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Article

Sodium Chloride Stress Induced Alterations in Germination, Growth and Biomolecules of Black Gram (*Vigna mungo* L.)

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Abstract: Salt stress is one of the most serious limiting factors for crop growth and production. In this study, the morphological and biochemical changes of black gram (*Vigna mungo* L.) under NaCl stress were investigated. The black gram seeds were treated with various concentrations viz., control, 10, 25, 50, 75, 100 and 150 ppm of NaCl. The well grown plants were utilized for analysis of various morphological and biochemical parameters. The morphological growth parameters such as germination percentage, seedling growth and photosynthetic pigments were decreased with increasing concentration of NaCl treatment, while metabolites such as sugar, protein, and proline content were increased at increasing concentrations of NaCl. This investigation demonstrated that decreasing of growth parameters and photosynthetic pigments was due to low osmotic potential at intracellular level generated by NaCl stress.

Keywords: sodium chloride; stress; growth; black gram; *Vigna mungo*.

1. Introduction

Salt stress is one of the most serious limiting factors for crop growth and production in the arid regions. About 23% of the world's cultivated lands are saline and 37% is sodic. Soils can be saline due to geo-historical processes or they can be man-made. The water and salt balance, just like in oceans and seas determine the formation of salty soils, where more salt comes in than goes out. Here, the incoming water from the land brings salts that remain because there is no outlet and the evaporation water does not contain salts. Soil salinity in agriculture soils refers to the presence of high concentration of soluble salts in the soil moisture of the root zone. These concentrations of soluble salts through their high osmotic pressures affect plant growth by restricting the uptake of water by the roots. Salinity can also affect plant growth because the high concentration of salts in the soil solution interferes with balanced absorption of essential nutritional ions by plants (Tester and Devenport, 2003).

It is well established that higher plants can withstand high salinity by either salt exclusion or salt inclusion. Salt excluders possess the ability to exclude salts from the entire plant or from certain organs. In such cases membrane selectivity favors the uptake of K^+ over Na^+, thus excluder crops are characterized by having low Na^+ and Cl^- content. Salt accumulators, on the other hand, are able to cope with the uptake of high salt concentrations through one of two strategies. The first, a common characteristic of halophytes, is through tolerating high levels of intercellular salts by resistant cell membranes. In such cases, high tissue Na^+ to K^+ ratio is evident. The second strategy is through removal of excess salt entering the plant, where root can take up salt ions but avoid their injurious effect (Badr and Shafei, 2002). To improve crop growth and production in the salt-affected soils, the excess salts must be removed from the root zone. Methods commonly used in reclamation such soils are scraping, flushing and leaching.

Population growth is increase on the one hand and land degradation by salinization has led plant scientists to the concept of developing salt-tolerant crops by genetic approaches (Cuartero *et al.*, 2006). However, the physiological, biochemical and molecular mechanisms of salt tolerance in plants are not yet sufficiently understood, and hence progress in developing salt tolerant crops has been slow.

Legumes are considered as the major source of protein and dietary amino acid for man and farm animals (Baudoin and Maquet, 1991). A major part of the human diet all over the world consists of cereals and legumes (Mandal and Mandal, 2000). According to the estimation of FAO, 70% of human food comprises cereals and legumes and the remaining 30% comes from animals (FAO, 1970).

Salinity reduces the ability of plant to take water and this quickly causes reduction in growth rate along with a suite of metabolic changes. Physiological criteria are tissue ionic contents and photosynthetic rate (Munns, and Scatchman, 1993). As regard the chlorophyll content of the salinized plant, it is apparent that the chlorophyll content was reduced as a result of increasing salinity (Ashraf, 1989), while biochemical ones include qualitative and quantitative changes in proteins, fats and carbohydrate patterns (Dubey1999; Khatkar and Kuhad, 2000). Besides, salt induced osmotic stress as well as sodium toxicity trigger to the formation of reactive oxygen species (ROS) such superoxide, hydrogen peroxide, hydroxyl radical and singlet oxygen, which can damage mitochondria and chloroplast by disrupting cellular structure (Mittler, 2001). The deleterious effects of salinity on plant growth are associated with low osmotic potential of soil solution, nutrient imbalance, specific ion effect or a combination of these factors (Marschner, 1995). In the pragmatic investigation, we carefully studied on changes of growth, photosynthetic pigments and bio-metabolites, such as sugar, protein and proline contents in black gram under induced NaCl stress.

