International Journal of Pharmacy and Biological Sciences-IJPBS™ (2019) 9 (3): 941-947
Online ISSN: 2230-7605, Print ISSN: 2321-3272
Research Article | Biological Sciences | Open Access | MCI Approved
UGC Approved Journal

Effect of NaCl Stress on Antioxidant Metabolism in Black Gram (*Vigna mungo L.*) Seedlings

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Received: 16 Mar 2019 / Accepted: 18 Apr 2019 / Published online: 1 Jul 2019
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Abstract
The effects of salt stress on seedling growth (percent seedling survival rate, shoot and root length) and the activity of antioxidative enzymes were investigated in two important black gram cultivars (Maruthi and LBG-752) during salt stress period. Seeds were grown at different concentrations of NaCl stress [(0.0 (control), 50, 100 and 150mM] for six days. Salt stress resulted reduced seedling growth in both cultivars. Nevertheless, the rate of decrease in seedling growth (percent seedling survival rate, shoot and root length) was significantly more in cultivar LBG-752 than cultivar Maruthi. Salt stress resulted a gradually significant increase in superoxide dismutase (SOD: EC 1.15.1.1), peroxidase (POX: 1.11.1.7), catalase (CAT: 1.11.1.6) and glutathione reductase (GR: EC 1.6.4.2) activities in both cultivars with increase in stress levels. However, a significant increase in superoxide dismutase (SOD: EC 1.15.1.1), peroxidase (POX: 1.11.1.7), catalase (CAT: 1.11.1.6) and glutathione reductase (GR: EC 1.6.4.2) enzyme activities in both cultivars with increase in stress levels. These results possibly suggest that the cultivar Maruthi showed a better protection mechanism against oxidative damage by maintaining higher constitutive and induced activities of antioxidant enzymes than the cultivar LBG-752.

Keywords
NaCl stress, Black gram, Antioxidative enzymes.

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INTRODUCTION
Soil salinity is one of the major abiotic stress affecting germination, crop growth and productivity. Soil or water salinity leads to cellular water deficit and toxicity in all cells (Greenway and Munns 1980, Munns 2002). A range of different salts affects crops and amongst these, NaCl is the most prominent salt. Addition of salts to water lowers its osmotic potential, resulting in decreased availability of water to root cells. Salt stress thus exposes the plant to secondary osmotic stress, which implies that all the physiological responses, which are invoked by drought stress, can also be observed in salt stress (Sairam et al 2002). Salt stress causes inhibition of growth and development, reduction in photosynthesis, respiration, protein synthesis and disturbs nucleic acid metabolism (Boyer 1965, Kaiser 1987, Lambers 1985, Levine et al., 1990).

Plant tolerance to abiotic stress is a complex phenomenon that involves physiological, biochemical and molecular processes. Plants have developed different strategies to withstand salt stress (Zhu 2001a). These include mechanisms, which facilitate ion exclusion/sequestration (Yeo and Flowers 1983), accumulation of compatible solutes and turgor maintenance (Bohnert and Jensen 1996), metabolic switches to more efficient C_4-type of photosynthesis and detoxification of free radicals (Cushman and Bohnert 2000). In plant cells, one such protective mechanism is an antioxidant system, composed of both non-enzymatic and enzymatic antioxidants (Foyer et al., 1994). The non-enzymatic antioxidants include the major cellular redox buffers, ascorbate and glutathione (GSH), as well as tocopherol, flavonoids, alkaloids and carotenoids which remove, neutralize and scavenge the AOS. Enzymatic AOS scavenging mechanisms in plants include superoxide. Gosset et al (1994a) reported that during stress the tolerant species shows more antioxidant activities than susceptible cultivar in pea and maize. An increase in the activities of (glutathione reductase) GR and ascorbate peroxidase (APX) was also detected under the influence of salt stress in pea and maize (Mittova et al 1998). All the aforesaid aspects of osmo adaptation are facilitated in plants by novel gene expression triggered by signal transduction in response to water/ionic stress (Zhu 2001b).

In recent decades, considerable improvements in salinity tolerance have been made in crop species through conventional selection and breeding techniques (Shannon et al 1999, Ashraf and Harris 2004). The initial approach at the proteomic level is a valuable tool for further determination of genes potentially involved in salt tolerance. Genetic markers induced in response to salt stress have been obtained for more than a decade (Rani et al 1994).

