(0.164)X1+(-0.26)X2 (in shoot)

For maskalai:

- Y = 129.8+(0.329)X1+(-0.155)X2 (in root)
- Y = 124.3+(0.226)X1+(-0.12)X2 (in shoot)

proline contents were positively correlated with increasing NaCl doses, whereas in pretreated sets, the contents were negatively correlated over non-pretreated control. The R^2 values in root and shoot were 0.9696 and 0.9697 in arhar, 0.8495 and 0.9086 in khesari and 0.8084 and 0.9502 in maskalai, respectively.

Effect of NaCl on the activities of superoxide dismutase: NaCl treatment on test seedlings showed increased activity of superoxide dismutase in both root and shoot (Fig. 2). In non-pretreated seedlings of arhar, the SOD activity was increased by 220% in root and 46% in shoot on an average, which was lowered down to 176% in root and 12% in shoot of halo primed seedlings. In non-pretreated maskalai, the increment of the superoxide dismutase enzyme activity was about 121% in root and 67% in shoot, on an average, which on halo priming of seeds was found to be reduced by 68% in root and 39% in shoot on an average. Non-pretreated khesari seeds on germination showed 166% increase in SOD activity in root and 55% in shoot, on an average, which was narrowed down after priming of seeds by 81% in root and 25% in shoot, on an average. Regression equations of non-pretreated sets.

For arhar:

- Y = 0.01216+(0.0002052)X1+(-0.0001235)X2 (in root)
- Y = 0.01417+(0.00005371)X1+(-0.00007245)X2 (in shoot)

For khesari:

- Y = 0.01817+(0.0001253)X1+(-0.0001748)X2 (in root)
- Y = 0.01495+(0.00003306)X1+(-0.0000472)X2 (in shoot)

For maskalai:

- Y = 0.01842+(0.000157)X1+(-0.00007195)X2 (in root)

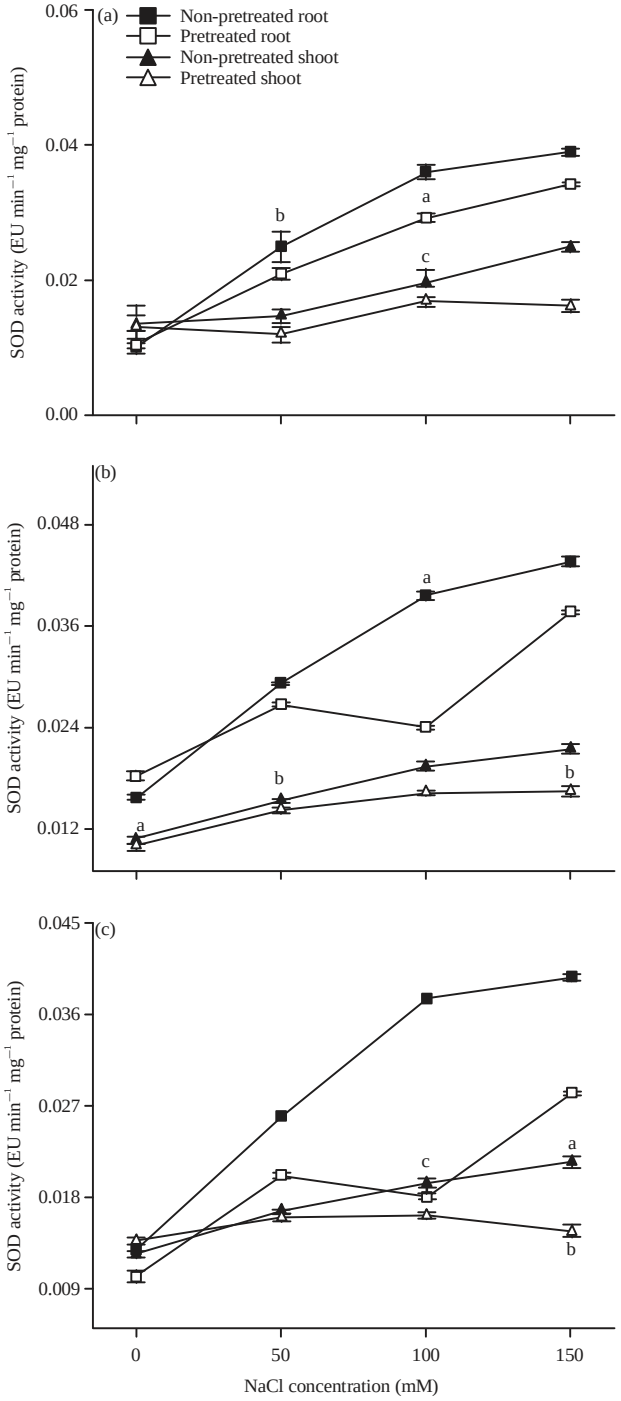

Fig. 2(a-c): Effect of NaCl on SOD activity in root and shoot of 21 days old non-pretreated and pretreated (with 50 mM NaCl) test seedlings, (a) Arhar, (b) Maskalai and (c) Khesari

Data values are expressed as means of three experiments conducted with two replicates in each treatment. a, b, c indicates statistically significant at $p \leq 0.001$, $p \leq 0.01$, $p \leq 0.05$, respectively over non-pretreated control

- Y = 0.01233+(0.00005809)X1+(-0.00005025)X2 (in shoot)

showed that superoxide dismutase activity was positively correlated with NaCl application. In pretreated samples, the superoxide dismutase activity decreased over non-pretreated control. The R^2 values for SOD activity in root and shoot were 0.9201 and 0.7796 in arhar, 0.8062 and 0.6225 in khesari and 0.8323 and 0.8945 in maskalai, respectively.