2. Material and Methods

2.1. Collection of Seeds

The seeds of black gram were obtained from Vamban Pulse Research Station, Vamban, Pudukottai, TN, India. The experiments were conducted at the Department of Botany, Annamalai University, Annamali nagar, Tamil nadu, India.

2.2. Salinity Treatment

The healthy non-dormant, homogenous seeds of black gram were subjected to surface sterilization with 0.01 mM mercuric chloride ($HgCl_2$) for 3 min with frequent shaking and then thoroughly washed with deionised water to remove the mercuric chloride. The seeds were treated with 0 (control), 10, 25, 50, 75, 100 and 150 ppm concentrations of non-iodized sodium chloride (NaCl) immersion for 3 h. After, the seeds were sown in petriplates in the laboratory.

2.3. Growth Phase

The germination percentage was calculated at 7^{th} day in petriplates in the laboratory condition. For further study, the plant samples were collected at 15^{th} day (DAS and analyzed growth parameters and biochemical estimations).

2.4. Biochemical Analyses

Photosynthetic pigments such as chlorophyll a & b and carotenoid contents were estimated according to the methods of Arnon (1949) and Kirk and Allen (1965) respectively. Soluble sugar (reducing) was estimated according to the method of Nelson (1994) using pure glucose as standard. Starch content was estimated by the method of Hansen and Moller (1974) using pure starch as standard. Protein content was estimated according to the method of Lowry et al. (1951) using bovine serum albumin as standard. Free proline was estimated according to the method of Bates et al. (1975) using pure proline as standard.

2.5. Statistical Analysis

Each treatment was analyzed and experiment was performed with 3 replications from petriplates and pot culture. The results are the presented as means and compared and analyzed by one way ANOVA with standard deviation.

3. Results and Discussion

3.1. Germination Percentage

Although most plants are tolerant during germination, salinity stress delays this process even though there may be no difference in the percentage of germinated seeds from one treatment to another (Maas and Poss, 1989). It is this observation that categorizes this developmental stage for most crops as ‘salt tolerant’. Even though salinity delays germination, higher salt concentrations will eventually reduce the percentage of germinated seeds (Mauromicale and Licandro, 2002). While most crops show enhanced tolerance to salinity during germination, this is not true for sugar beet, a crop categorized as salt tolerant which is somewhat sensitive to salinity at germination (Lauchli and Epstein, 1990). On the other hand, salt tolerant barley varieties germinated faster and showed a much higher germination percentage than the more sensitive ones (Tajbakhsh et al., 2006). Regardless, salt tolerance screening at germination provides little basis for assessing crop salt tolerance.

The germination percentage was gradually decreased with regarding to increasing concentrations compared to control under salinity induced by NaCl in both green gram and cowpea at 7th day of the present study. The highest reduction of germination was found at 150 mM treated population in the present study (Table 1). The increase in salinity not only decreased the germination but also delayed the germination initiation (Rahman et al., 2008). It can be hypothesized that the presence of NaCl even at low concentrations, which is penetrating ions, could have contributed to a decrease in the internal osmotic potential of germinating structures (Almasoori et al., 2001).