Black gram (*Vigna mungo* L.) is one of the important pulse crop in India. This crop comes up reasonably well in drought prone areas, where other crops invariably fail to grow. Information is lacking regarding to the relative levels of salt tolerance among the existing Black gram cultivars. Therefore, the objective of the present study was to evaluate the effects of salt stress on plant growth and activities of antioxidant metabolism in two different Black gram cultivars. Further, these lines are using in breeding for salt tolerance; and is seems to be effective and economic improvement.

MATERIAL AND METHODS

Experimental design

The seeds of Black gram (Vigna mungo L.) cultivars namely (Maruthi and LBG) were procured from Acharya N.G. Ranga Regional Agricultural Research Station, Nandyal, Kurnool District, Andhra Pradesh, India. The healthy seeds were surface sterilized with 0.1% mercuric chloride (w/v) solution for 1 minute and thoroughly rinsed with distilled water and germinated in Petri plates lined with filter papers. Salt stress was induced by using sodium chloride solution at different concentrations (50, 100 and 150mM) prepared from half strength Hoagland's nutrient solution. Half strength nutrient solution alone served as control. The Petri plates were kept at room temperature of 25± 4oC in dark for six days. After six day's total seedlings were used for experimental analysis. The growth of seedlings in terms of shoot and root length was measured and recorded.

ACTIVE OXYGEN SPECIES DETOXIFICATION SYSTEMS

Superoxide dismutase (SOD) (EC: 1.15.1.1)

The activity of superoxide dismutase was assayed by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium as described by Beauchamp and Fridovich, (1971).

Extraction:

0.5 gr of seedling material was homogenized in 50 mM phosphate buffer (pH 7.0) containing 1% poly vinyl pyrrolidine. The homogenate was filtered and then centrifuged in a refrigerated centrifuge at 15000×g for 15 min, and the supernatant obtained was used as source of enzyme. All steps in the preparation of enzyme extract were carried out at 4°C.

Assay

The reaction mixture (3 ml) consisting of 50mM phosphate buffer (pH 7.8), 13mM methionine, 75µM nitroblue tetrazolium, 0.1mM EDTA, 2µM riboflavin and 0.1 ml of enzyme extract. Riboflavin was added lastly, and test tubes were shaken and placed 30 cm below a light source (30 W fluorescent lamps). The reaction was started by switching-on the lights. The reaction was allowed for 30 min and then stopped by switching-off the lights. The tubes were covered with black cloth. The reaction mixture which was not exposed to light did not develop colour and served as control. The absorbance was measured at 560 nm in a Shimadzu-1601, UV-Vis spectrophotometer. Log A_{560} was plotted as a function of the volume of enzyme extract used in the reaction mixture. From the resultant graph, the volume of the enzyme extract corresponding to 50% inhibition of the reaction was

read and considered as one enzyme unit and expressed as unit g^{-1} fresh weight min^{-1}.

Catalase (CAT) (EC: 1.11.1.6) and Peroxidase (POD) (EC: 1.11.1.7)

Extraction

The plant material was placed in a precooled mortor and ground with cold 50 mM Tris-HCl buffer (pH 7.0). The extract was passed through muslin cloth and centrifuged in a refrigerated centrifuge at 1000×g to remove cellular debris. The supernatant solution was centrifuged again at 10,000×g for 20 min. The supernatant was passed through a Sephadex G-25 column and fractions containing enzyme were pooled and used as enzyme source for the assay of catalase and peroxidase. All the steps were carried out in cold room.

Catalase assay

Catalase activity was assayed as per the method of Barber, (1980). The reaction mixture consisted of enzyme extract, 5 mM H_2O_2 and 50 mM Tris-buffer, pH 7.0. After incubating it for 1 min at 25^0 C, the reaction was stopped by adding 1.0 ml of 2.5 N H_2SO_4. The residual H_2O_2 was titrated with 0.01 N $KMnO_4$. A blank was maintained with the reaction mixture at zero time. Catalase activity was expressed as mg H_2O_2 oxidized g^{-1} fresh weight min^{-1}.

Peroxidase assay

Total peroxidase activity in the extracts was assayed as described by Hammerschimdt *et al*., (1982). The reaction mixture (3 ml) consisted of 0.25% (v/v) guaiacol in 10 mM sodium phosphate buffer (pH 6.0) containing 10 mM hydrogen peroxide. 25 µl of the crude enzyme extract was added to initiate the reaction which was measured spectrometrically at 470 nm (Shimadzu 1601). Total peroxidase activity was expressed as the increase in absorbance at 470 nm min^{-1} g^{-1} F.W (0.01 OD = 1 EU). Proteins in the extracts were quantified by the method of Lowry *et al.*, (1951) using BSA as the standard as described earlier.

