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High Salinity Induces Differential Oxidative Stress Responses in Cowpea Genotypes at Early Seedling Stage

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Cowpea (*Vigna unguiculata* (L.) Walp.) is one of the most important grain legumes worldwide, and its production is affected by increasing soil salinity due to global climate change. In the present study, fourteen genotypes were tested for their salt tolerance at different concentrations of salinity; 0, 100, 150 and 200 mM of NaCl. The relative shoot length, relative fresh weight of whole plant, fresh root weight, relative water content, electrolyte leakage, malondialdehyde (MDA), proline and chlorophyll parameters were investigated. The results showed that growth of seedling, photosynthetic pigments parameters, relative water content were decreased significantly, and MDA, proline content and electrolyte leakage were increased under salinity stress compared to the control circumstances. This study revealed that KBC-2 and KM-5 amongst the fourteen genotypes showed salinity stress tolerance. These two salt tolerant genotypes KBC-2 and KM-5 can be used further in breeding program to develop salt tolerance in cowpea lines.

Introduction

Cowpea [*Vigna unguicalata* (L.) Walp.] is a diploid legume species (2n = 2x = 22) widely grown in Africa, Asia, southern Europe, the Middle East, western United States and South and Central America. Cowpea is cultivated on more than 14 million hectares (Singh *et al.*,

2003) and the worldwide grain production is estimated to be 5.4 million tons annually, Africa is the leading producer (FAOSTAT, 2016). It is a source of good-quality nutrition for human consumption (Frota *et al.,* 2008), and can be used as cover crop to protect soils eradiation. In the western part of the United States, a growing interest of cowpea as a cover crop has been noticed since it tolerates drought conditions (Agbicodo *et al.,* 2009). Cowpea is a spread legume representing about 80% of the total production of grains, as green vegetable and dry beans. Like bean, this crop is grown thoroughly in India, Africa and Brazil, constituting an important source of protein (23-30%) and carbohydrate (56-68%) (Bressani, 1993).

Agricultural productivity is severely affected by soil salinity and the damaging effect of salt accumulation in agricultural soils (Jaleel *et al.,* 2007). Saline environments can reduce a wide number of responses in plants, including readjustment of transport and metabolic processes, leading to growth inhibition. Most crop plants exhibit considerable hypersensitivity to saline environments because inter cellular accumulation of Na^+ is toxic to cellular metabolism, and for many salt-sensitive plants excess Na+ in the soil plays a major role in growth inhibition (Greenway and Munns, 1980).

Salinity is one of the major limiting factors that have been constraining agricultural production globally (Allakhverdiev *et al.*, 2000). In crop lands, salinity is due to an undesirable increase in the concentration of cations such as Mg^{2+}, K^+, Na^+ and Ca^{2+} and anions such as HCO^{3-}, Cl^-, SO_4^{2-}, and NO^{3-} (Wallender and Tanji 2011). However, salinity due to sodium chloride (NaCl) has been predominant (Ayers and Westcot, 1985); hence, tolerance to this type of salt was reported in this current investigation. Multiple factors such as rock weathering, poor quality of irrigation water, deforestation and inadequate fertilization - practices can worsen salinity on cultivated lands (Omami and Hammes, 2006). Studies have shown that salt stress can cause serious threats to cowpea production. Cowpea germination has been shown to be unfavorably affected by salt stress (Zahedi *et al.,* 2012). Salt-stressed cowpea plants exhibited a reduced plant growth and vigor (Mini *et al.,* 2015). Salt stress can impair plant physiology, photosynthesis, and absolutely important functions such as cell extension and division (Maas and Hoffman, 1977). These aforementioned factors could lead to a significant cowpea yield reduction (Dutta and Bera, 2014). Breeding for cowpea salt-tolerant cultivars is one of the most affordable solutions to tackle these issues. However, few studies have focused on addressing salt stress in cowpea in efforts to adequately providing breeders with critical information on the tolerance of cowpea genotypes to salinity. Our studies provide a detailed knowledge of physiological, morphological, and biochemical changes due to NaCl in fourteen cowpea genotypes during early seedling stage.