3.2. Seedling Growth

Salinity delays emergence and if the stress is severe enough, stand establishment can be reduced (Maas and Grattan, 1999). Crop tolerance during this sensitive growth-stage differs considerably among crops and like germination, does not correlate well with crop tolerances based on yield-response functions. Reduction in shoot growth due to salinity is commonly expressed by a reduced leaf area and stunted shoots (Lauchli and Epstein, 1990). Final leaf size depends on both cell division and cell elongation. Leaf initiation, which is governed by cell division, was shown to be unaffected by salt stress in sugar beet, but leaf extension was found to be a salt-sensitive process (Papp et al., 1983).

Table 1. Effect of different concentrations of NaCl on the morphological parameters of black gram (*Vigna mungo* L.)

NaCl Conc. (ppm)	Germination percentage	Root length (cm/seedling)	Shoot length (cm/seedling)	Fresh weight (g/seedling)	Dry weight (g/seedling)
Control	99.00 ± 2.5	7.29 ± 0.39	10.62 ± 0.53	1.230 ± 0.06	0.570± 0.02
10	95.00 ± 2.2	6.92 ± 0.36	9.16 ± 0.48	0.832 ± 0.05	0.220 ± 0.02
25	90.00 ± 2.1	6.12 ± 0.32	8.92 ± 0.43	0.763 ± 0.05	0.172 ± 0.02
50	81.00 ± 2.1	5.10 ± 0.30	8.64 ± 0.37	0.655 ± 0.05	0.118 ± 0.01
75	67.00 ± 1.8	4.26 ± 0.28	7.12 ± 0.32	0.593 ± 0.04	0.096 ± 0.01
100	41.00 ± 1.5	3.24 ± 0.24	6.28 ± 0.28	0.376 ± 0.03	0.088± 0.01
150	33.00 ± 1.5	2.72 ± 0.22	4.13 ± 0.25	0.232 ± 0.03	0.079± 0.01
F value	9.38**	12.86**	15.32**	10.12**	13.28**

Note: F values for the variance between treatments; ** Significant at 1% level.

Seedling growth was reduced with increasing concentration of NaCl when compared to control in green gram at 15th day (Table 1). It is due to salts accumulating in transpiring leaves at the excessive levels, exceeding the ability of the cells to compartmentalize salts in the vacuole. A disturbance in mineral supply, either an excess or a deficiency, induced by changes in concentrations of specific ions in the growth medium, might directly affected growth (Kavi et al., 1995).

There are two growth phases response to salinity (Munns and Scatchman, 1993). The first phase of growth reduction is quickly apparent and is due to the salt outside the roots. The growth reduction is presumably regulated by hormonal signals coming from the roots. Then, there is a second phase of growth reduction which takes time to develop and results from internal injury (Taffouo, 2009). This study showed that green gram was more sensitive by NaCl stress and reduce the seedling growth at 15th day.

3.3. Photosynthetic Pigments

Salinity drastically affects photosynthesis due to decreasing chlorophyll content and commonly showed adverse effects on membrane stability (Seeman and Sharkey, 1989; Hajar et al., 2006). Salinity reduced the chlorophyll a & b and carotenoid contents of green gram with increasing NaCl concentrations (Table 2). Increasing salinity decreased chlorophyll content in green gram seedlings (Yasar et al., 2008; Taffouo et al., 2009). In addition reactive oxygen species cause chlorophyll degradation and membrane lipid peroxidation, reducing membrane fluidity and selectivity due to NaCl salinity. The highest reduction of photosynthetic pigments was recorded at 150 mM NaCl concentration in both genotypes. The reduction of photosynthetic pigment in the present study might have been degradation of chlorophyll by chlorophyllase and reactive oxygen species generated during photorespiration under salinity. Salt induced osmotic stress as well as sodium toxicity trigger to the formation of reactive oxygen species, such superoxide, hydrogen peroxide, hydroxyl radical and singlet oxygen, which can damage mitochondria and chloroplast by disrupting cellular structure (Murakeozy, 2003). It is attributed to a salt-induced weakening of protein-pigment-lipid complex and due to the suppression of the specific enzyme which is responsible for synthesis of green pigments (Strogonove et al., 1970) or increases chlorophyllase enzyme activity (Stivesev et al., 1973).

