

Identification of salt-tolerant cowpea genotypes using ISSR markers and proteome analysis

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1. ABSTRACT

Soil salinity globally affects the productivity of staple food crops. Therefore, an understanding of the molecular mechanisms that lead to salt tolerance induced by antioxidant mechanisms can assist in the development of salt-tolerant crops. To decipher the molecular fingerprint of salt resistance, in this study, six salt-tolerant cowpea genotypes at the seedling

stage were assessed for their antioxidant responses, yield, genetic polymorphism and proteomics under salt stress. Leaves and roots showed distinct tissue-specific responses to salinity, and leaves showed a better protection against salt stress-induced oxidative stress than roots. Inter simple sequence repeat (ISSR) fingerprinting allowed molecular discrimination

between salt-tolerant cowpea genotypes. Proteome analysis of cowpea leaves under salt stress revealed up-regulation of ATP synthase, vacuolar ATPase, pentatricopeptide repeat protein, flavanone 3-hydroxylase and outer envelope pore protein. Thus, ISSR and proteome analysis allow the identification of salt-tolerant cowpea cultivars.

2. INTRODUCTION

On a global level, salt stress poses a serious threat for the cultivation of crops in arid and semiarid regions (1). Processes such as seed germination, vegetative growth, flowering and fruit set are adversely affected by high salt concentrations, ultimately resulting in diminished crop yield and quality (2). Cowpea (*Vigna unguiculata* (L) Walp) is a grain legume widely cultivated in arid and semiarid regions (3) and an important source of dietary protein in the developing countries of Asia and Africa. As a result of soil salinity, the average cowpea yield is significantly lower than the estimated potential yield in many cultivated areas (4,5). Therefore, the development of cowpea varieties with improved salt tolerance is a priority for cultivation in saline environments.

Plants growing in saline soils usually encounter two types of stresses, osmotic stress and ion toxicity (6). These stresses alter the electron transport chain in the mitochondria and chloroplasts and lead to the overproduction of active oxygen derivatives such as superoxide anion ($O_2^{\cdot-}$), singlet oxygen (1O_2), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH) (7,8). These oxygen species attack and damage macromolecules, leading to lipid peroxidation, protein degradation and DNA mutation in living cells (9). The toxic effects of these reactive oxygen species can be overcome by the activation of the antioxidant machinery. Several enzymatic and non-enzymatic antioxidants scavenge toxic oxygen species and protect the plants from oxidative damage. There is evidence that salt tolerance is positively correlated with increased levels of antioxidants (10-12).

Salt stress is accompanied by changes in gene expression that lead to changes in the protein profile. Differential expression of proteins has been observed under saline conditions in various plants including rice (13,14), potato (15), cucumber (16) and foxtail millet (17). However, only two studies have investigated the proteomics of salt-stressed cowpea leaves (18,19). Two-dimensional (2-D) gel electrophoresis, followed by mass spectrometry (MS), is a simple and powerful tool that can be used for comparative proteome analysis. This approach allows the study of the differences in the proteome expression profile under salt stress to identify novel salt-induced proteins that confer salt tolerance to cowpea plants.

For the development of salt-tolerant plants, it is essential to explore the molecular mechanism of salt tolerance. Also, knowledge on the genetic diversity of genotypes of the same species is important for breeding and developing salt-tolerant varieties. In the present study, cowpea genotypes that were identified as salt-tolerant and salt-sensitive at the early seedling stage through our previous work (20) were assessed for their antioxidant responses under salt stress. The yield and genetic polymorphism of the salt-tolerant genotypes were also studied. Proteomic analysis was conducted with the high-yielding cowpea genotype under salt stress to identify the proteins involved in salt tolerance and to gain better knowledge on the mechanisms of salt tolerance.

3. MATERIALS AND METHODS

3.1 Antioxidant studies

3.1.1. Plant material

Seeds of seven cowpea genotypes, obtained from the Central Arid Zone Research Institute (CARZI), Jodhpur and National Pulses Research Centre (NPRC), Vamban, India (Table 1), were surface-sterilised in 70% (v/v) ethanol for 2 min, rinsed thoroughly with sterile distilled water and allowed to germinate via the roll towel method (21). Fifteen sterilised seeds were placed in two rows on a pre-soaked germination paper towel. We placed a moistened germination paper over the first paper, leaving the seeds placed between the two towels. Subsequently, we rolled up the two towels with the seeds in-between and kept them vertically inside troughs containing 0 mM NaCl (distilled water) and 75 mM NaCl solutions in a germination room maintained at $28 \pm 1^\circ\text{C}$ and 80% relative humidity. Primary leaves and roots were harvested on the seventh day after sowing and used for antioxidant studies. Biochemical analyses of antioxidant parameters were replicated thrice with pooled samples.

3.1.2. Non-enzymatic antioxidants

Carotenoid content was estimated in fresh leaves and roots (22). Briefly, about 500 mg of fresh sample were homogenised with 10 mL of 80% acetone at 4°C and centrifuged at 2,500 rpm for 10 min at 4°C . The extraction was repeated until the residue became colourless. The supernatants were pooled, and the absorbance was read at 480, 645 and 663 nm with a spectrophotometer against 80% acetone as control. To calculate the carotenoid content, we used the following formula:

$$\text{Carotenoids (mg/g)} = (A_{480} + (0.114 \times A_{663}) - (0.638 \times A_{645})) \times V / 1000 \times W$$

Table 1. Source and salt tolerance level of the improved cowpea genotypes used in this study

Genotypes	source	Salt tolerance level
VCN1	National Pulses Research Centre, Vamban	Tolerant
VCN2	National Pulses Research Centre, Vamban	Tolerant
DC15	Central Arid Zone Research Institute, Jodhpur	Tolerant
KBC2	Central Arid Zone Research Institute, Jodhpur	Tolerant
VCP-09-001	National Pulses Research Centre, Vamban	Tolerant
IVT-VCP-09-013	National Pulses Research Centre, Vamban	Tolerant
CPD121	Central Arid Zone Research Institute, Jodhpur	Sensitive

Determination of α -tocopherol was conducted in fresh samples (23). About 1.0 g of the sample was ground with a solution containing 20 mL of petroleum ether and ethanol (2:1.6, v/v) and centrifuged. To 1 mL of supernatant, 200 μ L of 2% 2, 2'-dipyridyl in ethanol were added, and the mixture was kept in the dark for 5 min. Absorbance was measured at 520 nm, and a standard graph was generated with α -tocopherol.

Total phenols were extracted and quantified using Folin-Ciocalteu reagent (24). About 250 mg of fresh sample were extracted thrice with 80% ethanol. The supernatants were pooled and evaporated to dryness in a water bath, and the residue was dissolved in 5 mL of distilled water. An aliquot of 1 mL extract was made up to 3 mL with distilled water and mixed with 0.5 mL Folin-Ciocalteu reagent and 2 mL Na_2CO_3 (20 %). The solution was kept in boiling water bath for 1 min. After cooling, absorbance was measured at 650 nm, using gallic acid as standard.

3.1.3. Enzymatic antioxidants

For enzyme assays, 1.0 g fresh samples were homogenised in 3 mL 50 mM phosphate buffer (pH 7.0) that contained 0.1 mM EDTA and 1 mM of L-ascorbic acid. The homogenate was centrifuged, and the supernatant was used as enzyme source. Extraction was carried out at 4°C, and the protein content of the extract was measured by Lowry's method (25).

The catalase (CAT) assay was performed by adding 50 μ L enzyme extract to a solution containing 3 mL of 50 mM potassium phosphate buffer pH 7.0 and 20 mM H_2O_2 . The decrease in absorbance was observed at 240 nm (26), and CAT activity was expressed as U mg^{-1} protein. One CAT unit is defined as the μ moles of H_2O_2 oxidised per minute.

Peroxidase activity was measured using guaiacol (27). To 3 mL of 0.1 M phosphate buffer (pH 7.0), 0.05 mL of 20 mM guaiacol solution, followed by 0.03 mL of 12.3 mM of H_2O_2 solution, were added. To this, 0.1 mL of enzyme extract was added, and the increase in absorbance was measured at 436 nm at

different time intervals. The peroxidase activity was calculated as the rate of formation of the guaiacol dehydrogenation products (GDHP) and expressed as U g^{-1} protein. One unit of enzyme activity is defined as the amount of enzyme catalysing the formation of 1.0 μ M of GDHP min^{-1} mL^{-1} of extract.

The superoxide dismutase (SOD) assay was performed with a reaction mixture that contained 3 mL Tris HCl buffer (50 mM, pH 8.2) with 1 mM EDTA, 0.5 mL of tissue extract and 0.5 mL of 0.2 mM pyrogallol (28). The absorbance change at 420 nm was measured, and SOD activity was expressed as U g^{-1} protein. One SOD unit refers to the amount of enzyme that causes a 50% inhibition of pyrogallol autooxidation.

3.1.4. Determination of H_2O_2 and lipid peroxidation

Fresh samples (0.5 g) were homogenised with a mortar and pestle in 10 mL of 0.1% trichloroacetic acid (TCA) and centrifuged at 15,000 g for 15 min. The obtained supernatant was used for the estimation of H_2O_2 and lipid peroxidation. The supernatant (0.5 mL) was mixed with 0.5 mL of 10 mM phosphate buffer (pH 7) and 1 mL of 1 M KI to determine the H_2O_2 content (29). Absorbance was measured at 390 nm, and the H_2O_2 content was calculated from the standard graph generated with known amounts of H_2O_2 .