Effect of NaCl on H_2O_2 contents: In arhar, maskalai and khesari seedlings, H_2O_2 contents increased by NaCl treatment than that of non-pretreated control (Fig. 3), but the accumulation was more prominent in root than shoot. NaCl treatment showed an increase in H_2O_2 contents in arhar seedlings, on an average, to about 34% in root and 29% in shoot, in maskalai seedlings that increased about 7% in root and 3% in shoot whereas in khesari seedlings H_2O_2 contents increased to about 27 and 25% in root and shoot, respectively. This content decreased by only 2% in root and about 17% in shoot of arhar seedlings and 16% in root and 15% in shoot of khesari seedlings compared to non pretreated control, by the application of seeds pretreated with NaCl. While, the H_2O_2 contents got decreased to about 13% in root and 21% in shoot of pretreated maskalai seedlings, with respect to non-pretreated control. The regression equations for H_2O_2 contents (Y) were like:

For arhar:

- Y = 1.244+(0.00278)X1+(-0.0062)X2 (in root)
- Y = 1.21+(0.00357)X1+(-0.00125)X2 (in shoot)

For khesari:

- Y = 0.8298+(0.00207)X1+(-0.00135)X2 (in root)
- Y = 0.7632+(0.00189)X1+(-0.00115)X2 (in shoot)

For maskalai:

- Y = 1.308+(0.00062)X1+(-0.0046)X2 (in root)
- Y = 1.006+(-0.00008)X1+(-0.0039)X2 (in shoot)

The R^2 values for H_2O_2 contents in root and shoot were 0.9337and 0.7019 in arhar, 0.8863 and 0.8949 in khesari and 0.8811 and 0.8288 in maskalai, respectively.

Effect of NaCl on APX activity: The effect of NaCl on test seedlings showed increased activity of ascorbic acid

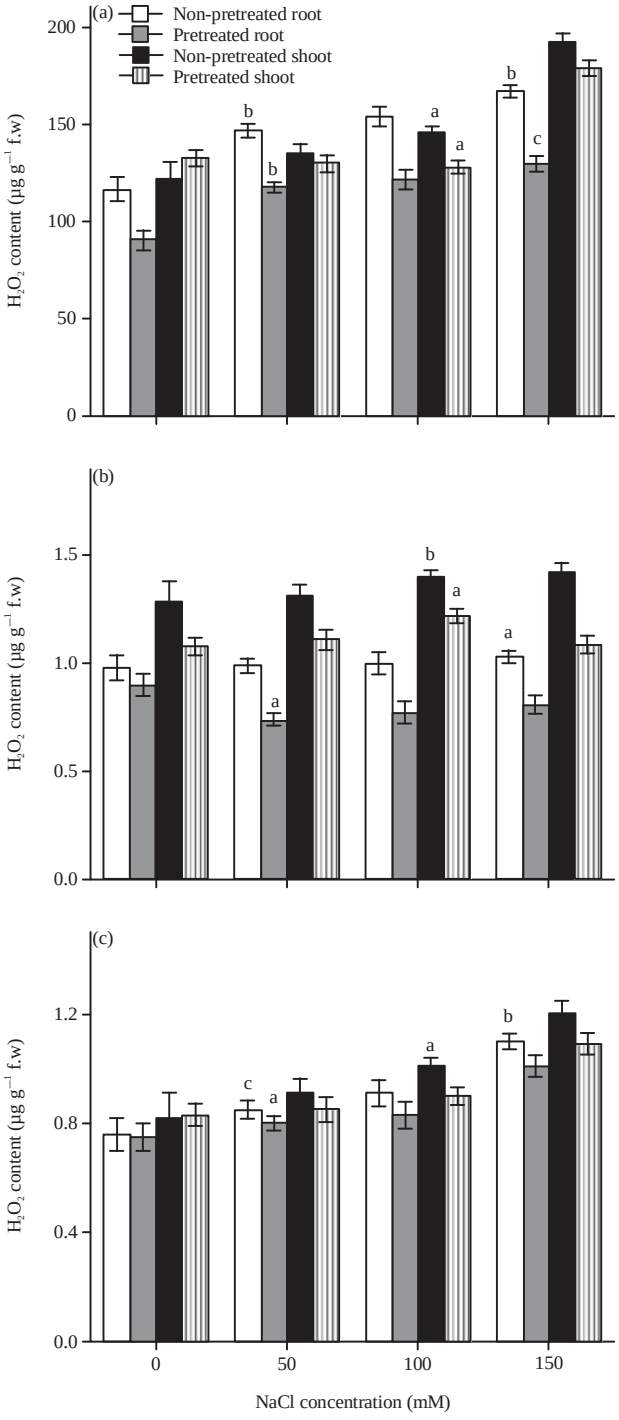

Fig. 3(a-c): Effect of NaCl on H_2O_2 contents in root and shoot of 21 days old non-pretreated and pretreated (with 50 mM NaCl) test seedlings, (a) Arhar, (b) Maskalai and (c) Khesari

Data values are expressed as means of three experiments conducted with two replicates in each treatment. a, b, c indicates statistically significant at $p \leq 0.001$, $p \leq 0.01$, $p \leq 0.05$, respectively over non-pretreated control

peroxidise (APX) (Fig. 4). The activity was increased on an average by 50% in root and 68% in shoot, on an average over non-pretreated control in arhar seedlings. When arhar seedlings were pretreated with NaCl, the increment in enzyme activity in root and shoot were found to be lowered down by 9 and 28%, respectively, on an average. In maskalai seedlings, the enzyme activity increased by NaCl treatment were about 38 and 168% in root and shoot, respectively, on an average, which were found ameliorated to about 33 and 120% in root and shoot, respectively, on an average, by using pretreated seeds. Whereas, in khesari seedlings, the activity increased by 25% in root and 170% in shoot, on an average, over control due to NaCl treatment. In halo primed sets the increment in enzyme activity in root and shoot were found to be decreased by around 17 and 109%, respectively, on an average. For APX activity (Y) in root, the regression equations were:

- Y = 0.0305+(0.00007)X1+(-0.00015)X2 (for arhar)
- Y = 0.03528+(0.000083)X1+(-0.000045)X2 (for khesari)
- Y = 0.0905+(0.00016)X1+(-0.0009)X2 (for maskalai)

For shoot, the regression equations were:

- Y = 0.03437+(0.000145)X1+(-0.000135)X2 (for arhar)
- Y = 0.0094+(0.000208)X1+(-0.00004)X2 (for khesari)
- Y = 0.02942+(0.000331)X1+(-0.000185)X2 (for maskalai)

The R^2 values for APX activity in root and shoot were 0.8047 and 0.9248 in arhar, 0.9810 and 0.9344 in khesari and 0.7816 and 0.9765 in maskalai, respectively.