Table 2. Effect of different concentrations of NaCl on the pigment contents (mg/g. fw) of black gram (*Vigna mungo* L.)

NaCl Conc. (ppm)	Chlorophyll a	Chlorophyll b	Total chlorophyll	Carotenoid
Control	0.836 ± 0.04	0.668 ± 0.03	1.504 ± 0.06	0.481 ± 0.03
10	0.694 ± 0.04	0.572 ± 0.03	1.266 ± 0.05	0.410 ± 0.03
25	0.614 ± 0.03	0.496 ± 0.03	1.110 ± 0.04	0.367 ± 0.02
50	0.528 ± 0.03	0.325 ± 0.02	0.853 ± 0.03	0.274 ± 0.02
75	0.496 ± 0.02	0.273 ± 0.02	0.769 ± 0.03	0.263 ± 0.01
100	0.325 ± 0.02	0.212 ± 0.01	0.537 ± 0.02	0.196 ± 0.01
150	0.252 ± 0.01	0.194 ± 0.01	0.446 ± 0.01	0.163 ± 0.01
F value	9.11**	8.75**	70.31**	5.36**

Note: F values for the variance between treatments; ** Significant at 1% level.

3.4. Reducing Sugar and Non-reducing Sugar Content in Shoot and Root

Carbohydrates such as sugars and starch accumulate under salt stress playing a leading role in osmoprotection, osmotic adjustment, carbon storage, and radical scavenging. The reducing sugar and starch content were increased in both shoot and root with increasing NaCl treatments compared to control in both green gram and cowpea (Table 3). Accumulation of sugar and starch was more in shoot than root. The accumulation of soluble carbohydrates in plants has been widely reported as a response to salinity or drought, despite a significant decrease in net CO_2 assimilation rate (Murakeozy et al., 2002). The accumulation of starch under abiotic stress has been reported previously (Huang and Liu, 2002) and it is tempting to speculate that starch synthesis from sucrose play a role in moderating the hyper-osmotic condition. Total soluble carbohydrates increased in salinized plants compared with control (Tawfik, 2008) that ability for water absorption under salt stress (Hajar, 1996). This indicates the carbohydrate metabolism was altered to adjust the osmosis in shoot and root at increasing concentrations of NaCl. However, it was decreased at higher doses such as 125 and 150 ppm due to severe salinity.

Table 3. Effect of different concentrations of NaCl (ppm) on the biochemical parameters of black gram (*Vigna mungo* L.)

NaCl Conc. (ppm)	Starch		Total free amino acid		Total protein		Reducing sugars		Non-reducing sugars	
	Root	Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root	Shoot
Control	4.232 ± 0.22	4.812 ± 0.22	2.986 ± 0.18	3.342 ± 0.19	3.091 ± 0.20	3.636 ± 0.18	8.326 ± 0.34	10.300 ± 0.36	4.461 ± 0.22	5.502 ± 0.24
10	3.586 ± 0.22	4.544 ± 0.22	2.346 ± 0.19	3.275 ± 0.20	2.886 ± 0.20	3.562 ± 0.17	8.006 ± 0.33	9.862 ± 0.36	4.260 ± 0.21	5.163 ± 0.24
25	3.18 ± 0.21	4.002 ± 0.22	2.292 ± 0.19	2.596 ± 0.18	2.462 ± 0.19	2.982 ± 0.16	7.600 ± 0.30	9.137 ± 0.34	4.150 ± 0.21	4.800 ± 0.22
50	2.752 ± 0.21	3.924 ± 0.21	1.436 ± 0.17	2.196 ± 0.17	2.296 ± 0.19	2.416 ± 0.14	6.367 ± 0.30	8.462 ± 0.34	3.150 ± 0.21	4.434 ± 0.22
75	2.632 ± 0.20	3.762 ± 0.20	1.214 ± 0.16	1.981 ± 0.16	1.632 ± 0.17	2.132 ± 0.14	6.432 ± 0.30	7.737 ± 0.34	2.928 ± 0.20	4.123 ± 0.22
100	2.436 ± 0.20	3.142 ± 0.20	1.186 ± 0.14	1.652 ± 0.15	1.386 ± 0.17	1.942 ± 0.13	5.924 ± 0.28	6.912 ± 0. 32	2.436 ± 0.20	3.314 ± 0.20
150	1.986 ± 0.20	2.743 ± 0.20	0.942 ± 0.11	1.342 ± 0.15	1.182 ± 0.14	1.386 ± 0.12	4.186 ± 0.26	5.650 ± 0.30	2.126 ± 0.20	3.102 ± 0.20
F value	12.68**	10.73**	12.69**	11.73**	13.71**	15.94**	9.27**	18.78**	20.63**	6.79**