Materials and Methods

Plant material and salt treatment

A total of fourteen cowpea genotypes viz., VBN-1, KBC-2, KBC-9, IT-38956-1, C-152, RC-19, GC-3, TUX-944, GC-9, KM-5, GC-4, VBN-3, GC-5 and RC-101 were used in the present study (Table 1). The seedlings were grown in seedling tray (6 seedlings/ tray) containing a mixture of 100 % cocopeat in greenhouse. Each tray containing 14 genotypes with 3 replications were maintained throughout the experiment. Watering of cowpea plants was done daily with 500 ml distilled water for up to 3 days and subsequently, irrigated with Hoagland nutritive solution (1:10 dilution in water) up to the 10^{th} day after sowing. The ten day old cowpea seedlings were treated with varying salt concentrations by irrigating the tray with 0 mM, 100 mM, 150 mM and 200 mM NaCl solution for 6 days. Thereafter, seedlings were subjected to morphological, physiological and biochemical analysis.

Morphological analysis during salt stress

For shoot length measurement, the shoots were put on graph papers and photographed. The images were investigated with the assistance of Image programming (Obiefuna and Ndubizu, 1979). To calculate the biomass and fresh root weight, ten whole seedlings and roots respectively, were considered for mean value calculation.

Evaluation of relative water content (RWC)

Leaf RWC was determined as described earlier with slight modifications (Turner *et al.,* 1981). The leaves from the control (unstressed) and stressed seedlings were sampled and fresh weight (FW) was recorded. The leaf samples were then soaked in water for 4 h at 25°C, surface dried and turgid weight (TW) was recorded. Next, the samples were dried in oven at 70°C until constant weight was achieved and dry weight (DW) was recorded. RWC was expressed in a percentage according to the following equation: RWC = (FW – DW)/(TW – DW)×100.

Determination of electrolyte leakage and lipid peroxidation

Electrolyte leakage, as an estimate of membrane stability, was determined as described previously (Bhushan *et al.,* 2007). Leaf tissues (100 mg) were collected, for each treatment, in triplicates and incubated for 4 h in 30 mL deionized water. An initial reading of the conductance was recorded ($C_{initial}$) using a conductivity meter (Labman Scientific Instruments, India). The tissues were then autoclaved at 121 °C for 20 min. The solution was then allowed to cool at 25 °C and the conductance was re-recorded (C_{max}). Electrolyte leakage was calculated as a percentage of Cinitial to Cmax according to the following formula: $(1- (C_{initial}/ C_{max}))\times 100$. The degree of lipid peroxidation level was determined in terms of malondialdehyde (MDA) content as described previously (Chakraborty *et al.*, 1992). The 200 mg of leaf tissue was homogenized in 2 mL of 5% trichloroacetic acid (TCA) and the homogenate was centrifuged at 10,000×g for 10 min. One part of the supernatant was added to a tube containing 4 parts of 0.5% (v/v) thiobarbituric acid prepared in 20% (v/v) TCA.

The mixture was heated in a water bath at 95 °C for 30 min and then cooled to 25 °C. The absorbance of supernatant was determined at 532 nm and 600 nm. MDA was calculated according to the following formula: $((A_{532}-A_{600}) \times \text{volume} \times 100) / (155 \times \text{FW}))$ and expressed in μmol mg−1 FW (Bhushan *et al.*, 2007).

Analysis of proline content

Extraction of free proline was performed as described (Bates 1973), with minor modifications. In brief, approximately 100 mg of leaf tissue was homogenized in 3% sulfosalicylic acid and centrifuged at 5000×g for 5 min. The proline content was quantified by mixing the supernatant with equal amount of glacial acetic acid and ninhydrin reagent. The mixture was then kept for 1 h at 95 °C in a water bath.

The reaction was terminated by placing the samples on ice. This was followed by addition of 4 mL of toluene in the mixture and shaken vigorously. The toluene layer was then taken to measure absorbance at 520 nm spectrophotometrically using toluene as blank. Free proline was quantified according to the following formula: 36.6 × A_{520} × volume / 2 × FW and expressed in µmol g−1 FW.

Quantitative determination of chlorophyll

Chlorophyll was extracted and estimated as described by Lichtenthaler (Lichtenthaler *et al.,* 1987). Approximately, 100 mg leaf sample was homogenized in 80% chilled acetone and the homogenate was centrifuged at 3000×g for 5 min at 4°C.

