



## Original article

Getting to the roots of *Cicer arietinum* L. (chickpea) to study the effect of salinity on morpho-physiological, biochemical and molecular traitsGurpreet Kaur<sup>a,b</sup>, Satish Kumar Sanwal<sup>b,\*</sup>, Nirmala Sehrawat<sup>a</sup>, Ashwani Kumar<sup>b</sup>, Naresh Kumar<sup>b</sup>, Anita Mann<sup>b</sup><sup>a</sup>Maharishi Markandeshwar (Deemed to be University), Mullana, Ambala, Haryana, India<sup>b</sup>ICAR-Central Soil Salinity Research Institute, Karnal, Haryana, India

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## ABSTRACT

The effect of saline irrigation ( $EC_{iw}$  6  $dS\ m^{-1}$  and 9  $dS\ m^{-1}$ ) on the roots of *Cicer arietinum* L. genotypes was examined at morpho-physiological, biochemical and molecular levels. Reduction in root growth due to salinity was observed, but less effect was seen on the roots of genotypes KWR 108, ICCV 10, CSG 8962, and S7 as compared to the other genotypes. Cell turgor was maintained in tolerant genotypes through optimum water relations and osmoprotectants (proline and total soluble sugars) than the sensitive cultivars. Salinity caused oxidative stress as increased hydrogen peroxide and malondialdehyde were noticed, where low accumulation was observed in tolerant genotypes due to the higher activity of enzymatic antioxidants (superoxide dismutase, catalase, ascorbate peroxidase, glutathione reductase and peroxidase).  $Na^+/K^+$  ratio increased, but more increment was reported in sensitive cultivars. Gene expression studies depicted that genes encoding pyrroline-5-carboxylate synthetase and pyrroline-5-carboxylate reductase got upregulated and that of proline dehydrogenase was downregulated and more fold change with respect to control was in the salt tolerant check CSG 8962 and the genotype KWR 108. Higher expression of the genes encoding reactive oxygen species scavenging enzymes namely, superoxide dismutase, catalase, peroxidase, and those involved in the ascorbate–glutathione cycle was noticed in KWR 108 and CSG 8962 than ICC 4463. Enhanced expression of sodium transporter *HKT1* due to salinity can be correlated with ion homeostasis maintenance. Cumulative effects of osmolytes, enzymatic antioxidants and maintaining ion homeostasis in root enable chickpea plants to survive in saline environments. © 2022 Published by Elsevier B.V. on behalf of King Saud University. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

## 1. Introduction

Chickpea is an important legume crop with high nutritional values and it will be one of the important food crop in changing climate era for food sustainability. Although global chickpea production has increased significantly from 6.4 to 14.7 MT during the last decade but the crop productivity is also being limited by abiotic stresses, especially salinity decreasing upto 10–15 % crop yield (Kaashyap et al., 2022). Chickpea being a cool-season legume is considered relatively salt-sensitive showing wide genotypic vari-

ation from seedling to maturity stage (Kaur et al., 2021; Mann et al., 2019). Salinity stress, a major abiotic factor, disrupts various physiological, biochemical and molecular processes which significantly affect the plant biomass and grain yield (Zörb et al., 2019). Salinity induces biphasic responses in plants, an early ion-independent osmotic phase leading to a reduction in water uptake and ionic toxicity that causes ion imbalance. Plants show various adaptive strategies to overcome the negative effects of salinity either through osmolyte accumulation that helps the plant to maintain cell turgor or by maintaining low  $Na^+$  ion concentration in the critical tissues, cells, and organs, or by enhancing the biosynthesis of antioxidants to balance the excessively generated reactive oxygen species (ROS), etc. (Munns and Tester, 2008; Zhao et al., 2020). The root is the primary sensor of abiotic stress facing the damages in terms of reduced lateral root formation with decreased water and nutrient uptake. Additionally, salinity increases the uptake of  $Na^+$  ions and its transportation from root to shoot (Bhat et al., 2020). Root plasticity under salt stress is the key to