Effect on total glutathione contents: In arhar, maskalai and khesari seedlings, total glutathione (GSH) contents increased by NaCl treatment (Fig. 5). The increase in GSH contents in arhar seedlings, on an average, to about 136% in root and 39% in shoot; in maskalai seedlings by about 86% in root and 81% in shoot and in khesari seedlings by about 103 and 67% in root and shoot, respectively over non-pretreated control. The content was narrowed down to about 52% in root and 37% in shoot of arhar seedlings, 37% in root and 47% in shoot of maskalai seedlings and 59% in root and 42% in shoot of khesari seedlings compared to non primed control, by the application of NaCl primed seeds. For GSH contents (Y), the regression equations stand out to be:

For arhar:

- Y = 78.5+(0.4787)X1+(-0.3663)X2 (in root)
- Y = 46.19+(0.4647)X1+(-0.4703)X2 (in shoot)

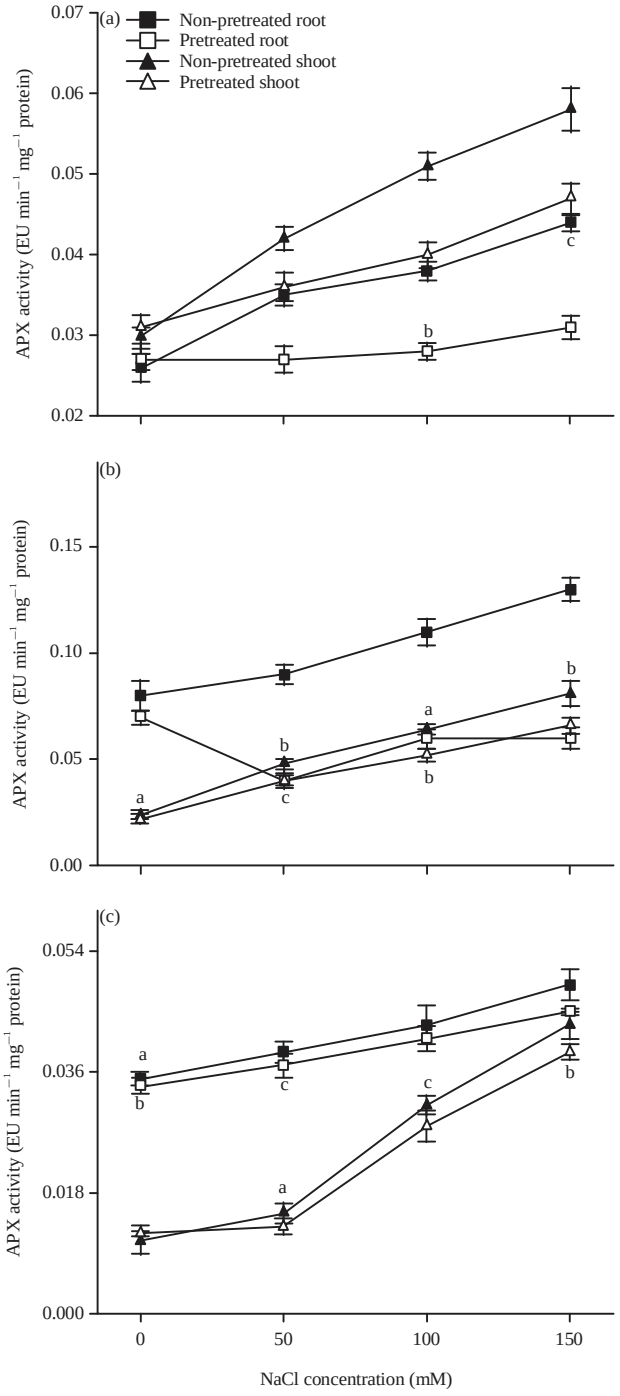

Fig. 4(a-c): Effect of NaCl on APX activity in root and shoot of 21 days old non-pretreated and pretreated (with 50 mM NaCl) test seedlings, (a) Arhar, (b) Maskalai and (c) Khesari

Data values are expressed as means of three experiments conducted with two replicates in each treatment. a, b, c indicates statistically significant at $p \leq 0.001$, $p \leq 0.01$, $p \leq 0.05$, respectively over non-pretreated control

For khesari:

- Y = 41.91+(0.7738)X1+(0.2425)X2 (in root)
- Y = 88.52+(0.468)X1+(-0.1284)X2 (in shoot)

For maskalai:

- Y = 59.93+(0.5241)X1+(-0.1803)X2 (in root)
- Y = 69.4+(0.5464)X1+(-0.3766)X2 (in shoot)

The R^2 values in root and shoot were 0.9615 and 0.9008 in arhar, 0.6715 and 0.7431 in khesari and 0.9939 and 0.9865 in maskalai, respectively.

Effect on ascorbate contents: The amount of ascorbate in root and shoot of arhar, maskalai and khesari seedlings were found to be decreased by NaCl treatment (Fig. 6). In non-pretreated seedlings the decrements in ascorbate contents were about, on an average, 15% in root and 18% in shoot in arhar; about 15% in root and 21% in shoot of maskalai; while in khesari seedlings that were about 13% in root and 20% in shoot over non pretreated control. Pretreatment of respective seeds with 50 mM NaCl, the rate of decrement of the ascorbate contents were narrowed down to about, on an average, 4% in root and get increased by 3% in shoot of arhar seedlings; to about 8% in root and 2% in shoot in maskalai seedlings. While in khesari seedlings, the ascorbate contents increased very little in root and the decrement narrowed down to 10%, on an average, in shoot over non-pretreated control. For ascorbate contents (Y), in case of arhar, regression equations were:

- Y = 0.9572+(-0.00153)X1+(0.00145)X2 (in root)
- Y = 1.493+(-0.00261)X1+(0.00325)X2 (in shoot)

In case of khesari:

- Y = 0.9293+(-0.00119)X1+(0.00165)X2 (in root)
- Y = 0.8315+(-0.00132)X1+(0.0008)X2 (in shoot)

In case of maskalai:

- Y = 0.8705+(-0.00194)X1+(0.0027)X2 (in root)
- Y = 1.499+(-0.00198)X1+(0.0014)X2 (in shoot)

The R^2 values in root and shoot were 0.7850 and 0.9162 in arhar, 0.8019 and 0.9436 in khesari and 0.9015 and 0.9591 in maskalai, respectively.