Glutathione reductase (GR) (EC: 1.6.4.2)

The plant material was extracted in 100 mM potassium phosphate buffer (pH 7.0). The homogenate was centrifuged for 10 min at 10,000×g in a refrigerated centrifuge. The supernatant was passed through Sephadex G-25 column; active fractions were collected and used as enzyme source for the assay of glutathione reductase.

Assay

Glutathione reductase activity was assayed as per the method of Foster and Hess, (1980). The reaction mixture consists of enzyme extract, 100 mM potassium phosphate buffer; (pH 7.0) containing 1.0 mM EDTA, 150µM NADPH and 500µM oxidized glutathione. The enzyme activity was measured at 340 nm. Activity was calculated using the extinction coefficient for NADPH of 6.22 mM^{-1} cm^{-1} and expressed as mmol NADPH oxidized mg^{-1} protein min^{-1}.

Statistical Analysis

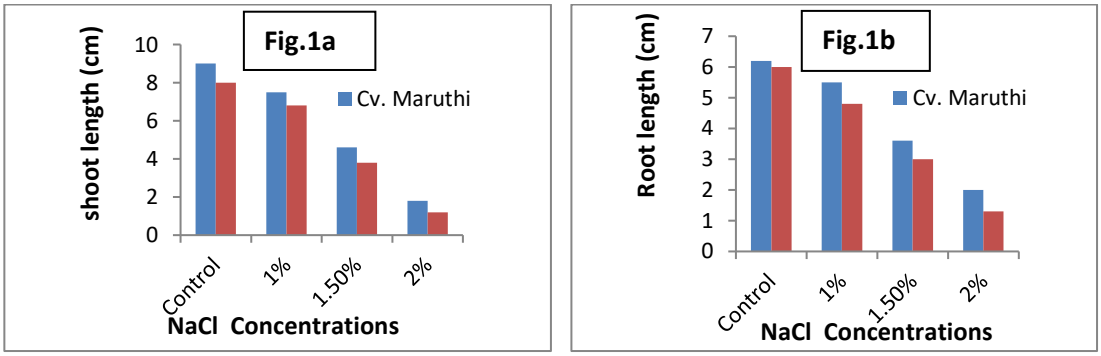
The data obtained in all cases were subjected to Analysis of Variance (ANOVA), and the mean values were compared by Duncan's Multiple Range (DMR) test at 0.05% level (Duncan, 1955).

RESULTS

Seedling growth:

The growth of the seedlings in terms of root and shoot length of all studied cultivars in control and stressed plants were measured and results were depicted in **figure 1A and 1B**. Salt stress showed significant inhibition of root and shoot length in two cultivars with increasing NaCl stress. However, the percent inhibition was high in cv. LBG and low in cv. Maruthi.

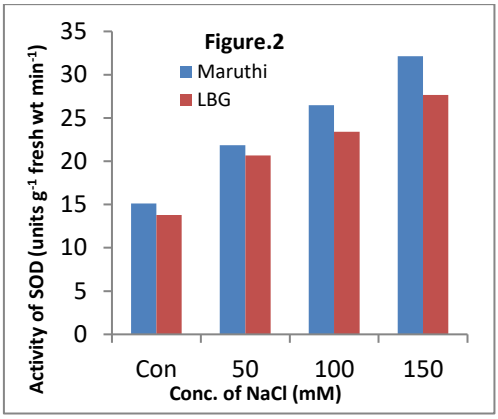
Figure 1: Shoot length and root length in 6-day old seedlings of two black gram cultivars under control and NaCl stress. (a) Root length (cm) and (b) shoot length (cm). Values are mean from five replications. Vertical bars indicate ±S.D.



Superoxide dismutase:

The enzyme superoxide dismutase activity was assayed in the seedlings of control and salt stressed plants on day-6 and the results are presented in **figure 2**. Superoxide dismutase activity was significantly elevated in the stressed seedlings over controls in both the cultivars. The superoxide dismutase activity did not appreciably increase at 50mM NaCl stress in both cultivars, but recorded a significant elevation in its activity at 100 and 150mM NaCl treatments in both cultivars. Nevertheless, the magnitude of increase in SOD activity was relatively more in the cultivar Maruthi, than in LBG, at 100 and 150mM NaCl levels on the 6th day after induction of stress.