The supernatant was collected and absorbance was measured spectrophotometrically at 663, 645 and 470 nm. Contents of chlorophyll a (Chl a), chlorophyll b (Chl b) in the leaf samples were calculated according to following formulae and expressed in mg g−1 FW: Chl a (mg g−1) = ((12.7 × A_{663}) – (2.69 × A_{645}) v/w); Chl b (mg g−1)=((22.9 × A_{645}) – (4.68 × A_{663}) v/w); Crd (mg g−1) = ((1000 × A_{470}) – ((3.27 × Chl a) + (1.04 × Chl b)) / 227 v/w); Total Chl (mg g−1)=(20.2 × A_{645} + 8.02 × A_{663}).

Statistical analysis

The entire experiment was conducted at College of Agricultural Biotechnology, Baramati during the year 2019. The entire experiment was repeated 3 times to validate the preceding results. All the obtained information was evinced as mean (n = 10) followed by standard error (mean ± SE) format using SPSS 21 (Windows version) software. Furthermore, difference among the various treatments was determined by employing ANOVA (analysis of variance) and post hoc Tukey's HSD (honest significant difference) test at 0.05 level of significance.

Results and Discussion

Morphological changes in cowpea seedlings

To examine the effect of high salt stress-induced morphometric alterations, ten days old seedlings were exposed to salt stress for duration of six days at 0 Mm, 100 mM, 150 mM and 200 mM NaCl concentrations. Seedlings of all the fourteen genotypes showed the visible stress symptoms such as leaf chlorosis and leaf rolling. However, KBC-2 and KM-5 showed minimal chlorosis and rolling even on the sixth day. The seedlings of KBC-9, RC-19, TUX-944, GC-9, and RC-101 showed severe visible symptoms, but KBC-2, IT-38956-1 and KM-5 showed minimal symptoms even at 200 mM NaCl (Fig. 1). Several researchers have reported similar results in cowpea under salinity stress (Mishra *et al.,* 2014; Taffouo *et al.,* 2009).

Effect of high salt stress on biomass and shoot length

To assess the effect of high salinity on cowpea growth, the biomass (fresh weight of whole plant), fresh root weight and shoot length of seedlings were measured (Fig. 2, 3, 4). Increase in the NaCl concentration resulted in significant decrease in the fresh weight, fresh root weight and relative shoot length of several genotypes such as KBC-9 and RC-101. However, genotype KBC-2 and KM-5 showed no statistical difference in the biomass, fresh root weight and relative shoot length compared to the controls (Fig. 2, 3, 4). This suggests that severe salinity reduced growth progressively from shoot along the vertical gradient to the cowpea plant. The previous several study reports revealed reduction in shoot, root and biomass of plant exposed to salt, drought, aluminum and cadmium stress (Chowardhari *et al.,* 2019; Awasthi *et al.,* 2017; Saha *et al.,* 2016; Mishra *et al.,* 2014).

Table.1 Description of cowpea genotypes

S. No.	Genotypes	Salient features
1.	VBN-1	A variety released in 1998 for cultivation in rainfed areas of Tamil Nadu and Karnataka; late type (90-100 days), yellowish white flower, long pods, large size creamy white seed; yield potential: 950-1100 kg/ha.
2.	KBC-2	A late maturing variety released in 1998 for cultivation in Karnataka and Tamil Nadu; asynchronous maturity, long pods, light brown seeds; tolerance to rust; yield potential: 600-1200 kg/ha.
3.	KBC-9	A medium duration (80-85 days) variety released in 2018 for cultivation in southern states (Karnataka, Tamil Nadu, Andhra Pradesh and Kerala); resistant to collar rot, moderate resistance to yellow mosaic virus (YMV); good and stable yield (1100-1200 kg/ha).
4.	IT-38956-1	A grain purpose older variety released for cultivation in Karnataka.
5.	RC-19	An old variety released in 1993 for cultivation in Rajasthan; early (65-70 days) and synchronous maturity, moderately resistant to YMV; white seed with good yield potential (900-1000 kg/ha).
6.	GC-3	A nationally adapted, drought tolerant and disease resistant variety released in 1997 for states of central zone (Gujarat and Maharashtra), south zone (Karnataka and TN), north-east plain (Jharkhand) and north-west plain (Delhi); short stature, late maturing (90-95 days) and good yield potential (750-1150 kg/ha).
7.	TUX-944	-NA-
8.	C-152	An older variety having medium maturity duration (85-90 days) released in 1985 for cultivation in Karnataka; light coloured seed; susceptible to rust.
9.	GC-9	-NA-
10.	KM-5	A high-yielding variety released after C-152 for cultivation in northern transitional zone of Karnataka; its superiority over C-152 owes to its greater height and more number of branches; resistant to powdery mildew and rust.
11.	GC-4	An early (58-70 days) variety with synchronous maturity released in 1998 for cultivation in Gujarat; moderately resistant to YMV with good yield potential (900-1000 kg/ha).
12.	VBN-3	A variety released after VBN-1 for cultivation in Tamil Nadu; early type (75-80 days), light purple flower, long pods, large size light brown seed; yield potential: 1100-1300 kg/ha
13.	GC-5	A long-duration (94-100 days) and high yielding (1100 kg/ha) variety released in 2005 for the state of Gujarat; moderately resistant to YMV.
14.	RC-101	An extra early (60-65 days) and determinate variety released in 2001 for cultivation in the state of Rajasthan; non-viny, white seeded with good yield potential (750-850 kg/ha); drought tolerant and suitable for low rain fall areas.