Lipid peroxidation was measured as the amount of malondialdehyde (MDA) (30). To 1 mL supernatant, 4 mL of 0.5% TBA in 20% TCA were added. The mixture was incubated at 95°C for 30 min, cooled in an ice bath, and centrifuged; the absorbance of the supernatant was recorded at 532 and 600 nm. The MDA concentration was calculated as the difference between absorbance and molar extinction coefficient ($155 \text{ mM}^{-1}\text{cm}^{-1}$).

3.2. Pot experiment

3.2.1. Preparation of pots and experimental setup

Red soil (EC = 0.13 dSm^{-1} , pH = 7.3, organic carbon = 3.2 g kg^{-1} , available nitrogen = 179 kg ha^{-1} ,

available phosphorus = 10 kg ha⁻¹, available potassium = 161 kg ha⁻¹, iron = 4.24 ppm, manganese = 23.42 ppm, zinc = 0.38 ppm, copper = 2.02 ppm), collected at Madurai, was used as reference soil without salt stress. Coastal alluvial soil (EC = 6.8 dSm⁻¹, pH = 7.4, organic carbon = 3.6 g kg⁻¹, available nitrogen = 235 kg ha⁻¹, available phosphorus = 40 kg ha⁻¹, available potassium = 561 kg ha⁻¹, iron = 14.4 ppm, manganese = 15.18 ppm, zinc = 1.08 ppm copper = 4.44 ppm), collected from Ramnad, served as salt-affected soil. The soils were air-dried and passed through a 5-mm mesh screen and thoroughly mixed. Based on the soil test values, site-specific fertiliser requirements were calculated using the Decision Support System for Integrated Fertilizer Recommendation (DSSIFER) software (31) and applied to both treatments. Subsequently, 18 plastic pots were filled with 5 kg of reference soil each, while another 18 pots were filled with 5 kg of saline soil. The drainage holes were sealed to prevent salt leaching. Seeds of six different cowpea genotypes were sterilised with 70% (v/v) ethanol and rinsed thoroughly with distilled water. Three seeds were sown in each pot and watered daily to field capacity with ground water. The pots were kept in the open field under uniform environmental conditions. After 20 days, seedlings were thinned to one plant per pot. Three replicates were used, with each replicate representing one seedling.

3.2.2. Grain yield and quality parameters

The fully matured pods were harvested from each plant separately, and pod number and length were recorded. The number of seeds per pod was determined and recorded. Subsequently, the seeds were separated and dried. Hundred-seed weight and yield per plant were calculated. The dried grains were ground to a fine powder, and grain quality parameters (crude protein, crude fat, ash content, crude fibre and carbohydrates) were estimated according to the methods outlined in the AOAC (32). Grain moisture content was estimated by drying the sample in a crucible kept in an oven at 105°C for 3 h. Crude protein was estimated via the Kjeldahl method. Crude fat was extracted using a soxhlet apparatus; based on the extract, the amount of crude fat was calculated. To estimate the ash content, about 2 g of the sample were weighed into a crucible and kept in a hot air oven for 3 h at 100°C. The crucible with the dried sample was then placed into a muffle furnace at 550°C until the sample turned white and was free of carbon. The crucible was then removed from the furnace, cooled in a desiccator and reweighed, followed by the calculation of the ash content. To determine crude fibre, 2 g of the sample were treated with 1.25% H₂SO₄, followed by washing and subsequent treatment with 1.25% NaOH solution. The residue was washed with hot water and dried in an oven to constant weight. The dried residue was then combusted in a muffle furnace, and the percentage

of crude fibre was calculated. The sums of all these parameters were subtracted from 100 to obtain the carbohydrate content.

3.3. Inter simple sequence repeat (ISSR) analysis

Genomic DNA of the six cowpea plants grown in reference soil was extracted via the mini-prep CTAB method (33). The purity of the extracted DNA was checked based on A₂₆₀/A₂₈₀ ratios and estimated using a Nano-Drop spectrophotometer; the DNA concentration was normalised to be 50 ng µL⁻¹ for PCR.

A total of nine ISSR primers, synthesized by Bangalore Genei Pvt. Ltd., Bangalore, India, were used for the polymorphism survey. Polymerase chain reaction (PCR) was carried out in a 15-µL reaction mixture containing 1 µL of genomic DNA (50 ng), 0.50 µL of primer (20 mM), 1.2 µL of dNTPs (2.5 mM), 1.50 µL of Tris HCl buffer (10 mM, pH 8.3), 0.18 µL of Taq DNA polymerase (three units), 0.20 µL of MgCl₂ (2 mM) and 10.42 µL of sterile distilled water. The thermal cycler was set up for an initial denaturation at 94°C for 5 min, followed by 39 cycles of denaturation at 94°C for 1 min, annealing at 46-48°C for 45 secs, extension at 72°C for 2 min and final extension at 72°C for 10 min. The obtained PCR product was separated by agarose gel (1%) electrophoresis; the gel was stained with ethidium bromide and documented. A 1-kb DNA ladder was used as size marker, and ISSR banding patterns were scored as present (1) or absent (0); the scores were used to construct a dendrogram based on Jaccard's similarity coefficient with the unweighted pair group method (UPGMA), using the NTSYS software version 2.02.

3.4. Protein extraction and 2-D gel analysis

Seeds of the VBN2 genotype were surface-sterilised and allowed to germinate via the roll towel method (21) inside troughs containing distilled water (control) and 75 mM NaCl solution (salt stress). The primary leaves were taken from the seedlings on the seventh day after sowing for protein extraction and 2-D gel analysis (19).

About 200 mg of the sample were finely ground in liquid nitrogen. To this, 200 mg of polyvinylpyrrolidone (PVPP) were added, and the mixture was ground in 5 mL of extraction buffer containing 40 mM Tris-HCl (pH 7.5), 10 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 250 mM sucrose, 1 mM dithiothreitol (DTT) and 1% (v/v) Triton X-100. The mixture was vortexed for 1 h and centrifuged at 15,000 × g for 15 min at 4°C. An aliquot of 10 mL 10% (w/v) trichloroacetic acid (TCA) in cold acetone was added to the supernatant, and the mixture was kept for 12 h at -20°C. The precipitated protein

was centrifuged at $15,000 \times g$ for 15 min at 4°C , and the pellet was washed four times with ice-cold acetone and vacuum-dried. The dried pellet was then dissolved in 300 μL lysis buffer (7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 2% (v/v) IPG buffer pH 4-7 and 1% (w/v) DTT) for 1 h at room temperature. Subsequently, the suspension was centrifuged at $12,000 \times g$ for 10 min at 25°C , and the supernatant was collected for 2-D electrophoresis. The method of Bradford (34) was applied to determine the protein content.

In a further step, 400 μg of extracted protein were mixed with rehydration buffer containing 8 M urea, 2% (w/v) CHAPS, 2% (v/v) IPG buffer (pH 4-7), 0.3% (w/v) DTT and 0.002% (w/v) bromophenol blue and loaded onto an 18-cm, pH 4-7 linear gradient IPG strip in a rehydration tray. The strips were rehydrated at room temperature for about 12-14 h and subjected to isoelectric focussing (IEF) using an Ettan IPGphor II IEF system (GE Healthcare, USA) with 500 V for 1 h, followed by 1,000 V for 1 h and finally 3,000 V at 20°C for 24 h. The focused strip was equilibrated twice for 15 min with equilibration buffer at room temperature. The first equilibration was conducted in a solution with 50 mM Tris-HCl (pH 8.8), 6 M urea, 2% (w/v) sodium dodecyl sulphate (SDS), 30% (v/v) glycerol, 0.002% (w/v) bromophenol blue and 1% (w/v) DTT. The second equilibration was performed with the same buffer modified by replacing DTT by 2.5% (w/v) iodoacetamide. Subsequently, SDS-PAGE electrophoresis was performed on a 12% (v/v) gel using an Ettan DALTsix (GE Healthcare) electrophoresis unit. The equilibrated IPG strip was rinsed with an electrode buffer, placed on top of SDS gel and overlaid with 2 mL of molten agarose solution. The gel was electrophoresed at constant current (25 mA) until the tracking dye reached near the bottom; subsequently, the gel was removed and fixed overnight with acetic acid: methanol (1:4) solution and stained with colloidal Coomassie Blue R-250.

Scanning of the stained gels was performed with Image Scanner III and image visualisation with Image Master 2D Platinum (GE Healthcare, USA). The 2-DE gels obtained from the selected two biological replicates of control and salt-treated leaf samples were used for image analysis. After automated detection and matching, the abundance ratio was calculated as the % volume of spots under stress divided by the % volume of spots under control. Protein spots that changed by more than 1.5-fold between control and salinity-treated tissues were selected and manually excised from the gel. These spots were subjected to in-gel trypsin digestion and MALDI-TOF/MS. The mass spectral (MS) data was used to identify the proteins from the NCBI database (taxon Viridiplantae) using the MASCOT search engine. Parameters such as peptide mass tolerance limit of ± 150 to ± 200 ppm, one missed cleavage, carbamidomethylation (C) as fixed

modification and oxidation (M) as variable modification were used to search for sequences. The searches that showed the highest MASCOT score with maximum sequence coverage were selected.

3.5. Statistical analysis

Data obtained for the antioxidant studies and pot experiments were analysed for significance by ANOVA, and mean values and standard deviations were determined. Data means were compared using Duncan's test ($p < 0.05$). Pearson's correlation analysis was also conducted. All statistical analyses were performed using the software package SPSS v. 20.

4. RESULTS

4.1. Antioxidants

Antioxidant parameters were studied for one salt-sensitive (CPD121) and six salt-tolerant (VBN2, VBN1, DC15, KBC2, VCP-09-001 and IVT-VCP-09-013) cowpea genotypes. These cowpea genotypes were characterised as salt-tolerant and -sensitive at the seedling stage based on our previous study (20). The study showed significant variations in all antioxidant parameters under salt stress in both leaf and root tissues.