Note: F values for the variance between treatments; ** Significant at 1% level.

3.5. Protein Content in Shoot and Root

Proteins that accumulate in plants under saline conditions may provide a storage form of nitrogen that is re-utilized later (Nelson, 1994) and may play a role in osmotic adjustment. In the present investigation, protein content was increased in shoot and root with increasing NaCl in green gram compared with respect control plants (Table 3). Between shoot and root, former one had more protein than later at lower concentrations. They may be synthesized *de novo* in response to salt stress or may be present constitutively at low concentration (Pareek-Singla and Grover, 1994). The synthesis of protein was more at 125 mM induced by NaCl. It has been concluded that a number of proteins induced by salinity are cytoplasmic which can cause alterations in cytoplasmic viscosity of the cells (Hasegawa et al., 2000).

3.6. Proline Content in Shoot and Root

Accumulation of free proline under abiotic stress has been reported for several plants (Potluri and Devi, 1996). Proline is frequently involved in osmotic protection in higher plants and has been reported to be associated with salt tolerance (Flowers et al., 1986; Kavi et al., 1995). Proline content of black gram was increased with increasing NaCl treatments even at higher concentration (150 mM) compared to control (Table 4). Accumulation of proline was more in shoot than root. A considerable increase was observed in proline levels during germination period in the seedling of *Phaseolus vulgaris* subjected to NaCl treatment (Fattah, 2007). Increased amount of proline is considered to be an indication of tolerance to salt stress because proline is thought to function either as an osmoregulator or a protector of certain enzymes (Aspinall and Paleg, 1981). Accumulation of proline was more in root than shoot due to roots directly contact with NaCl impregnated soil sphere.

Table 4. Effect of different concentrations of NaCl (ppm) on the proline accumulation of black gram (*Vigna mungo* L.)

NaCl Conc. (ppm)	**Proline**	
	Root	**Shoot**
Control	2.986± 0.18	2.743± 0.20
10	3.346± 0.20	2.962± 0.22
25	3.492± 0.20	3.158± 0.24
50	4.436± 0.20	3.736± 0.26
75	5.214± 0.22	4.114± 0.28
100	5.786± 0.22	4.883± 0.32
150	6.153± 0.24	5.327± 0.34
F value	11.80**	12.37**

Note: F values for the variance between treatments; ** Significant at 1% level.

4. Conclusions

In this investigation, salinity decreased seedling growth and photosynthetic pigments at 15^{th} day due to decreasing osmotic potential level in root and shoot led to decreasing photosynthesis in both green gram and cowpea. However, physiological system regulate the osmotic potential by increasing synthesis of reducing sugar, starch, protein at 100 and 125 mM NaCl in black gram, higher dose 150 mM showed decreasing trend of these biomolecules due to acute salinity of NaCl. Meanwhile, osmoregulator proline was increased even at higher doses, and regulates osmosis and cytoplasm viscosity. Because proline may contribute to osmotic adjustment at the cellular level, it may acts as an enzyme protectant and stabilizing the structure of macro-molecules and also acts as a major reservoir of energy and nitrogen for utilization upon exposure to salinity.

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