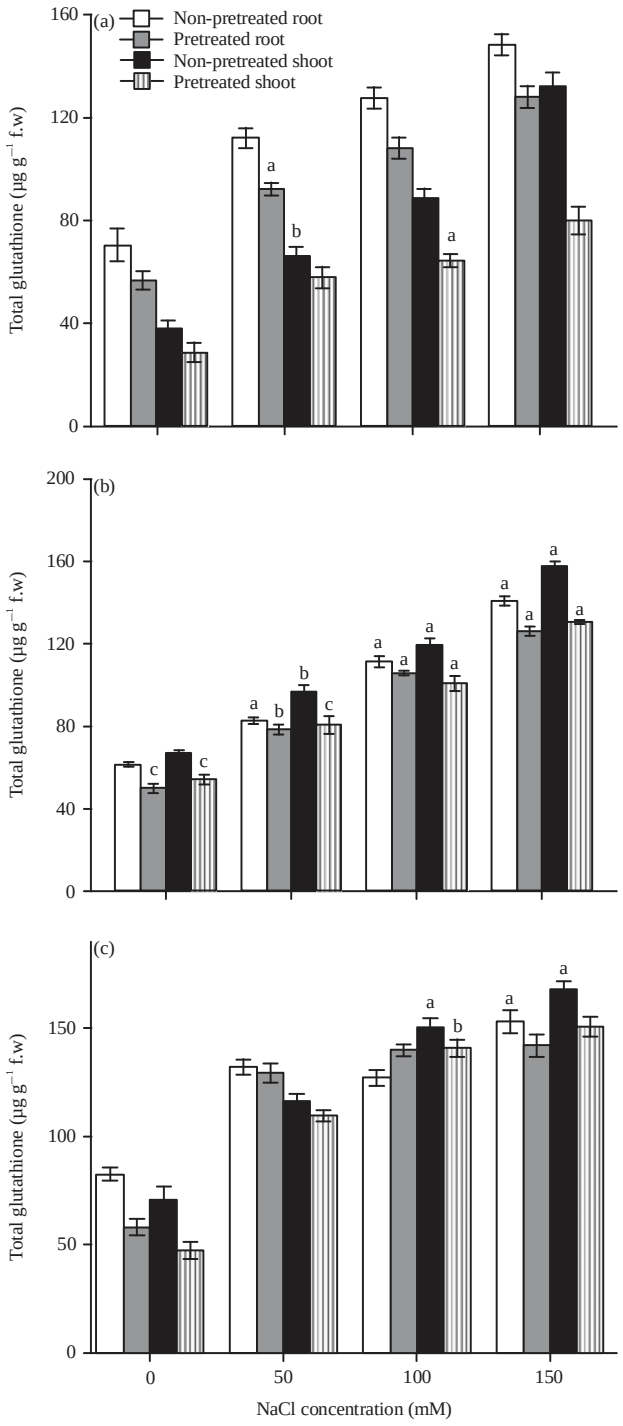

Fig. 5(a-c): Effect of NaCl on GSH in root and shoot of 21 days old non-pretreated and pretreated (with 50 mM NaCl) test seedlings, (a) Arhar, (b) Maskalai and (c) Khesari

Data values are expressed as means of three experiments conducted with two replicates in each treatment. a, b, c indicates statistically significant at $p \leq 0.001$, $p \leq 0.01$, $p \leq 0.05$, respectively over non-pretreated control

Effect on GR, GPx and GST activities: A considerable increment in the activity of glutathione reductase (GR) was observed in the test seedlings under NaCl treatment (Fig. 7). The test seedlings experienced an increase of about 155, 354, 370% in the root and 63, 86, 128% in shoot (arhar); 92, 165, 204% in the root and 10, 84, 116% in the shoot (maskalai) and 150, 172, 316% in root and 16, 129, 142% in shoot (khesari) under 50, 100 and 150 mM NaCl treatment in GR activity respectively. Pretreatment of seeds with NaCl showed partial decrement in the enzyme activity in all the test seedlings. In the roots of arhar and khesari seedlings, rate of increment were narrowed down to 23 and 92%, on an average, while shoots recorded narrowed increment in GR activity by 75 and 34%, respectively over non-pretreated control. Maskalai experienced less inhibition in enzyme activity after pretreatment that were, on an average about, 58% in root and 14% in shoot over non pretreated control. Regression equations in roots for GR activity (Y), were like:

- Y = 0.0713+(0.000456)X1+(-0.0013)X2 (for arhar)
- Y = 0.05052+(0.000573)X1+(-0.000485)X2 (for khesari)
- Y = 0.4042+(0.003857)X1+(-0.003685)X2 (for maskalai)

In shoots:

- Y = 0.01196+(0.0000482)X1+(-0.000162)X2 (for arhar)
- Y = 0.03558+(0.000399)X1+(-0.000295)X2 (for khesari)
- Y = 0.371+(0.002397)X1+(-0.002735)X2 (for maskalai)

For GR activity the R^2 values in root and shoot were 0.7259 and 0.7227 in arhar, 0.8868 and 0.8899 in khesari and 0.9572 and 0.8781 in maskalai, respectively.