4.1.1. Non-enzymatic antioxidants in leaves and roots under salt stress

Our data show that leaf carotenoid contents significantly increased under salt stress in the genotypes KBC2, VCP-09-001 and IVT-VCP-09-013, but decreased in VBN1 and CPD121 (Figure 1). A notable increase of 70% was seen in the carotenoid content of the genotype KBC under salt treatment. Salt stress had a decreasing effect on the carotenoid content in the roots of all genotypes. A drastic reduction in root carotenoid concentration was observed in the salt-sensitive genotype, CPD121. The addition of NaCl to the growth medium caused a strong and significant increase in the tocopherol content of leaves and a decrease in the roots of all genotypes (Figure 1). Generally, the amount of tocopherol was higher in leaves than in roots. An irregular pattern was observed in the total phenol content in both leaf and root tissues under salt stress (Figure 1). In VBN1, DC15, KBC2, IVT-VCP-09-013 and CPD121, total leaf phenol levels significantly decreased, but increased in VBN2. Total root phenol levels were higher in salt-stressed DC15 and VCP-09-001 genotypes, but lower in KBC2 and CPD121 compared to the control.

4.1.2. Enzymatic antioxidants under salt stress

Leaf catalase (CAT) activity was decreased by salt stress (Figure 2). A significant reduction was

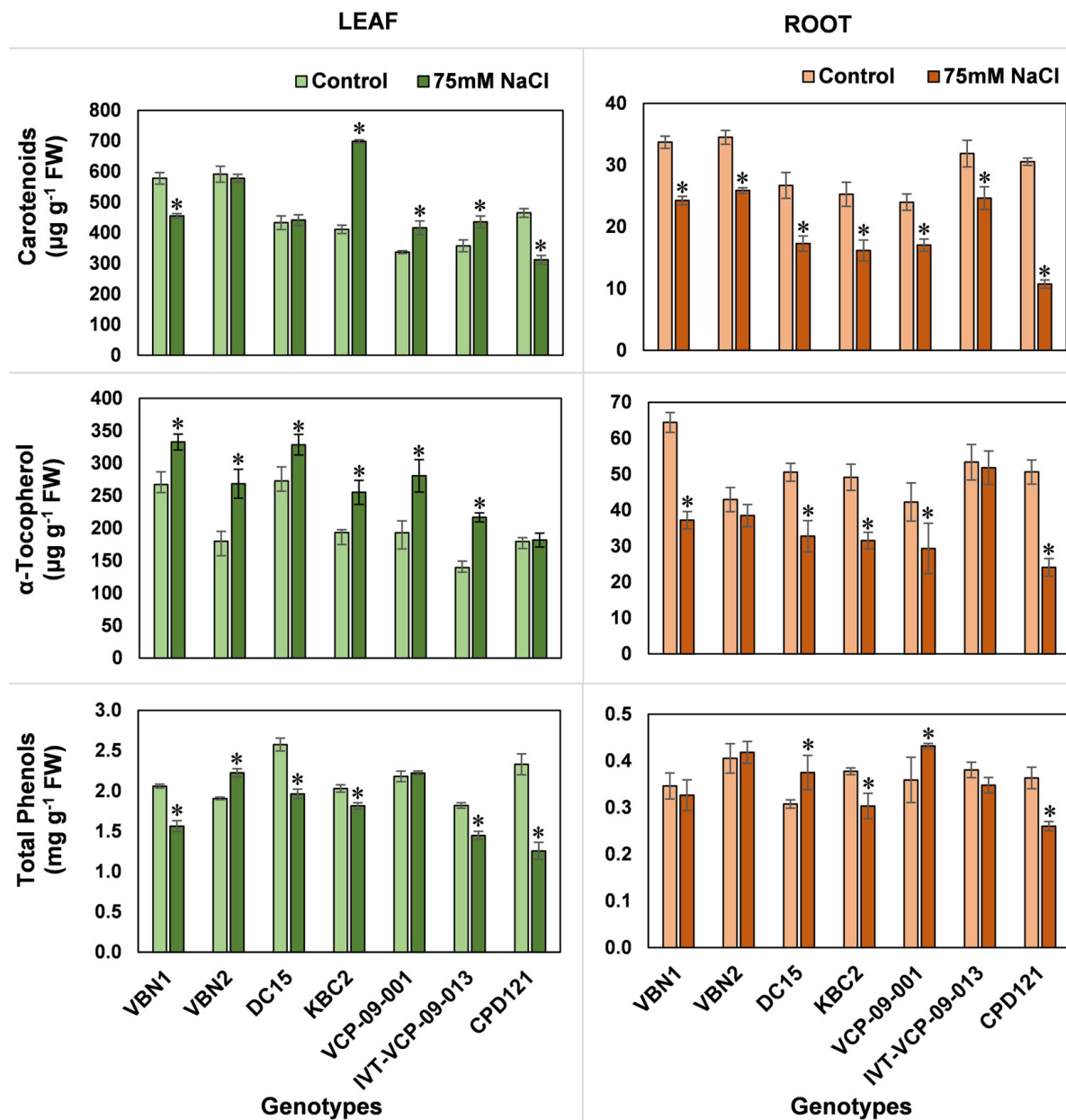


Figure 1. Salt induced changes in non-enzymatic antioxidants in cowpea genotypes. Vertical bars represent the mean value \pm SD of three replicates. * Indicates that a significant difference with $p < 0.05$ exists between control and salt stressed tissues of the same genotype.

observed in the three genotypes DC15, KBC2 and CPD121, while for the other four genotypes, a non-significant reduction was found. The CAT activity in roots of salt-tolerant seedlings under salt stress showed an increasing trend, while root CAT activity remained unchanged in CPD121. The activity of peroxidase (POD) was significantly higher in roots than in leaves (Figure 2). The percentage increase in POD activity over the control was greater in leaves than in roots of salt-stressed seedlings. A more than two-fold increase in leaf POD activity was presented by the genotypes VBN1, VBN2, VCP-09-001 and IVT-VCP-09-013.

Furthermore, our results showed that SOD activity in leaves decreased in VBN1, KBC2 and CPD121 and increased in VBN2, DC15 and IVT-VCP-013 (Figure 2). Except for VBN1 and CPD121, the roots of all genotypes showed a significant increase in SOD activity (Figure 2).

4.1.3. Hydrogen peroxide and lipid peroxidation

The H_2O_2 produced as a result of oxidative stress is involved in the peroxidation of membrane phospholipids, with the production of malondialdehyde (MDA). To study the extend of lipid peroxidation, H_2O_2

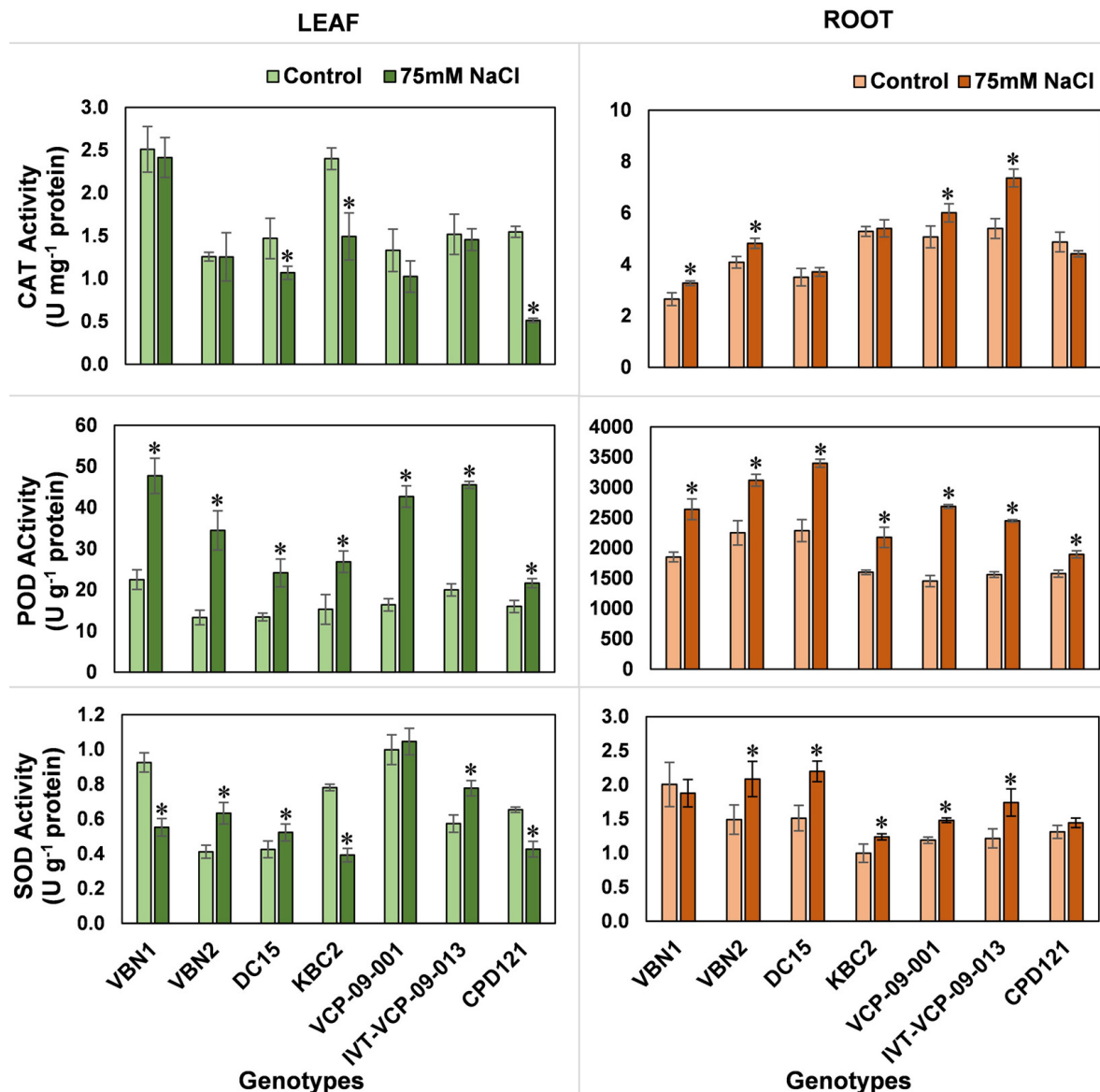


Figure 2. Salt induced changes in enzymatic antioxidants of cowpea genotypes. Vertical bars represent the mean value \pm SD of three replicates. * Indicates that a significant difference with $p < 0.05$ exists between control and salt stressed tissues of the same genotype.