*Modified from Project Report (2018-19), All India Network Research Project on Arid Legumes, IIPR, Kanpur 208 024.

NA: Information not available

Fig.1 Salt stress induced morphological changes in developing cowpea seedlings. Cowpea genotypes were grown and monitored under control and salt stress conditions (100, 150,200 mM) for 6 days. The experiments were carried out in triplicates and data represented is the mean of the replicates. Histograms with different alphabets depict the statistical significance at P value < 0.05

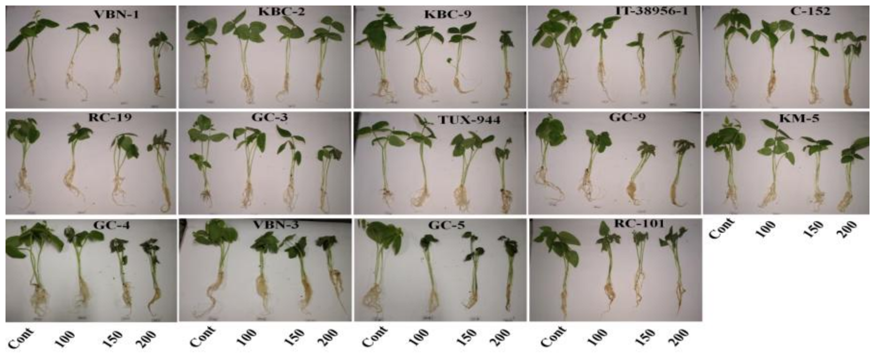


Fig.2 Assessment of physicochemical parameters (relative fresh weight) under control and salt stress conditions among all cowpea genotypes. The experiments were carried out in triplicates and data represented is the mean of the replicates. Histograms with different alphabets depict the statistical significance at P value < 0.05

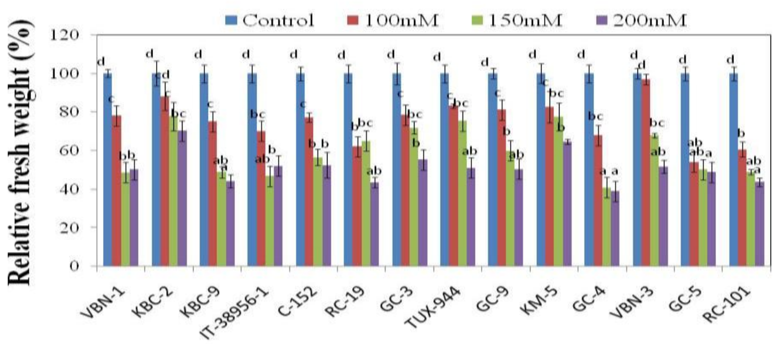


Fig.3 Assessment of physicochemical parameters (relative fresh root weight) under control and salt stress conditions among all cowpea genotypes. The experiments were carried out in triplicates and data represented is the mean of the replicates. Histograms with different alphabets depict the statistical significance at P value < 0.05