In arhar, maskalai and khesari seedlings, the activity of glutathione peroxidase (GPx) found to be increased by NaCl treatment (Fig. 8). The NaCl treatment showed an increase in enzyme activity in root of arhar by about 106% and in shoot by about 126%, on an average than non-pretreated control. After pretreatment, appreciable recovery was also observed in the test seedlings and the increment was narrowed down in root by about 17 and 61% in shoot, on an average. The maskalai seedlings demonstrated proportionately less enzyme activity by NaCl treatment that was about 70 and 78%, on an average, in root and shoot, respectively, over the non-pretreated control. Pretreatment of seeds of Gpx cultivar with NaCl narrowed down the elevated enzyme activity on an average by 52% in root and 67% in shoot. Whereas, khesari seedlings showed moderate increment in the enzyme activity under

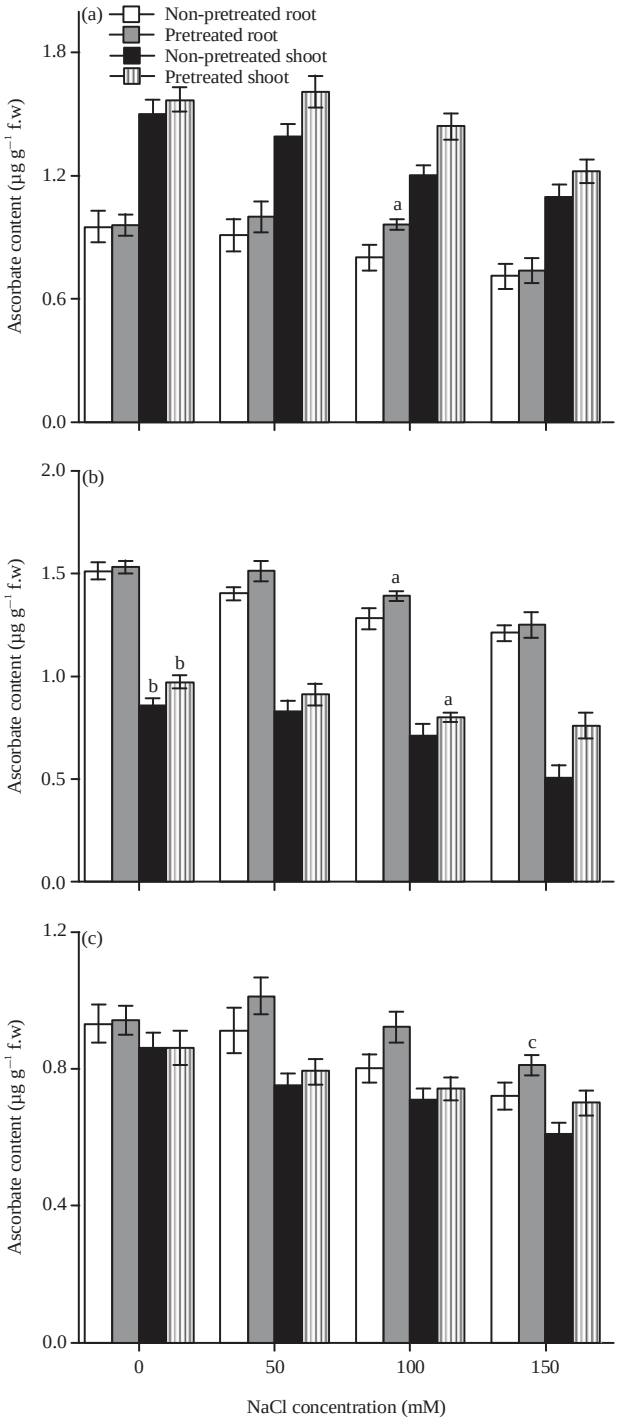

Fig. 6(a-c): Effect of NaCl on ascorbate content in root and shoot of 21 days old non-pretreated and pretreated (with 50 mM NaCl) test seedlings, (a) Arhar, (b) Maskalai and (c) Khesari

Data values are expressed as means of three experiments conducted with two replicates in each treatment a, b, c indicates statistically significant at $p \leq 0.001$, $p \leq 0.01$, $p \leq 0.05$, respectively over non-pretreated control

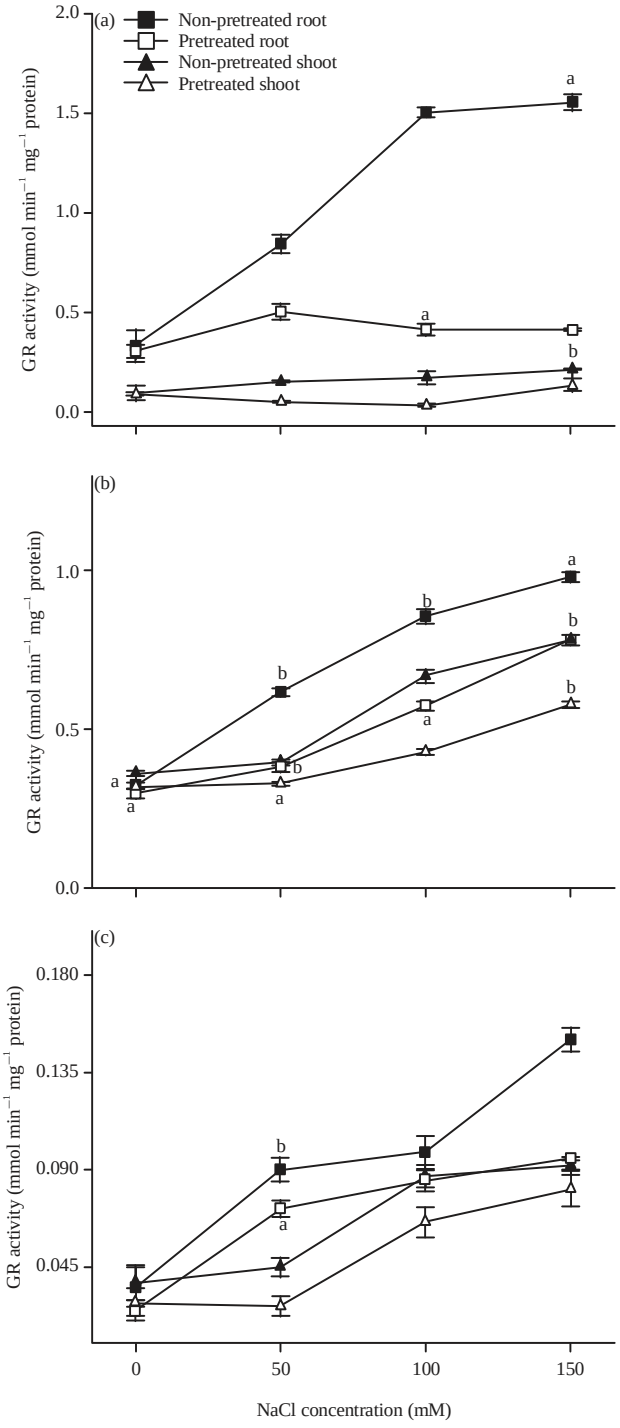

Fig. 7(a-c): Effect of NaCl on GR activity in root and shoot of 21 days old non-pretreated and pretreated (with 50 mM NaCl) test seedlings, (a) Arhar, (b) Maskalai and (c) Khesari