and MDA contents were determined in leaves and roots of the seven cowpea genotypes (Figure 3). Except for VCP-09-001, all genotypes showed a significant increase in leaf H_2O_2 contents, with extremely high levels in the genotype CPD121. Root H_2O_2 content showed an increasing trend, but a statistically significant increase was noticed in five genotypes (VBN1, DC15, KBC2, IVT-VCP-09-013 and CPD121) out of the seven genotypes studied (Figure 3). For the genotype VCP-09-001, we observe no significant changes in H_2O_2 levels in both leaves and roots.

Irrespective of the genotype, a significant increase was noted in the MDA content under salt stress in both leaves and roots (Figure 3). In leaves,

the MDA content in the control plants ranged between 30.36 and 36.57 $\mu M g^{-1}$, while in the salt-stressed leaves, levels from between 38.84 and 44.12 $\mu M g^{-1}$ were observed. The MDA content in control roots ranged between 13.06 and 19.46 $\mu M g^{-1}$, while in salt-stressed roots, MDA levels were between 19.32 and 28.81 $\mu M g^{-1}$. The percentage increase in MDA was higher in roots than in leaves, indicating higher lipid peroxidation in root membranes, which are in direct contact with the salt solution. The salt-sensitive genotype CPD121 showed a comparatively higher MDA content than salt-tolerant genotypes.

Correlation analysis was conducted to understand the influence of the antioxidant

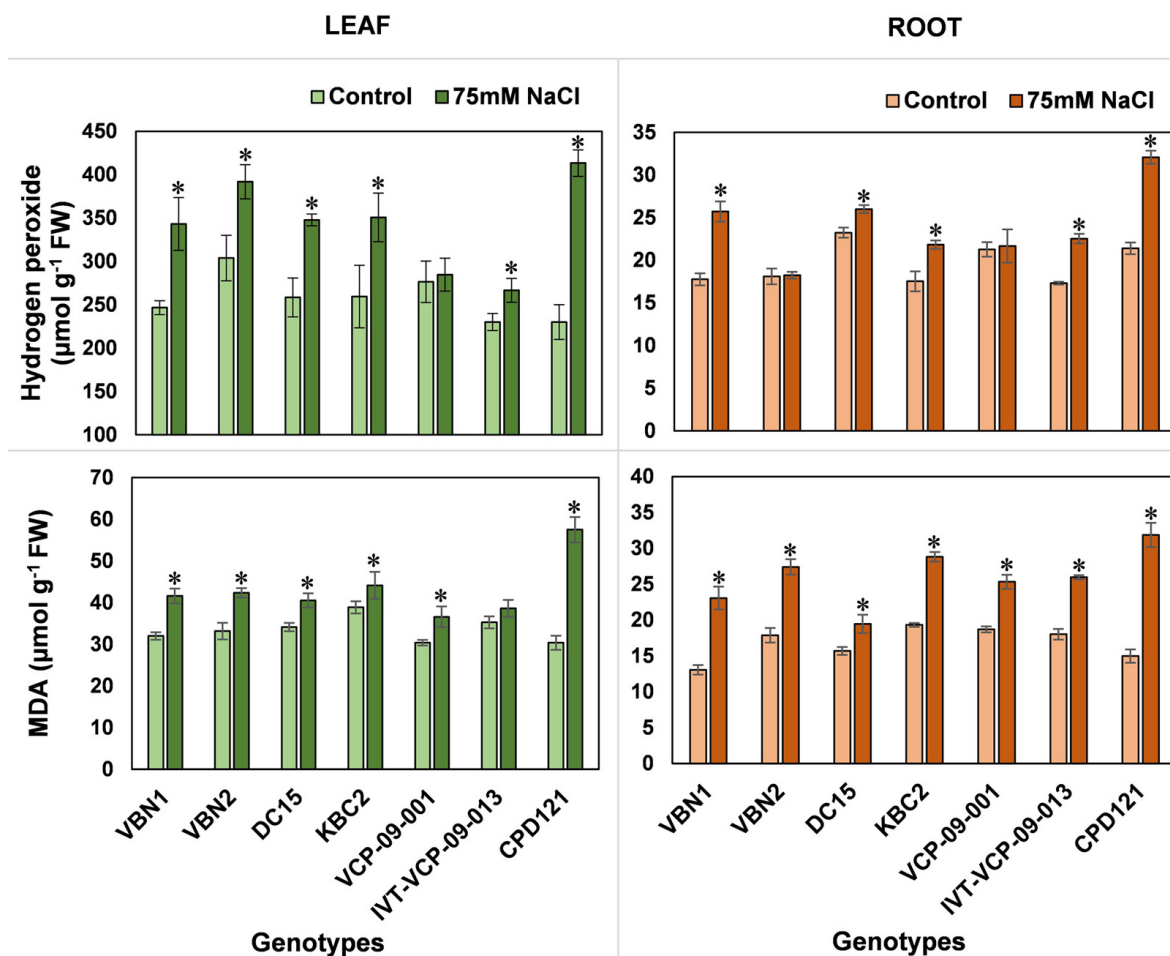


Figure 3. Increase in H_2O_2 and MDA contents in cowpea genotypes under salt stress. Vertical bars represent the mean value \pm SD of three replicates. * Indicates that a significant difference with $p < 0.05$ exists between control and salt stressed tissues of the same genotype.

Table 2. Correlation coefficients of the non-enzymatic and enzymatic antioxidants against H_2O_2 and MDA

Parameter	LEAF		ROOT	
	H_2O_2	MDA	H_2O_2	MDA
Carotenoids	-0.513*	-0.662**	-0.525*	-0.678**
α -Tocopherol	-0.726**	-0.696**	-0.357	-0.648**
Phenols	-0.679**	-0.566**	-0.713**	-0.759**
CAT	-0.655**	-0.619**	-0.221	-0.365
POD	-0.735**	-0.513*	-0.551**	-0.679**
SOD	-0.433*	-0.337	0-.574**	-0.598**

The total number of data used is 7 (genotypes) \times 3 (replications) =21 for each parameter, * Significant at $p < 0.05$, ** Significant at $p < 0.01$

components towards H_2O_2 and MDA contents. The percentage increase/decrease in the parameters (Figs. 1-3) for the salt-stressed and control plants were calculated and used for correlation analysis. In leaf tissue, carotenoids, α -tocopherol and phenols were negatively correlated with both H_2O_2 and MDA (Table 2), indicating that higher levels of these antioxidants

impede lipid peroxidation and the formation of MDA in leaves. In roots, carotenoids and phenols were significantly correlated with H_2O_2 and MDA. Leaf CAT and POD activity were negatively correlated with both H_2O_2 and MDA (Table 2). In roots, POD and SOD activities showed a negative correlation with both H_2O_2 and MDA. Generally, SOD generates H_2O_2 from

Table 3. Effect of salt stress on yield parameters of salt tolerant cowpea genotypes

Parameter	Genotypes	Treatment		% Reduction
		Control	Salt stress	
No. of pods per plant	VBN1	9.33 ± 0.58	8.33 ± 0.58	10.7 ^{ab}
	VBN2	14.00 ± 2.00	11.00 ± 1.00*	21.4 ^{bc}
	DC15	11.67 ± 1.53	8.33 ± 0.58*	28.6 ^c
	KBC2	9.33 ± 0.58	7.00 ± 1.00*	25.0 ^c
	VCP-09-001	10.00 ± 1.00	9.00 ± 0.00	10.0 ^{ab}
	IVT-VCP-09-013	11.33 ± 0.58	10.33 ± 0.58	8.8 ^a
Pod length (Cm)	VBN1	16.60 ± 0.61	12.22 ± 0.75*	26.6 ^c
	VBN2	15.05 ± 1.72	13.61 ± 0.38	9.5 ^{ab}
	DC15	16.33 ± 0.68	14.22 ± 0.60*	12.9 ^b
	KBC2	16.71 ± 1.05	12.44 ± 0.71*	25.5 ^c
	VCP-09-001	16.51 ± 1.12	15.58 ± 0.37	5.7 ^a
	IVT-VCP-09-013	14.80 ± 0.34	12.78 ± 0.46*	13.7 ^b
No. of seeds per pod	VBN1	9.27 ± 0.55	8.70 ± 0.26	6.1 ^a
	VBN2	8.80 ± 0.87	8.47 ± 0.81	4.2 ^a
	DC15	9.40 ± 1.11	8.97 ± 0.38	4.6 ^a
	KBC2	11.75 ± 0.07	10.67 ± 0.21	9.2 ^{ab}
	VCP-09-001	10.90 ± 0.44	9.27 ± 0.49*	15.0 ^b
	IVT-VCP-09-013	11.40 ± 1.10	9.63 ± 0.15*	15.5 ^b
Hundred seed weight (g)	VBN1	11.62 ± 0.13	9.25 ± 0.21*	20.5 ^d
	VBN2	12.24 ± 0.22	11.32 ± 0.18*	7.5 ^b
	DC15	10.19 ± 0.20	9.86 ± 0.19	3.3 ^a
	KBC2	10.03 ± 0.17	9.83 ± 0.13	2.0 ^a
	VCP-09-001	13.09 ± 0.18	11.51 ± 0.29*	12.1 ^c
	IVT-VCP-09-013	10.01 ± 0.10	8.95 ± 0.17*	10.6 ^{bc}
Yield per plant (g)	VBN1	10.04 ± 0.26	6.83 ± 0.14*	31.9 ^a
	VBN2	14.94 ± 0.44	10.53 ± 0.65*	29.5 ^a
	DC15	11.14 ± 0.32	7.37 ± 0.18*	33.8 ^a
	KBC2	10.84 ± 0.50	7.35 ± 0.45*	32.2 ^a
	VCP-09-001	14.23 ± 0.15	9.57 ± 0.25*	32.7 ^a
	IVT-VCP-09-013	12.96 ± 0.44	8.92 ± 0.31*	31.2 ^a