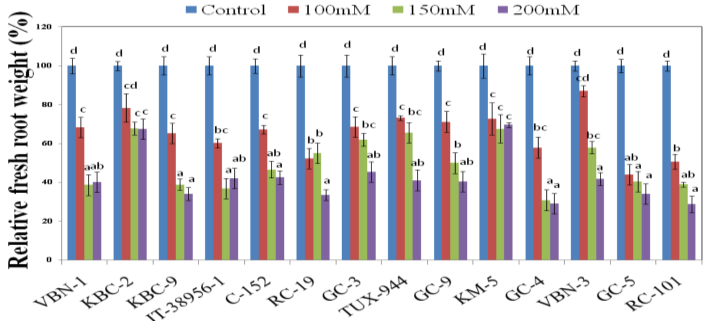


Fig.4 Assessment of physicochemical parameters (relative shoot weight) under control and salt stress conditions among all cowpea genotypes. The experiments were carried out in triplicates and data represented is the mean of the replicates. Histograms with different alphabets depict the statistical significance at P value < 0.05

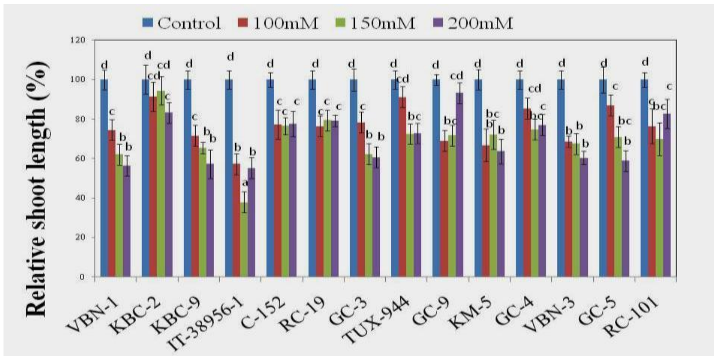


Fig.5 Assessment of biochemical parameters (relative water content) under control and salt stress conditions among all cowpea genotypes. The experiments were carried out in triplicates and data represented is the mean of the replicates. Histograms with different alphabets depict the statistical significance at P value < 0.05

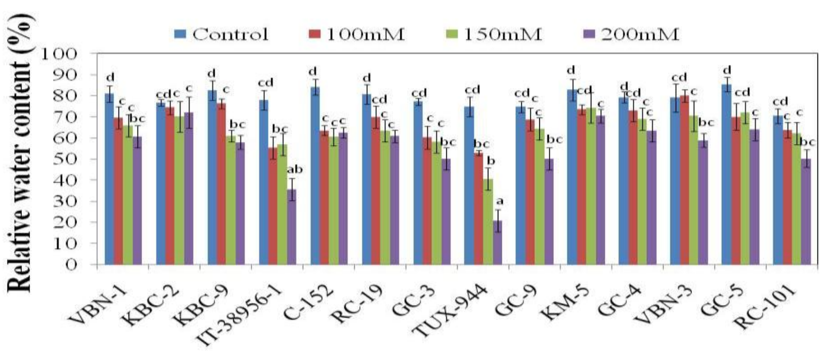


Fig.6 Assessment of biochemical parameters (electrolyte leakage) under control and salt stress conditions among all cowpea genotypes. The experiments were carried out in triplicates and data represented is the mean of the replicates. Histograms with different alphabets depict the statistical significance at P value < 0.05

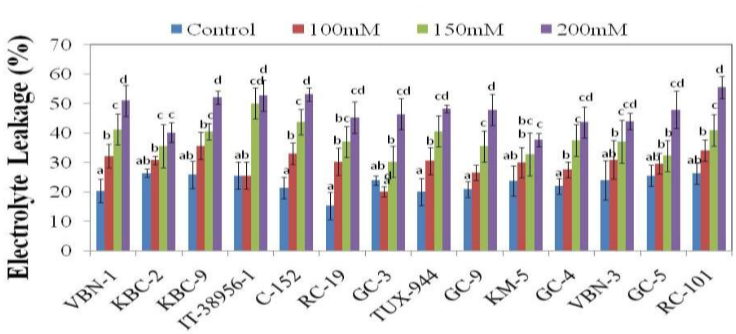


Fig.7 Assessment of biochemical parameters (MDA) under control and salt stress conditions among all cowpea genotypes. The experiments were carried out in triplicates and data represented is the mean of the replicates. Histograms with different alphabets depict the statistical significance at P value < 0.05