Data values are expressed as means of three experiments conducted with two replicates in each treatment. a, b, c indicates statistically significant at $p \leq 0.001$, $p \leq 0.01$, $p \leq 0.05$, respectively over non-pretreated control

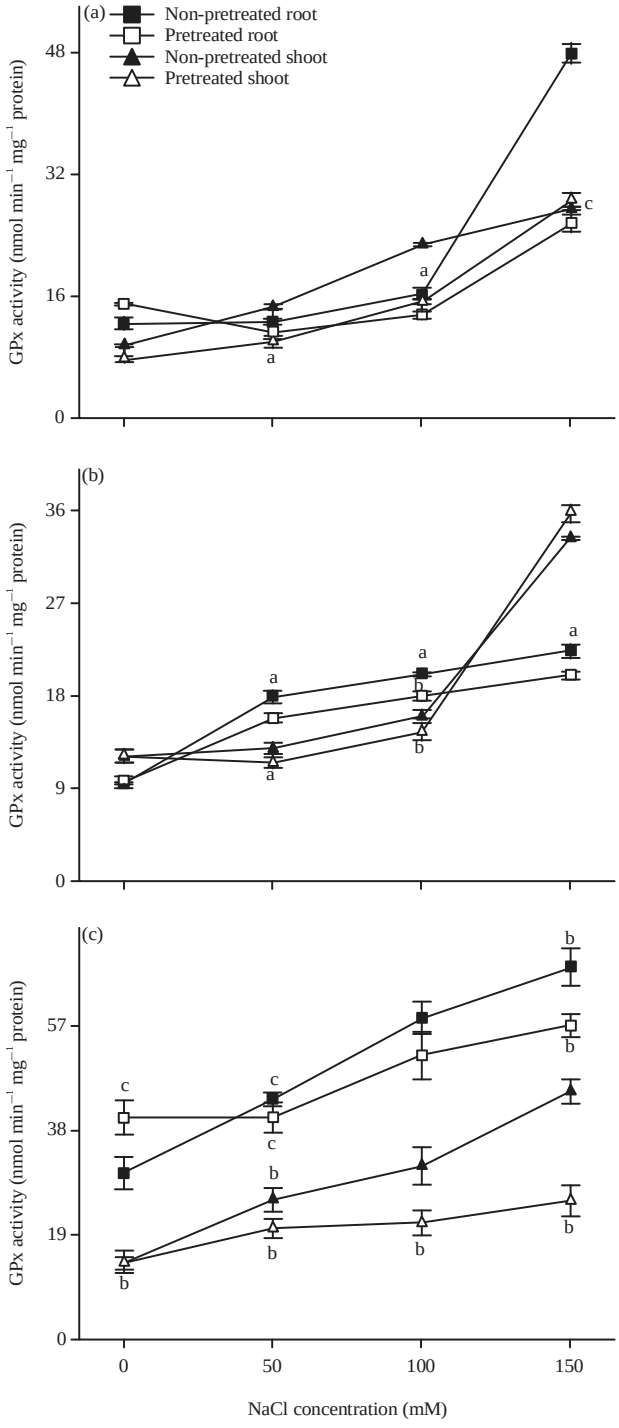

Fig. 8(a-c): Effect of NaCl on GPx activity in root and shoot of 21 days old non-pretreated and pretreated (with 50 mM NaCl) test seedlings, (a) Arhar, (b) Maskalai and (c) Khesari

Data values are expressed as means of three experiments conducted with two replicates in each treatment. a, b, c indicates statistically significant at $p \leq 0.001$, $p \leq 0.01$, $p \leq 0.05$, respectively over non-pretreated control

NaCl treatment that were about 87% in root and 142% in shoot, on an average, over control. After pretreatment, a good recovery was also observed in the said test seedlings and the increment was narrowed down by about 56% in root and 43% in shoot, on an average, over control. Regression equations were:

For arhar:

- Y = 0.8845+(0.01301)X1+(-0.006395)X2 (in root)
- Y = 1.085+(0.01526)X1+(-0.01658)X2 (in shoot)

For khesari:

- Y = 36.88+(0.186)X1+(-0.06798)X2 (in root)
- Y = 19+(0.1346)X1+(-0.1783)X2 (in shoot)

For maskalai:

- Y = 0.8064+(0.01404)X1+(-0.00027)X2 (in root)
- Y = 1.189+(0.007409)X1+(-0.003195)X2 (in shoot)

respectively. The R^2 values in root and shoot were 0.9273 and 0.6301 in arhar, 0.8692 and 0.8394 in khesari and 0.7022 and 0.8902 in maskalai, respectively.