All values are expressed as mean ± SD of three replications. * Indicates that significant differences exists between control and salt stress treatment of the same genotype at $p < 0.05$.

superoxide; hence, increasing SOD levels should increase H_2O_2 content. However, the contradictory results obtained in this study suggest an alternate powerful detoxification system available for the removal of H_2O_2 in leaf and root tissues. The significant negative correlation of leaf and root POD against H_2O_2 (Table 2) suggests that POD plays a significant role in the removal of H_2O_2 in leaves and roots.

4.2. Salt stress affects grain yield and quality parameters

The results of the pot experiment showed that salt stress significantly reduced the grain yield of all genotypes. With respect to yield parameters, variations were noted between genotypes. The three genotypes VBN2, DC15 and KBC2 presented a significant reduction in pod number per plant when subjected to salt stress, whereas other genotypes showed a non-significant reduction (Table 3). The least reduction in po number per plant (8.8%) was observed for the genotype IVT-VCP-09-013. Four genotypes showed a significant reduction in pod length, with the reduction percentage ranging between 5.7 and 26.6 (Table 2). Genotype VCP-09-001 showed the lowest reduc-

tion in pod length, while the genotypes VCP-09-001 and IVT-VCP-09-013 showed significant reductions in seed number per pot. A minimal reduction in hundred-seed weight was observed in KBC2 (2.0%) and DC15 (3.3%) (Table 3). Grain yield per plant was significantly reduced in all genotypes (Table 3). Under salt stress, VBN2 showed the highest yield of 10.53 g per plant, followed by VCP-09-001 (9.57 g); the values ranged between 29.5 and 33.8%. There was no significant difference in the reduction percentage of grain yield among the genotypes, indicating the same level of salt tolerance.

Grain quality analysis revealed that salt stress resulted in a significant change only in crude fat and crude fibre contents, while other parameters were less affected (Figure 4). Salt-stressed VBN1 showed a significant decrease, while DC15 showed an increase in protein levels when compared with the control (Figure 4). The other four genotypes showed no significant changes in grain protein content under salt stress. Under salt stress, crude fat decreased significantly in all genotypes except VBN2 (Figure 4). An increasing trend was observed for ash content under salt stress, but a significant increase

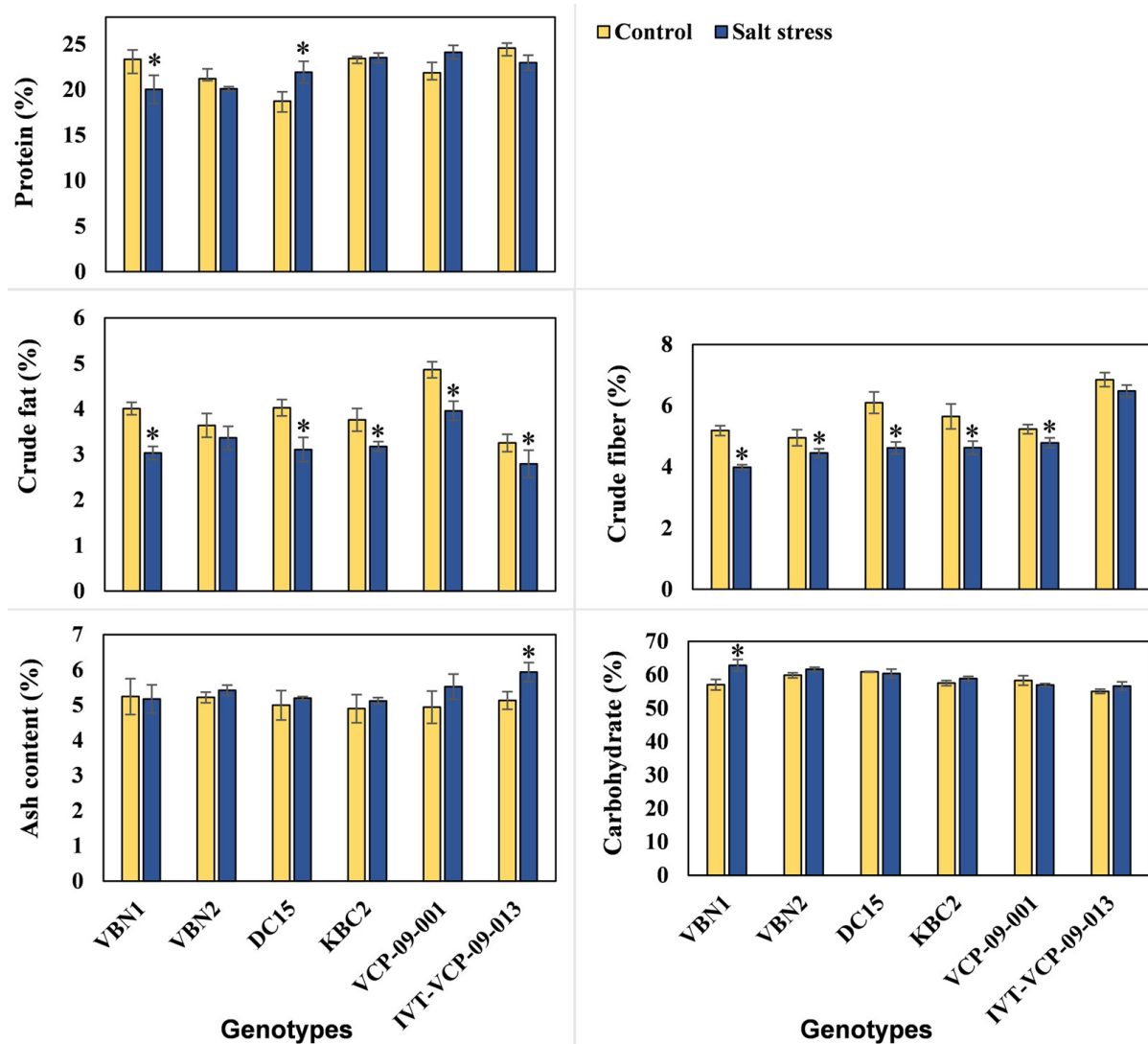


Figure 4. Variations in grain quality parameters of salt-tolerant cowpea genotypes under salt stress. Vertical bars represent the mean value \pm SD of three replicates. * Indicates that significant differences with $p < 0.05$ exist between control and salt stressed tissues of the same genotype.

was presented only by IVT-VCP-09-013 (Figure 4). All genotypes except IVT-VCP-09-013 showed a significant decrease in crude fibre content under salt stress (Figure 4). The carbohydrate content of grains either increased or decreased under salt stress, but a significant change was observed only in VBN1 (Figure 4).

4.3. Genetic discrimination of salt-tolerant cowpea genotypes

Genetic polymorphism was analysed using nine inter simple sequence repeat (ISSR) markers. The ISSR primers were selected based on previous studies of cowpea genetic diversity (35) and were initially checked for reproducibility and clarity of polymorphic banding patterns. The nine primers

yielded 57 bands, of which 41 were polymorphic, with a total polymorphism of 72% (Table 4). The ISSR marker UBC-809 showed 100% polymorphism, and UBC-812 produced the maximum number of bands (Figure 5). The ISSR data were used to generate a dendrogram based on UPGMA analysis (Figure 6). The estimates of genetic similarity, based on Jaccard's coefficient, ranged from 0.44 to 0.94. At the similarity level of 0.63, three main clusters were formed. The first cluster consisted of the genotypes VBN1 and IVT-VCP-09-013, while DC15, KBC2 and VCP-09-001 were grouped in the second cluster; VBN2 formed the third cluster. The genotypes KBC2 and VCP-09-001 had a high similarity coefficient of 0.94, while VBN2 formed a unique cluster at the similarity coefficient of 0.44, indicating that it is highly divergent from the other five genotypes (Figure 6).