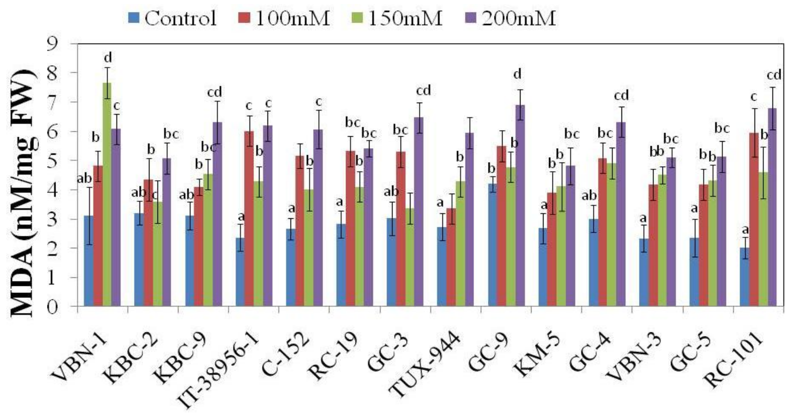


Fig.8 Assessment of biochemical parameters (proline) under control and salt stress conditions among all cowpea genotypes. The experiments were carried out in triplicates and data represented is the mean of the replicates. Histograms with different alphabets depict the statistical significance at P value < 0.05

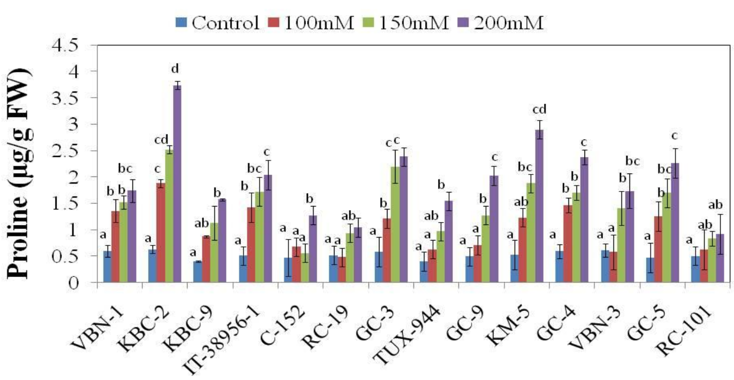


Fig.9 Evaluation of photosynthetic pigments (chlorophyll a) among all cowpea genotypes

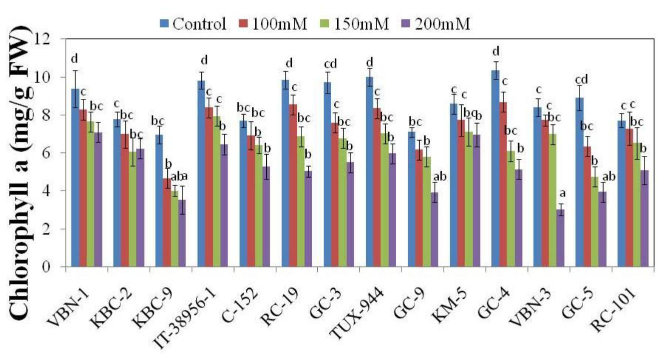


Fig.10 Evaluation of photosynthetic pigments (chlorophyll b) among all cowpea genotypes

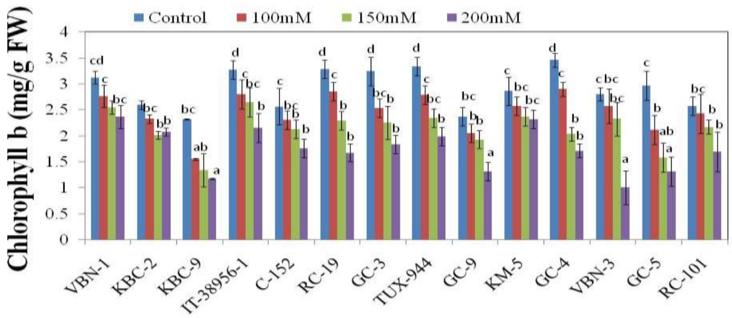
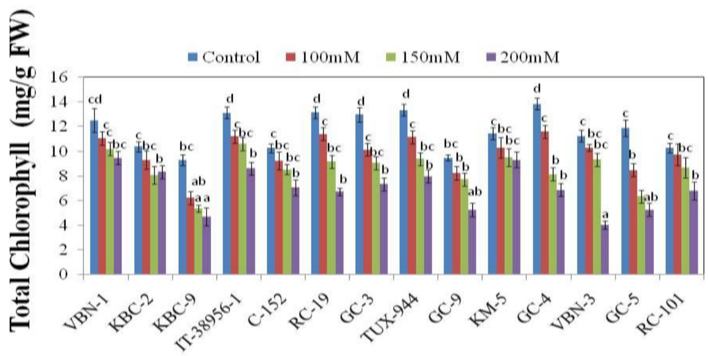