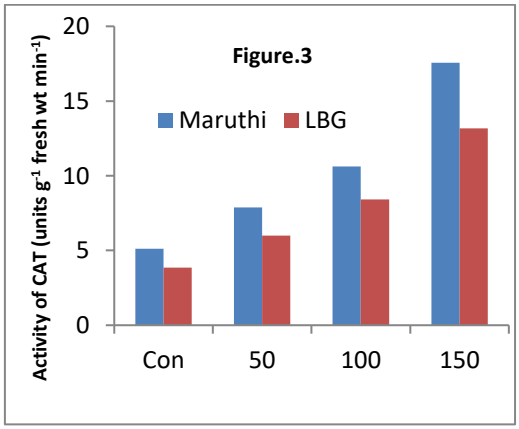
Figure 2: The activities of superoxide dismutase (SOD, unit's gm^{-1} fw min^{-1}) in seedlings of both control and stressed black gram cultivars on 6th day.



Catalase:

The activity of catalase was assayed in the seedlings of both control and NaCl stressed plants of Maruthi and LBG on day-6 and the data was represented in **figure 3**. Relative to controls, the activity of catalase was registered an increase in the stressed seedlings of both cultivars. The activity did not appreciably increase during low salt stress treatment at 50mM NaCl stress on day-6 in the cultivars, however it was significantly elevated during moderate and severe stress treatments in both cultivars. The rate of increase in enzyme activity was dependent on intensity of stress. Nevertheless, a greater per cent increase was recorded for the cv. Maruthi compared to cv. LBG at all stress regimes.

Figure 3: The activities of catalase (CAT, mg of H_2O_2 oxidised gm^{-1} fw min^{-1}) in seedlings of both control and stressed black gram cultivars on 6th day.

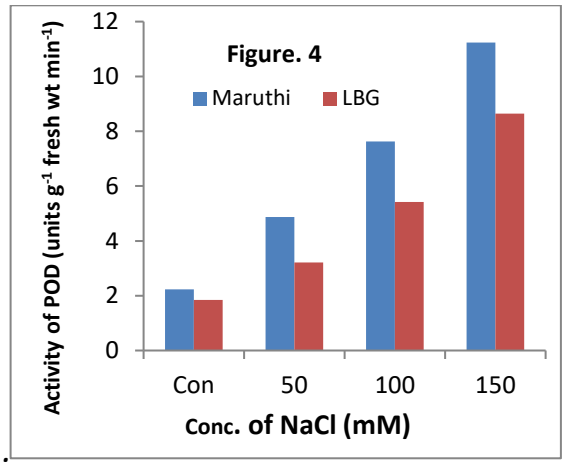


Peroxidase:

The peroxidase activity was assayed in the seedlings of control and salt stressed cultivars of black gram on day-6, and the results are presented in **figure 4**. The peroxidase activity was elevated in the stressed plants of both cultivars. The activity was increased at

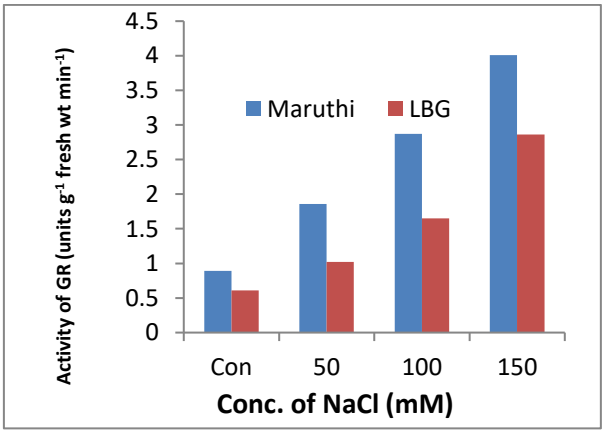
50 mM NaCl stress treatment, but significantly increased at 100 mM and 150 Mm NaCl on day-6 in both the cultivars. However, the per cent increase in POD activity was relatively more in cv. Maruthi than in cv. LBG.