The activity of glutathione-s-transferase (GST) increased in root and shoot of all the test seedlings by NaCl treatment (Fig. 9). In arhar seedlings increment in enzyme activity was about 63% in root and 45% in shoot on an average. This activity increased to about 120% in root and by 153% in shoot, on an average, by pretreatment of seeds with NaCl. In maskalai, the activity was increased by 157% in root and by 144% in shoot, on an average, by NaCl treatment. In pretreated seedlings further increment in enzyme activity was observed, in root by about 224% and in shoot it increased by three fold, on an average over non-pretreated control. Whereas, in khesari seedlings the increment of GST activity by NaCl treatment was about 217% in root and by 42% in shoot. The activity was found to get ameliorated and increased by about 245% in root and in shoot it increased by five folds on an average, after priming the seeds with NaCl. Regression equations were:

For arhar:

- Y = 22.09+(0.2363)X1+(0.9352)X2 (in root)
- Y = 43.94+(0.2344)X1+(1.092)X2 (in shoot)

For khesari

- Y = 4.771+(0.09855)X1+(0.08855)X2 (in root)
- Y = 5.284+(0.04341)X1+(0.2218)X2 (in shoot)

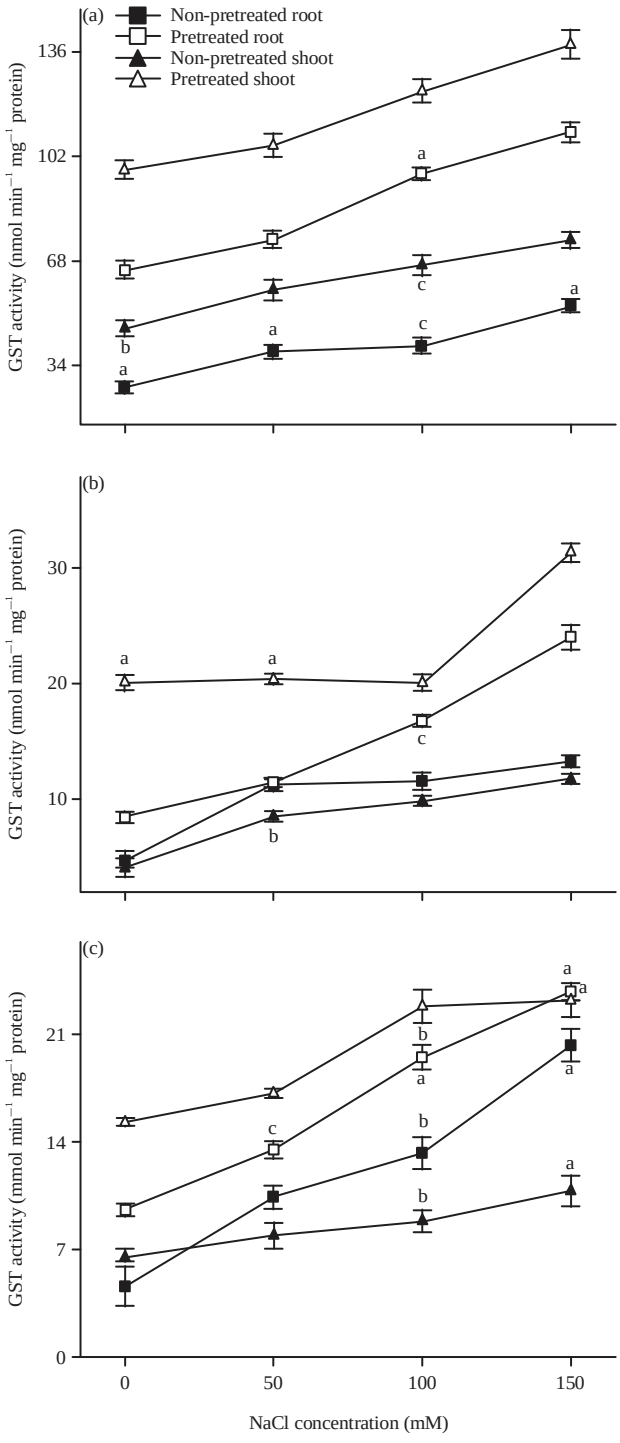

Fig. 9(a-c): Effect of NaCl on GST activity in root and shoot of 21 days old non-pretreated and pretreated (with 50 mM NaCl) test seedlings, (a) Arhar, (b) Maskalai and (c) Khesari

Data values are expressed as means of three experiments conducted with two replicates in each treatment. a, b, c indicates statistically significant at $p \leq 0.001$, $p \leq 0.01$, $p \leq 0.05$, respectively over non-pretreated control

For maskalai:

- $Y = 4.376+(0.07799)X1+(0.09965)X2$ (in root)
- $Y = 4.252+(0.05774)X1+(0.288)X2$ (in shoot)

The R^2 values in root and shoot were 0.9693 and 0.9893 in arhar, 0.9872 and 0.9644 in khesari and 0.8704 and 0.9230 in maskalai, respectively.

DISCUSSION

In the study, the authors speculated that the pretreated arhar, maskalai and khesari seedlings accumulated proline at lower proportion than non-pretreated seedlings. Maximum accumulation of proline was observed in non-pretreated seedlings of arhar followed by khesari and minimum in maskalai seedlings, indicating the tolerant nature of maskalai and the sensitive nature of arhar under NaCl stress. Elevated level of proline has been reported to confer increased tolerance to hyperosmotic stress that might have been the possible role of proline in non-pretreated plants[29].

A significant increase in SOD activity was observed in response to direct salt stress. The rate of increment of SOD activity in response to salinity was higher in root than that in shoot in all of the three test cultivars. Similar increase in SOD activity has been reported in *Jatropha* and mungbean[30,31]. However, NaCl pretreated arhar, maskalai and khesari seedlings showed decreased SOD activity in both root and shoot, which have also been reported in mungbean[31]. While maximum SOD activity was observed in root of arhar, the SOD activity was minimum in root of maskalai. After pretreatment, the increased activity of SOD that was observed in all the test seedlings during direct salt treatment got decreased both in roots and shoots, probably due to cellular detoxification. In the test seedlings, the H_2O_2 contents was found to increase under salinity, the contents being more in root than in shoot of all the three test cultivars. Maximum accumulation of H_2O_2 was observed in arhar and least in maskalai which could be correlated with the SOD activity obtained i.e. in arhar, maximum SOD activity resulted in more H_2O_2 conversion and minimum accumulation took place in maskalai attributing towards less toxic cellular environment which probably was an indication of its tolerance against salinity. Further, pretreatment with sublethal dose of NaCl caused a marked decrease in H_2O_2 level in the seedlings of arhar, maskalai and khesari. Therefore, the authors conclude that to restore normal growth and metabolism of the test seedlings by altering the activities of antioxidant enzymes it became possible by priming the seeds with sublethal dose of NaCl prior to treatment with lethal concentrations of NaCl. Parallel result was observed in salt stressed rice[29].