Fig.11 Evaluation of photosynthetic pigments (total chlorophyll) among all cowpea genotypes



Effect of salinity on plant water status

Salinity-imposed osmotic stress leads to cell turgor loss and cell volume change and therefore, we examined the water status of the seedlings. Initially, RWC was found to be decreased in all the cultivars by NaCl stress. Significant decrease in RWC was observed in all genotypes except KBC-2, KM-5 and GC-4. The maximum decrement in RWC was observed in TUX-944 (~54%) and IT 38956-1 (~42%), but minimum in KBC-2(~4%) and KM-5 (~12%) (Fig. 5). The previous study revealed that the salinity decreased RWC and water retention capacity, while increased water saturation deficit and water uptake capacity in mung bean (Kabir *et al.,* 2004). The RWC is an indicator of water status in leaves. Control plant seedling maintained a high water status as compared with stressed seedling. Earlier reports (Mishra *et al.,* 2014; Gouiaa *et al.,* 2012) also suggested similar results.

Effect of salinity on membrane integrity

We critically monitored the status of membrane integrity of the seedlings exposed to salt stress in terms of the extent of electrolyte leakage. Electrolyte leakage showed significant increment in most of the genotypes with increasing salt concentration. Minimum increment (~38–40%) of electrolyte leakage was observed in KM-5 and KBC-2, whereas C-152 and RC-101 showed maximum increase (~55–53%) (Fig. 6). Several previous studies have reported that various stresses such as salt, drought, aluminum, high temperature and cadmium account for the production of reactive oxygen species (ROS), leading to membrane damage (Chowardhara *et al.,* 2019 Awasthi *et al.,* 2017; Saha *et al.,* 2016; Chakraborty and Pradhan, 2010). Stress induced membrane fluidity is a key factor for membrane damage (Ruelland & Zachowski, 2010), and increased membrane fluidity is closely related to electrolyte leakage (Bhushan *et al.,* 2007). Therefore, high salt induced minimum membrane damage was observed in the genotypes KBC-2 and KM-5, thus it showed minimum increment in electrolytic leakage. Several workers (Tuna *et al.,* 2008, 2007; Kaya *et al.,* 2007; Ghoulam *et al.,* 2002; Lutts *et al.,* 1996, 1999) reported that electrolyte leakage increased in the leaves of tomato plants grown under salt stress.

Effect of salinity on lipid peroxidation

The NaCl stress induced peroxidation of membrane lipids, which was determined by MDA concentration. The MDA has been widely used as a selection criterion to assess salt injury in various plants (Jaleel *et al.,* 2007 Katsuhara *et al.,* 2005). The MDA concentration was significantly increased in the stressed seedlings. Minimum lipid peroxidation was observed in KBC-2 (~20 %) and KM-5 (~20 %), as against RC-101 (~50 %) and IT 38956-1 (~40 %) (Fig. 7). These results suggest that KBC-2 and KM-5 could better maintain cell membrane homeostasis and integrity under high salt stress. The MDA which is often used as an indicator of oxidative damage and effects of oxidative stress on membrane integrity are frequently evaluated through the increases in MDA concentration, is produced during peroxidation of membrane lipid by decomposition of polyunsaturated fatty acid (Sanchez-rodriguez *et al.,* 2010; Pinheiro *et al.,* 2004; Bor *et al.,* 2003). In our present study, we observed that the minimum MDA content was increased by about 2 fold at 200 mM NaCl concentrations in KBC-2 and KM-5. The level of MDA is considered as a biomarker of stress and increased as severity of stress increased. Guimaraes *et al.,* (2011) showed that increased MDA content might result in electrolyte leakage, indicating a loss of membrane integrity. These obtained results are similar to those obtained by Sadak *et al.,* (2017) on chickpea plant and by El-Awadi *et al.,* (2017) on faba been plant.