Figure 4: The activities of peroxidase (POD, unit's gm^{-1} fw min^{-1}) in seedlings of both control and stressed black gram cultivars on 6th day



Glutathione reductase

Glutathione reductase activity was assayed in the seedlings of two black gram cultivars under control and NaCl stressed conditions were studied on day-6 and results are tabulated in **figure 5**. The enzyme glutathione reductase was significantly increased in the seedlings of all stressed plants. The rate of increase in enzyme activity was found to be dependent on salt concentration in both cultivars. But the per cent increase in the enzyme activity was more in cv. Maruthi than in cv. LBG. The enzyme activity was increased by 9-fold in Maruthi at 150mM NaCl stress level, where as it was 3-fold in LBG at the same level of stress on day-6. In general, the enzyme activity increased to a greater extent in cv. Maruthi when compared to cv.LBG at 150 mM NaCl level of stress during the 6th day.



DISCUSSION

To avoid oxidative damage under unfavourable conditions such as high/low temperatures, water deficit, salinity etc., plants possess efficient antioxidant mechanism. Plants possess antioxidant systems in the form of enzymes such as superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), ascorbate peroxidase (APX), glutathione reductase (GR), dehydroascorbate (DHAR) and metabolites *viz*., ascorbic acid, glutathione, α-tocopherol, carotenoids and flavanoids etc. The role of these antioxidative enzymes are responsible for scavenging of ROS in plants (Moran *et al*., 1994; Popp and Smirnoff *et al*., 1995). The increase of antioxidant enzymes and metabolites are reported to increase under various environmental stresses (Hernandez *et al*., 1995; Yu

and Rengel, 1999). Role of cellular antioxidant system in relation to water and/or temperature stress tolerance has been reported by many workers (Moran *et al*., 1994; Sairam *et al*., 1998). Some investigators have also reported on the generation of ROS and plant antioxidants in relation to salt stress (Hernandez *et al*., 1993; Gueta-Dahan *et al*., 1997).

Scalet *et al*., (1995) suggested that the higher POD activity in tolerant plant species enable plants to protect themselves against oxidative stress, whereas such activity was not observed in sensitive plants (Pastori *et al*., 1989). The salt induced enhancement of POD activity in S1 cultivar indicated that it had a higher capacity for the decomposition of H_2O_2 generated by SOD. Elevated SOD activity without an accompanying increase in the ability to scavenge H_2O_2 can result in enhanced cytotoxicity by the destructive hydroxyl radical generated from H_2O_2 in a metal catalyzed Haber-Weiss reaction (Gossett *et al*., 1994 b). Lechno *et al*., (1997) noticed the enhanced activities of catalase and GR in cucumber leaves under NaCl stress. Burke (1984) suggested that GR could play a key role in the protection against oxidative stress. In contrast Cavalcanti *et al*., (2004) reported the negative correlation between the accumulations of SOD, CAT, POD activities which do not conferred protection against oxidative damage in salt-stressed cowpea leaves.

Salt tolerant varieties exhibit protection mechanism against increased radical production by maintaining the specific activity of antioxidant enzymes (Dionisio-Sese and Tobita, 1998). Several studies indicated that acquisition of salt tolerance was a consequence of improved resistance to oxidative stress (Hernandez *et al*., 1993, 2002, Gueta-Dahan *et al*., 1997). The antioxidative enzyme activity was found to be higher in salt tolerant cultivars than the susceptible ones (Sairam *et al*., 1997, 2002; Sudhakar *et al*., 2001; Hernandez *et al*., 2003). Rout and Shaw (2001) have been suggested that salt-tolerant plants besides being able to regulate the ion and water movements also have a better antioxidative system for effective removal of ROS. Similarly, in the present study a higher accumulation of antioxidative enzymes was observed in tolerant cv. Maruthi than in the susceptible cv. LBG, suggesting that higher antioxidant enzyme activity have a role in imparting tolerance to tolerance against various environmental stresses.

In the present investigation, the diverse responses of SOD, CAT, POD and GR enzyme activities to NaCl stress on six-day salt stressed seedlings suggest that oxidative stress may be an influential component of environmental stresses on Black gram. Salinity led to significant increases in SOD, CAT, POD and GR activities in both black gram cultivars but the activity was higher in cv. Maruthi compared to cv. LBG. Similar increase in the activities of these enzymes have been reported in other tolerant cultivars subjected to salt stress (Hernandez *et al*., 1993; Ramanjulu *et al*., 2001; Sudhakar *et al*., 2001). Gossett *et al*., (1994 a, b) has been reported that high antioxidant levels could be associated with salt tolerance.

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