Table 4. Polymorphism among salt tolerant cowpea genotypes obtained by ISSR primers

Primer	Sequence	Number of bands	Number of polymorphic bands	% Polymorphism
UBC-807	(AG) ₈ T	6	5	83
UBC-808	(AG) ₈ C	4	2	50
UBC-809	(AG) ₈ G	7	7	100
UBC-811	(GA) ₈ C	7	6	86
UBC-812	(GA) ₈ A	10	7	70
UBC-834	(AG) ₈ YT	6	4	67
UBC-836	(AG) ₈ YA	7	5	71
UBC-841	(GA) ₈ YC	5	3	60
UBC-856	(AC) ₈ YA	5	2	40
Total		57	41	72

Single letter abbreviation for base positions Y=(C,T)

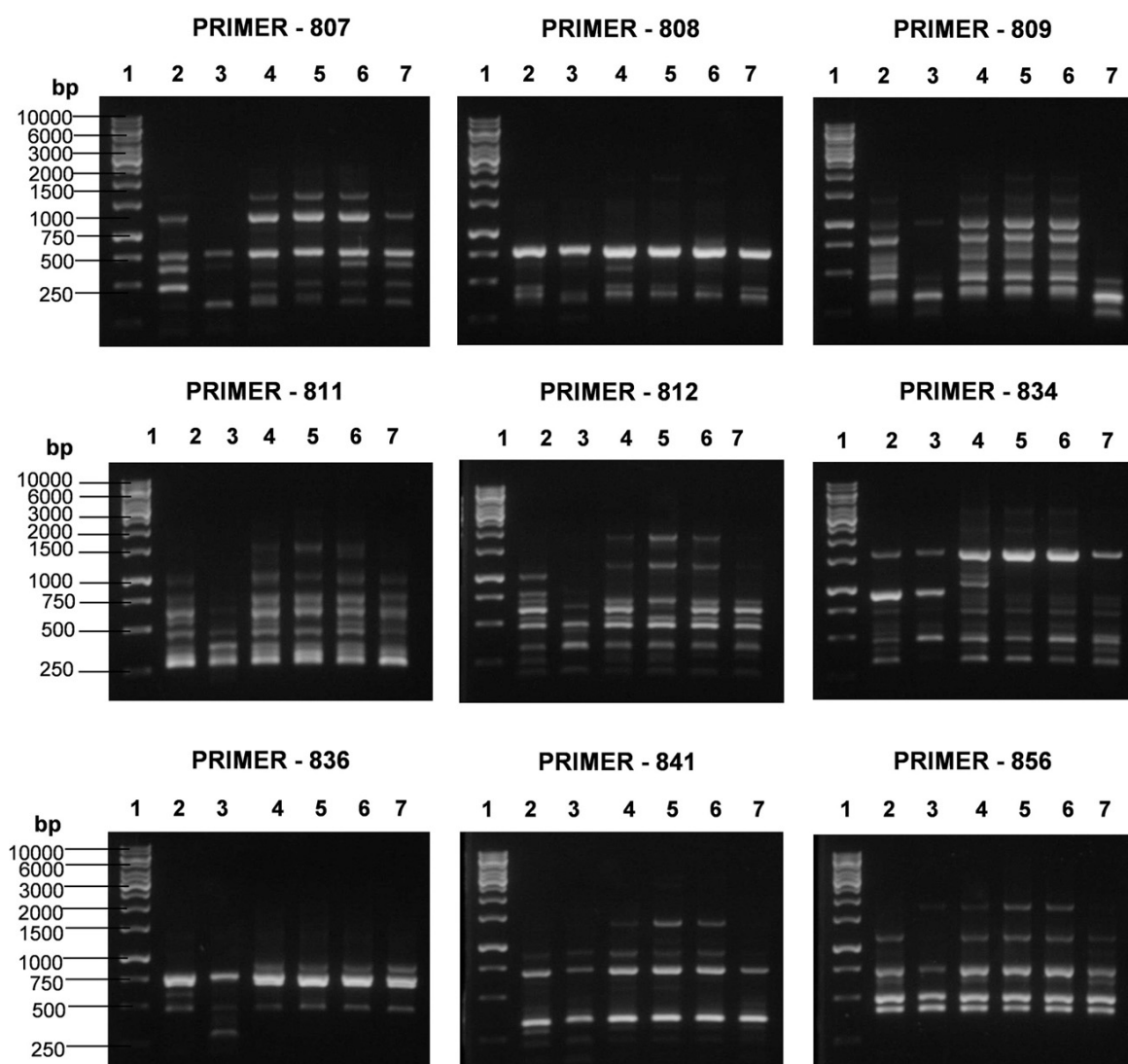


Figure 5. ISSR fingerprinting of the salt-tolerant cowpea genotypes. ISSR analysis was done with six cowpea genotypes using nine UBC primers. Lane 1 represents the 1 kb DNA ladder, 2- VBN1, 3- VBN2, 4- DC15, 5- KBC2, 6- VCP-09-001 and 7- IVT-VCP-09-013.

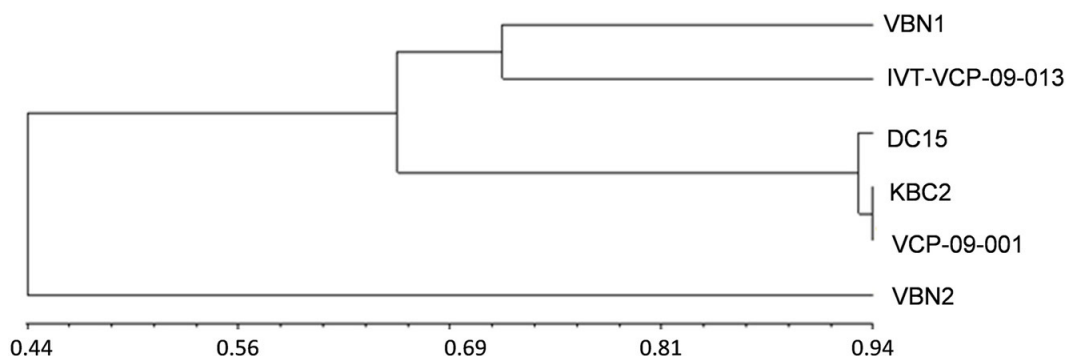


Figure 6. Dendrogram showing the genetic relationship between six cowpea genotypes.

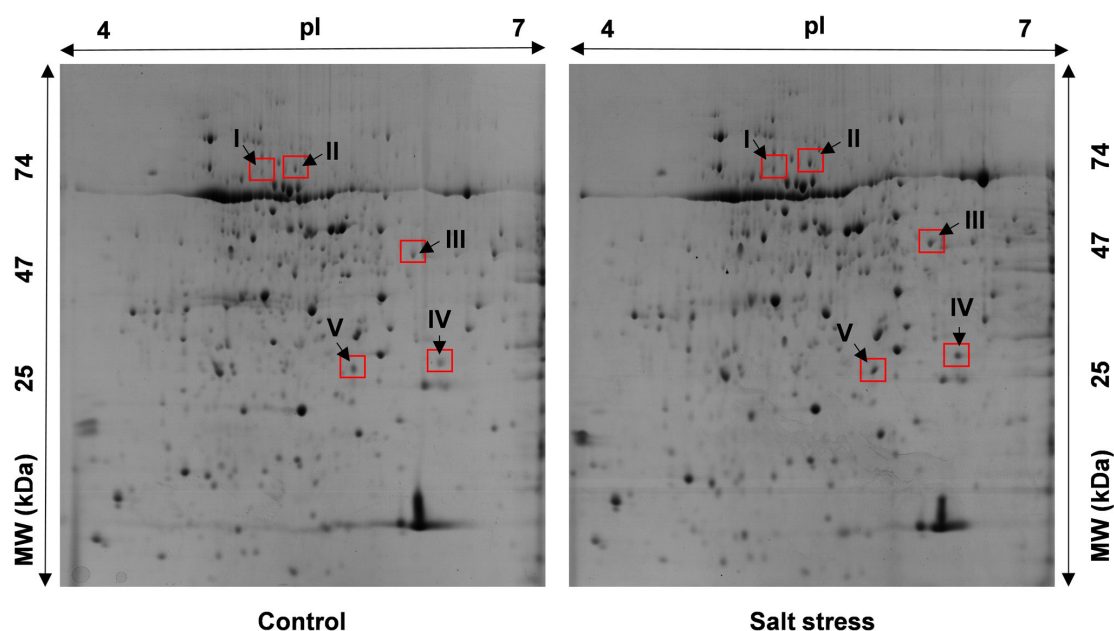


Figure 7. Two dimensional gels of proteins expressed in VBN2 cowpea leaf grown under control (0 mM NaCl) and salt stress (75 mM NaCl) conditions. Upregulated protein spots are numbered I to V.

4.4. Two-dimensional gel analysis and protein identification

The protein profile revealed the presence of approximately 175 clear and consistent protein spots (Figure 7), of which five spots showed a 1.7-fold or more upregulated expression (Figure 8). All identified proteins were tabulated with identity, NCBI accession number of homology protein, score, experimental and theoretical pI value and molecular weight (Table 5). The upregulated protein (spot I) matched with the mitochondrial ATP synthase beta sub unit of *Vigna radiata* (Table 5), which is one of the subunits of the catalytic core of ATP synthase. Mitochondrial ATP synthase catalyses the ATP synthesis, coupled with proton transport, during the mitochondrial electron transport chain. Hence, the upregulated protein is

involved in energy production under salt stress. Spot II matched with the V-type proton ATPase catalytic subunit A of *Vigna radiata* (Table 5). A mascot score of 98, with 38% protein sequence coverage, was obtained. The vacuolar-type H⁺-ATPase in plants is a large multimeric enzyme complex and involved in the pumping of protons across the membrane via primary active transport, playing a critical role in the maintenance of homeostasis in the vacuoles of plant cells. The V-type proton ATPase catalytic subunit A contains a nucleotide-binding motif and functions to bind and hydrolyse ATP.