Effect of NaCl on proline accumulation

Accumulation of osmolytes especially proline is a well-known adaptive response in plants against environmental stress conditions including high salt stress. The proline content significantly increased in all the genotypes treated with salt stress compared to respective controls. Here, KBC-2 (30 %) showed the highest accumulation, whereas RC-101 did not record any induction of proline under 200 mM NaCl stress (Fig. 8). The compatible solutes, particularly proline, contribute a significant role in osmotic adjustment and structural stability during stress (Wahid *et al.,* 2007). Indeed, cellular machinery releases the compatible solutes to maintain the redox potential under high salt stress. Increased accumulation of proline was thus in agreement with these findings, as maximum proline accumulation was observed in KBC-2 (Fig. 8). Accumulation of proline in response to various stresses in leaf have been reported previously in many studies (Eraslan *et al.,* 2007; Tarakcioglu and Inal, 2002; Aziz *et al.,* 1999), which (proline) is responsible for detoxifying ROS by forming a stable complex with them, and thus inhibits the process of lipid peroxidation (Xiong and Zhu, 2002). It is increasingly clear that plants accumulate proline to avoid water loss under stress conditions and maintain the cell water balance. The increased proline accumulation at early stage of salt treatment could be positively correlated with the preventive maintenance of RWC. The accumulation of the proline in plant tissues in response to different abiotic stresses may play an important role against oxidative damages (Alia *et al.,* 2001), take part in cellular osmotic adjustment (Yamada *et al.,* 2005), and stabilize the membrane and protein 3D structure (Kavi-kishor *et al.*, 2005; Ashraf and Foolad 2007). Proline is a compatible osmolyte, and performs multiple functions in stress adaptation, recovery and signaling, stabilization of proteins and protein complexes in the chloroplast and cytosol and protection of the photosynthetic apparatus in plants (Szabados, and Savoure, 2009). Ashraf and Foolad (2007) suggested that the application of proline successfully improved stress tolerance in plants.

Effect of salt stress on photosynthetic pigments

To assess the effects of high salt on the photosynthetic capabilities, the status of photosynthetic pigments *viz.*, chlorophyll a, chlorophyll b and total chlorophyll was analyzed. The seedlings exhibited a drastic decline in photosynthetic pigments with increasing salt concentration. The genotype CG-5 showed the highest reduction (57%) of chlorophyll *a*, followed by VBN-3 (50%) and KM-5 (20%) at 200 mM NaCl stress (Fig. 8). Similar phenomenon was observed in case of chlorophyll *b* and total chlorophyll, upon exposure to NaCl stress (Fig. 10 and 11). This result indicated that minimum degradation of chlorophyll content in KM-5 genotype under salt stress showed its better photosynthetic ability. Tuna *et al.*, (2007) reported that the both chlorophyll a and b contents of maize plant decreased in response to salinity stress. The reductions in different photosynthetic pigment components in response to salt stress were confirmed by Sadak *et al.*, (2015) and Ismail *et al.*, (2019) on faba bean plant. The decline in photosynthesis due to salinity stress could be due to lower stomata conductance, inhibition of photochemical capacity, depression in carbon uptake and metabolism and a combination of all these factors (Mundree *et al.,* 2002). Moreover, the inhibitory effects of salt stress on chlorophyll pigments could be due to suppression of specific enzymes responsible for the synthesis of the green pigments or due to increased chlorophyllase activity in wheat and mustard (Mishra *et al.,* 2006; Kiani *et al.,* 2005). Photosynthetic pigments decreased in chickpea grown under salt stress (Beltagi 2008) and photosynthesis was reduced to 60% (Murumkar and Chavan, 1993).

In conclusion the salt stress is second most adversity after drought in agriculture production, and it leads to drastic change in physiological, biochemical and molecular behavior of plants. In terms of physiological parameters, stunted growth, chlorosis, loss of water content and membrane damage were observed in salt sensitive cultivars. The screening of the fourteen cultivars revealed that KBC-2 and KM-5 were salt tolerant even at higher level of salinity. The two KBC-2 and KM-5 salt tolerant genotypes can be used in pre-breeding program to develop salt tolerance in cowpea lines.

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