The APX activity was found to increase under salinity, the activity being more in root than in shoot of all the three test cultivars. Maximum activity of APX was observed in arhar and least in maskalai which can be correlated with SOD activity, i.e. in arhar, maximum SOD activity resulted in more APX conversion and minimum accumulation took place in maskalai attributing towards less toxic cellular environment which probably was an indication of its tolerance against salinity. Further, pretreatment with sublethal dose of NaCl caused a marked decrease in APX activity in all the tested seedlings. The authors conclude that to restore normal growth and metabolism of the test seedlings by altering the activities of antioxidant enzymes, it becomes possible to promote better growth by priming the seeds with sublethal dose of NaCl prior to treatment with lethal concentrations of NaCl. Similar ameliorative effects have also been reported in arsenic stressed wheat[10].

In the test seedlings treated with salt, there was elevation in the activity of APX. Up regulation of APX activity has also been reported in arsenic exposed rice[32], mung bean[33] and wheat[10]. The increased activities of APX provide circumstantial evidence for enhanced production of oxygen free radicals. APX activity in all the tested seedlings was found to increase more in shoots than in roots due to its abundance in chloroplastids. The rate of increment of APX activity under NaCl stress was maximum in maskalai whereas least in arhar and maximum accumulation of APX conferred its probable sensitive nature towards salinity.

A significant increase in GSH contents upon salt exposure was observed in all the studied legume cultivars. Thus, GSH protected the tested cultivars from oxidative damage by a greater level of ascorbate-glutathione pool. Similar observation has been reported in arsenic stressed lentils[34]. GSH contents were more in root than in shoot. In arhar, the rate of increment in GSH contents was maximum followed by khesari and least in maskalai. Thus, maskalai experienced least adverse effect of salinity due to its partial tolerance towards salt. The maximum GSH contents were observed in arhar which probably tried to generate more reducing environment conferring its ability to acclimatize under high NaCl concentrations. After pretreatment with sublethal dose of NaCl, the level of GSH was altered in all the tested legume cultivars, that resulted maintenance of low GSH/GSSG ratio due to release of stress.

It was demonstrated that ascorbate contents of the test seedlings were profoundly decreased with direct salt treatment. The maximum decrease in ascorbate contents was observed in shoot of maskalai, followed by khesari and least in arhar seedlings. However, since ascorbate acted as the substrate for APX, maximum decrease was found in shoot than root and pretreatment with sublethal dose of NaCl increased the level of ascorbate than those of non-pretreated test seedlings which was evident from low APX activity. Similar reports were observed in wheat exposed to arsenic stress[10].

Treatment with NaCl resulted in significant increase in GR activity in the test seedlings. This was because GR played an important role in the detoxification of salt induced ROS, possibly via the glutathione-ascorbate cycle. Similar trend was reported in arsenic stressed wheat[10]. The decreased activity of the enzyme by pretreatment of seeds with NaCl may be related with increased pool of antioxidant metabolites which acted as scavengers of various reactive species that influenced gene expression. The sole role for increased GR activity was to maintain a proper ratio of oxidized (GSSG) to reduced (GSH) glutathione. In non-pretreated seedlings, GR activity was more in root than shoot of all the tested legume seedlings. The rate of increment in GR activity was maximum in arhar, followed by khesari and maskalai seedlings, resulting in increased level of GSH contents which could be correlated with the increased GR activity. This indicated the ability of all the three tested legume cultivars to combat oxidative stress of variable amounts under saline condition.

The increase in GPx activity in both root and shoot of non-pretreated test seedlings could be correlated with the results obtained in arsenic stressed rice varieties[35]. The rate of increment was more in root than shoot. Maximum activity was shown by arhar with minimum activity in maskalai seedlings under salt exposure supporting the maximum necessity for detoxification and degradation of APX in arhar and minimum in maskalai. After pretreatment, with sublethal dose of NaCl altered results were observed in all the test seedlings that showed reduced production of cellular APX, thus less requirement of degradation of APX in the pretreated seedlings.

An increase in GST activity was observed in non-pretreated test seedlings of all the cultivars with the application of salt. The increment was more prominent in root than shoot. Maximum enzyme activity was observed in maskalai conferring maximum vacuolar sequestration of GSH thereby, reducing oxidative stress ensuring better tolerance. On the contrary, minimal activity was seen in arhar seedlings indicating least transport of GSH into the vacuoles as a result of which osmotic balance was affected indicating its probable sensitivity to saline environments. The ameliorative effect of pretreatment with sublethal dose of NaCl could improve the tolerance of salt stress in pretreated test seedlings by further increasing the enzyme activity. The obtained results were in accordance with the observations demonstrated by Saha *et al.*[36] in NaCl treated mungbean (*Vigna radiata* L. Wilckzek) and in maize seedlings[36,37].

Conducted study discloses the role of cost-effective priming technique in the tested legume cultivars that may help the poor farmers to grow legumes in salt affected agricultural fields with normal growth and productivity. However, field evaluation with pretreated seeds is required before its recommendation to the farmers.

CONCLUSION

Presented data concluded that salinity induced damage in ascorbate-glutathione cycle was recovered by priming of seeds with sublethal dose of NaCl. The obtained results showed altered metabolic status of all non-enzymatic as well as enzymatic antioxidant defense machinery in the test seedlings. Amongst the three legume cultivars, arhar (*Cajanus cajan* L. Mill sp. cv T120) was found to be the most sensitive under salt stress followed by Khesari (*Lathyrus sativus* L. cv. Nirmal) and least in maskalai (*Vigna mungo* L. Hepper cv. WBU109).

SIGNIFICANCE STATEMENT

This study discovers the possible role of seed preconditioning in the three tested legume cultivars that can be beneficial for the poor farmers to grow legumes in saline prone agricultural fields to increase legume productivity. This study will help the researchers to uncover the critical areas of seedling growth under saline conditions adopting farmer friendly seed priming technique that many researchers were not able to explore in the tested legumes. Thus, referred cost effective methodology will help in the growth and development of legume cultivars in saline prone agricultural lands.

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