The protein sequence of spot III had 51% similarity with a pentatricopeptide repeat (PPR) superfamily protein of *Monsoniae marginata* (Table 5). The PPR proteins are distinguished by the presence

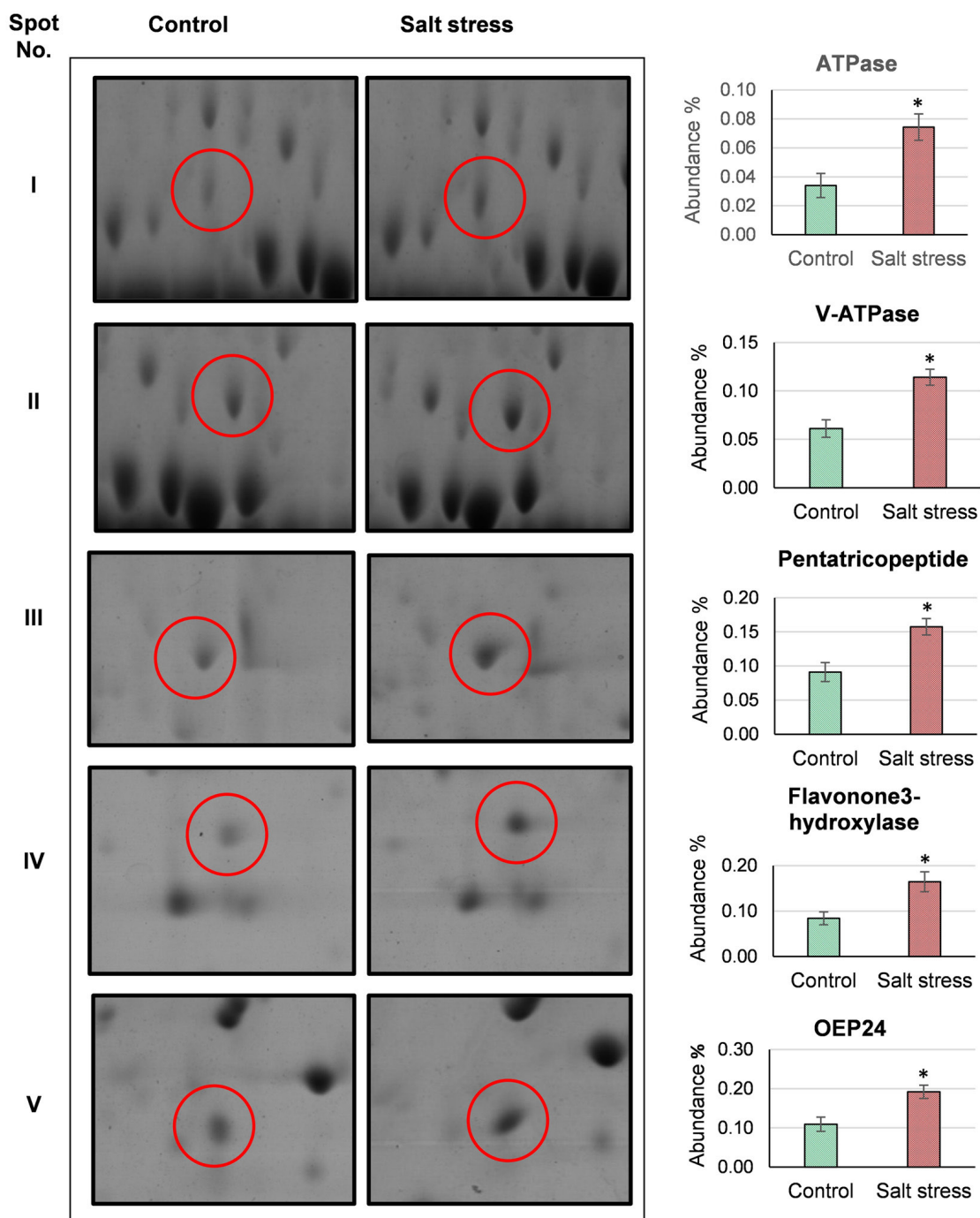


Figure 8. Enlargement of the five spots (I to V) within the 2-DE gels of VBN2 cowpea leaf. The spots within the red circles indicate the upregulated protein spots which showed significant increased intensity changes during salt stress (75 mM NaCl).

of tandem degenerate PPR motifs consisting of 35 degenerate amino acids. These proteins facilitate the processing, splicing, editing, stability and translation of RNAs. An abundant expression of flavanone 3-hydroxylase (F3H) was observed in this study. The protein sequence of spot IV matched with flavanone 3-hydroxylase of *Secale cereal*, with a mascot score of 60 and a sequence coverage of 50% (Table 5). The

F3H is a key enzyme involved in the biosynthesis of flavonoids, which are phenolic compounds involved in plant defence mechanisms. Flavonoids are powerful non-enzymatic antioxidants. Spot V showed similarity with the sequence of the outer envelope pore protein 24 (OEP24) of *Nicotiana tomentosiformis* (Table 4). The current proteome study thus revealed the abundance of certain proteins under salt stress, related to energy

Table 5. Upregulated proteins in cowpea (VBN2) leaf under salt stress identified by MALDI-TOF/MS and MASCOT peptide mass fingerprinting

S. No	Description	Accession No. (NCBI)	Sequence coverage (%)	Score	Function	Theor. PI/ MW (kDa)	Exp. PI/ MW (kDa)
I	Predicted: ATP synthase beta subunit, (<i>Vigna radiata</i>)	gi 951027555	33	49	Energy production (mitochondria)	5.90/ 59.78	5.1/ 72
II	V- ATPase subunit A (<i>Vigna radiata</i>)	gi 955078628	38	98	Acidification of intracellular compartments	5.30/ 68.92	5.3/ 74
III	Pentatricopeptide repeat protein (<i>Monsonia emarginata</i>)	gi 817499522	51	51	RNA processing (mitochondria, chloroplast)	8.29/ 50.49	6.1/ 47
IV	Flavanone 3-hydroxylase (<i>Secale cereale</i>)	gi 461939648	50	60	Secondary metabolism/ defense	6.92/ 15.13	6.3/ 27
V	Predicted: Outer envelope pore protein 24 (<i>Nicotiana tomentosiformis</i>)	gi 697149619	51	67	Chloroplast membrane transport	7.77/ 24.24	5.7/ 25

The spot numbers correspond to those given in Figure 7

metabolism, ion homeostasis, defence and transport; this can potentially contribute towards salt tolerance in cowpea.

5. DISCUSSION

The antioxidant responses of salt-sensitive and salt-tolerant cowpea genotypes at the early seedling stage were studied to understand the antioxidant mechanisms in cowpea leaves and roots. Leaves and roots presented distinct responses to salinity in relation to carotenoids, α -tocopherol, CAT and SOD (Figs. 1 and 2). The differences in the responses between leaves and roots of salt-stressed cowpea seedlings are due to the variation in the types of cellular and organelle metabolism in these organs. The responses of POD activity in leaves and roots of salt-treated seedlings were similar. The salt-sensitive genotype CPD121 showed comparatively lower levels of enzymatic and non-enzymatic antioxidants compared to those of salt-tolerant genotypes (Figs. 1 and 2).

Among the non-enzymatic antioxidants analysed, α -tocopherol contents of leaves significantly increased in all genotypes, but did not change in CPD121 under salt stress when compared to that of the control (Figure 1). In contrast to leaves, α -tocopherol contents in roots remarkably decreased under salt stress. In plants, α -tocopherol is mostly distributed along with chlorophyll in chloroplasts, possibly because of the specificity of tocopherols in scavenging singlet oxygen radicals in photosystem II (36). Our study revealed higher levels of α -tocopherol in the leaves compared to roots in un-stressed plants. Thus, tocopherol appears to be a more shoot-specific antioxidant in cowpea seedlings. The negative correlation of leaf α -tocopherol against H_2O_2

and MDA (Table 2), observed in this study, confirms that α -tocopherol minimises membrane damage and offers protection against ROS produced in leaves. Carotenoid levels decreased in the roots of all salt-stressed cowpea genotypes (Figure 1). In leaves, KBC2 showed a remarkable increase in carotenoid levels under salt stress compared to the other genotypes. This genotype showed the highest salt tolerance index for chlorophyll a and total chlorophyll in our previous study (20). Interestingly, the high level of carotenoids, along with tocopherol, under salt stress in KBC2 might have contributed to the higher stability of chlorophyll molecules in this genotype. Also, the correlation study, which showed a decrease in MDA content with a relative increase in carotenoids (Table 2), revealed the antioxidant role of carotenoids under salt stress in cowpea leaves.

To cope with oxidative damage, plants finely regulate enzymatic antioxidants (37). In our study, the decreased CAT activity was compensated by the elevated POD activity in leaves in combating H_2O_2 (Figure 2). Therefore, we suggest a major role of POD in detoxifying H_2O_2 in salt-stressed cowpea plants. This is confirmed by the increase in POD activity in both leaves and roots under salt stress (Figure 2) and by the negative correlation with H_2O_2 (Table 2). Increased POD activities have been reported earlier in some cowpea varieties under salt stress (38-40); in our study, an increased SOD activity was also observed in roots when treated with salt.

Leaves expressed higher H_2O_2 contents than roots under salt stress. Also, this study showed a decrease in the activity of leaf CAT, which is a key enzyme in the detoxification of H_2O_2 (Figure 2). For effective antioxidant protection, a balance between the antioxidant enzymes has to be maintained (7). Hence,

increased POD activity might have down-regulated CAT activity, and increased photorespiration and decreased CAT activity might have contributed to the increased H_2O_2 levels in leaves. Interestingly, although H_2O_2 accumulation was high, the MDA content was lower in the leaves compared to the roots (Figure 3), which can be explained by the higher α -tocopherol content in leaves under salinity. Tocopherols scavenge lipid peroxyl radicals and protect polyunsaturated fatty acids, thus preventing membrane damage (41). Also, the lower stress impact observed in the leaves might be due to the systemic signalling of the ROS from roots to leaves, causing the leaves to activate better antioxidative protection against salt stress (42).

Pod length and hundred-seed weight were the parameters most affected by salt stress. Seed number per pod was least affected by salinity. Pod number per plant was significantly decreased in three genotypes (Table 3). As established in previous studies, the decrease in pod number might be due to stress-induced embryo abortion during flowering and pod setting (43). The decrease in hundred-seed weight, observed in this study (Table 3), reflects that the seed filling duration is decreased under salt stress, leading to smaller seeds. The observed early senescence of the salt-stressed plants confirms this hypothesis. Comparable results have been reported in studies under drought stress in soybean (44). Although differences in the grain yield were observed among the six genotypes under salt stress, the percentage decrease in yield did not show significant differences among genotypes (Table 3). This confirms that these genotypes have the same level of stress tolerance during the harvest stage. The grain yield of genotype VBN2 was higher than that of other genotypes under salt stress (Table 3). This variety is already being cultivated as a high-yielding cowpea in many fertile areas in South India, especially in Tamil Nadu. Our study suggests that the cowpea variety VBN2 is better suited for cultivation in salt-affected soils.

Salt stress caused significant changes in crude fat and crude fibre contents (Figure 4). The other biochemical parameters, such as crude protein, ash and carbohydrate levels, were not affected. Salt stress can impose source limitations on grain filling because all major processes such as photosynthesis, protein synthesis, energy production and lipid metabolism are affected (45). Also, the observed low K^+/Na^+ ratio under salt stress in our previous study (20) might have impaired the function of K^+ in transporting the carbon sources to the site of seed filling. This might be the reason for the reduction in the crude fat and fibre contents in the grains. Protein and carbohydrate contents were not affected under salt stress (Figure 4), since these are the major storage metabolites in cowpea grains. Due to genetic influence, the limited supply of source materials might have been used with

priority for the synthesis of these components. Also, the early senescence of the leaves observed in this study shows that nitrogen and other nutrients are mobilised from these leaves to the seeds for protein and starch synthesis, possibly explaining the lower variations in these components.

Knowledge of genetic diversity is essential for developing systematic breeding and conservation strategies. Several DNA profiling techniques are now available for diversity studies of plants. Among these, ISSR (inter simple sequence repeat) analysis is a simple DNA marker technique extensively used for detecting polymorphisms in numerous plant species (46). Variations in the microsatellite regions present in plant genomes are the targets for the ISSR method of DNA fingerprinting and the assessment of genetic diversity. This technique is carried out using a single primer and requires no prior sequence information. Only a small amount of DNA template is sufficient, and the results are storable and reproducible (47). Hence, ISSR fingerprinting was conducted in this study.

Genetic fingerprinting revealed a high polymorphism among the genotypes and generated 72% polymorphic bands, although they were all categorised as salt-tolerant (Table 1). Similarly, a high level of polymorphism was observed using ISSR markers in Algerian cowpea landraces (35). The dendrogram generated by ISSR molecular data showed that VBN2 formed a unique cluster, at the coefficient of 0.44, indicating that it is quite different compared to the remaining genotypes (Figure 6). However, no unique profile in the antioxidant or grain quality was observed for VBN2 when compared with other genotypes. However, the number of pods per plant in VBN2 differed from that for other genotypes. In VBN2, the number of pods per plant was 14, whereas in other genotypes, it ranged between 9.33 and 11.67. Pod lengths of the genotypes DC15, KBC2 and VCP-09-001, which were grouped in the same cluster, showed almost similar values of 16.33, 16.71 and 16.51 cm, respectively. The cultivars KBC2 and VCP-09-001 had the highest similarity, and most of the root antioxidant (carotenoid, tocopherol, CAT, POD) and grain quality (protein, ash, crude fibre) parameters of these two genotypes showed almost similar profiles. The remarkable molecular discrimination between these cowpea genotypes indicates that they are good candidates for breeding studies.

Proteome profiling with the high-yielding VBN2 genotype revealed the up-regulation of the mitochondrial ATP synthase beta subunit, involved in ATP synthesis under salinity conditions (Table 5). The ATP, the main source of energy, is indispensable for many metabolic pathways in higher plants. Energy provision is essential for plants for the synthesis of compatible solutes and is one of the strategies of

plants to cope with salt stress (48,49). Our previous study has revealed an increased accumulation of the compatible solute proline and of proteins under salt stress (20). The study also showed that these metabolites accumulated in higher levels in salt-tolerant genotypes compared to salt-sensitive ones. In agreement with the current result, up-regulation of ATP synthase has been reported in the proteomic studies of salt-stressed plants (50-52). Therefore, up-regulation of ATP synthase provides salt tolerance to cowpea plants.

The up-regulation of ATP synthase might be linked with the abundance of the outer envelope pore protein 24 (OEP24), which was observed in this study (Table 5). The OEP24 is a chloroplast outer membrane porin which facilitates the transport of triose phosphates, hexose-phosphates, sugars, ATP, phosphates, dicarboxylates such as 2-oxoglutarate and charged amino acids (53). The up-regulation of OEP24 in this study suggests the increased demand of metabolite flux across the chloroplast membranes. Previous studies have reported that C4 plants express an abundance of OEP24 compared to C3 plants as an adaptation to higher CO₂ assimilation rates (54-56). Although cowpea is a C3 plant, the up-regulation of OEP24 will be helpful in increasing the transport of solutes across the chloroplast membrane to meet the high nutrient requirements under stressful conditions.

Proteome analysis showed an abundance of the V-type proton ATPase (V-ATPase) catalytic subunit A under salt stress. Several studies have also reported the up-regulation of V-ATPase subunits in different crops (57-60). The V-ATPase helps to sequester Na⁺ from the cytosol to the vacuole (61). Detoxification of Na⁺ from the cytosol by sequestration into the vacuole is an important strategy adopted by many salt-tolerant plants. The sequestration of Na⁺ in salt-tolerant genotypes might have contributed to the lower ion toxicity and hence the lower accumulation of H₂O₂ in salt-tolerant genotypes, as observed in this study. The salt-sensitive genotype CPD121 accumulated extremely high levels of H₂O₂ and MDA in both leaf and root tissues. Thus, this study suggests that Na⁺ sequestration plays a vital role in salinity tolerance in cowpea plants.

Our study revealed a possible role of pentatricopeptide repeat (PPR) protein in salt tolerance by its relative abundance under salt stress. Some PPR proteins were found to confer salt, drought and cold tolerance in *Arabidopsis* (62-64). Up-regulation of flavanone 3-hydroxylase (F3H) was also observed in this work. The F3H is a key enzyme at a diverging point of the flavonoid pathway, leading to the production of dihydroflavonols, various isoflavones and anthocyanins. These phenolic secondary metabolites

can mostly be attributed to free radical scavenging and antioxidant activities, thus overcoming oxidative stress (65). This study showed that leaf phenolic content in the salt-sensitive genotype CPD121 decreased drastically under salt stress, whereas the salt-tolerant VBN2 showed an increase in phenolic content (Figure 1). Also, the leaf phenolic content was negatively correlated with both H₂O₂ and MDA levels. Thus, the abundance of F3H in the VBN2 genotype confers salt tolerance, with increased antioxidant activity and the prevention of membrane damage. The current proteome data thus strongly establish the up-regulation of proteins involved in salt tolerance mechanisms in cowpea. To the best of our knowledge, we are the first to report the up-regulation of pentatricopeptide repeat protein, flavanone 3-hydroxylase and outer envelope pore protein in salt-stressed cowpea leaves.

6. CONCLUSIONS

Collectively, it is noteworthy to understand the biochemical fingerprints of cowpea seedlings under salt stress and the underlying salt tolerance mechanisms. Although the cowpea cultivar VBN2 is already being cultivated in several fertile areas of South India, its potential to perform in salt-affected areas is established by this study. The observed high genetic polymorphism suggests that the cowpea genotypes VBN2, VBN1, DC15, KBC2, VCP-09-001 and IVT-VCP-09-013 can be used for breeding and the development of better high-yielding salt-tolerant cowpea varieties. These findings open up new windows by pointing out salt stress-associated proteins that could be extensively studied and used for developing salt-tolerant cowpea varieties.

7. ACKNOWLEDGEMENTS

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Abbreviations: ATP, adenosine tri phosphate; CAT, catalase; CHAPS, 3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate; CTAB, cetyltrimethylammonium bromide; DNA, deoxyribonucleic acid; 2-D, two-dimensional; 2-DE, two-dimensional electrophoresis; dNTP, deoxynucleotide triphosphate; EC, electrical conductivity; GDHP, guaiacol dehydrogenation products; IEF, isoelectric focussing; ISSR, inter simple sequence repeat; MALDI-TOF/MS, matrix-assisted laser desorption/ionization- time-of-flight mass spectrometer; MDA, malondialdehyde; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; SDS, sodium dodecyl sulphate; SOD, superoxide dismutase; TBA, thiobarbituric acid; UPGMA, unweighted pair group method; VBN1, VBN2, DC15, KBC2, VCP-09-001, IVT-VCP-09-013, CPD121, genotypes of cowpea.

Key Words: Cowpea Genotypes, Antioxidant, Lipid peroxidation, Grain Quality, Polymorphism, Vacuolar ATPase, Pentatricopeptide Repeat Protein, Flavanone 3-hydroxylase

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