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copied with stressful conditions, which prevents the accumulation of salts in the root and hence maintains water uptake from such soils (Arif et al., 2019; Kong et al., 2017). Root responds to salinity by altered regulation of genes and proteins that changes the transport processes, composition of the cell wall, shape, and size of the cell and hence changes the architecture of the root (Byrt et al., 2018). Root traits are directly related to the shoot's physiological traits to cope with stress responses, viz., adaptations at the biochemical level through osmotic adjustment, ROS detoxification and adaptations at the molecular level to maintain physiological growth under salt stress conditions. Under stressed conditions, these complex interactive systems adjust homeo-statically to minimize the negative impacts of stress and maintain metabolic equilibrium. Optimal absorption and translocation of water and essential nutrients under abiotic or biotic stresses are particular features of the robust root system. Transcriptomic analysis of chickpea roots revealed that some important genes related to cationic peroxidases, phosphatidylinositol phosphate kinase, asparticase, NRT1/PTR, DREB1E and ERF etc got significantly up-regulated in the tolerant genotype. Further, authors reported that some important genes, namely, dirigent proteins, expansin and casparian strip membrane proteins, which are associated with cell wall modification and root morphogenesis, might confer tolerance towards salinity in chickpea (Kaashyap et al., 2018). Stress signaling and adaptation at root system define the response at upper plant parts, therefore, this experiment was designed to screen the root characters in chickpea under saline conditions.

## 2. Materials and methods

### 2.1. Plant material and experimental set up

Seeds of ten chickpea genotypes, BG 1103, S7, DCP 92-3, ICCV 10, KWR 108, BG 256, K 850, JG 16, ICC 4463 along with the salt-tolerant check CSG 8962 (Karnal chana 1) (pedigree given in supplementary table 1) were collected from the repository of ICAR-Central Soil Salinity Research Institute, Karnal. Seeds were treated with the *Rhizobium* culture ( $10^8$  cfu/ml) purchased from Chaudhary Charan Singh Hisar Agricultural University Hisar. The experiment was conducted for two consecutive years during Rabi cropping season of 2018-2019 and 2019-2020, respectively, at ICAR-Central Soil Salinity Research Institute, Karnal, in 20 Kg capacity porcelain pots filled with acid-washed sand in randomized complete block design with three replications. Natural saline water and best available water which served as control were used for irrigation purpose and the composition of both the types of water is reported in our earlier work (Kaur et al., 2021). Based on bulk density and volume, pots were saturated with 3.12 L of respective irrigation water before sowing. Natural saline water was diluted to  $EC_{iw} \sim 6$  dS  $m^{-1}$  and  $EC_{iw} \sim 9$  dS  $m^{-1}$  and treatment-wise irrigation along with nitrogen-free nutrient solution Wilson and Reisenauer (1963) was given at regular intervals on the basis of 100 % evapotranspiration (ET). Sampling for all parameters was done at the flowering stage.

### 2.2. Morphological parameters

Uprooted plants were washed to remove the sand particles and measured the length and fresh weight of the roots. Dry weight was measured after drying them at 65 °C for 72 h in an oven.

### 2.3. Relative water content (RWC)

The method of Barr and Weatherley (1962) and formula  $[(FW-DW)/(TW-DW)]*100$  was applied for RWC (%) estimation, where

FW: fresh weight, TW: turgid weight measured after immersing the samples in double distilled water for 4 h and DW: dry weight measured after drying the samples at 72 °C for 48 h.

### 2.4. Water potential ( $\psi_w$ )

Fresh root sample (0.5 g) was finely chopped, and WP4C Dew-point Potentiometer (METER Group, Inc. USA) was used to measure the water potential ( $\psi_w$ ). The instrument works on the chilled-mirror dew point technique and displays the reading as -MPa (Haghverdi et al., 2020).

### 2.5. Osmotic potential ( $\psi_s$ )

Osmolality (c) was determined according to the method given by Cuin et al., (2009). Fresh root samples (1 g) were collected and frozen at -20 °C. Crushed samples were squeezed to extract the sap, and osmolality was measured with 5  $\mu$ l of the sap using Vapor Pressure Osmometer (Model 5600, ELITech Group, Belgium) after calibrating it with the osmolality reference standards of NaCl (Wescor Inc, USA). Osmolality reading (mmol/kg) was converted to osmotic potential by applying the Vant'Haff equation (Hessini et al., 2019).

$$\psi_s \text{ (MPa)} = -c \times 2.58 \times 10^{-3}$$

### 2.6. Membrane stability

The method given by Dionisio-Sese and Tobita (1998) was used to determine membrane stability. Root samples (100 mg) were incubated in 20 ml of doubled distilled water at room temperature for 2 h. Electrical conductivity was measured before and after boiling the samples. Percentage membrane stability was calculated by applying the formula:

$$\text{Membrane Stability (\%)} : [(EC \text{ after boiling}) / (EC \text{ before boiling} + EC \text{ after boiling})] * 100$$

### 2.7. Measurement of oxidative stress indicators

Malondialdehyde (MDA) and hydrogen peroxide ( $H_2O_2$ ) were determined according to the method of Loreto and Velikova (2001). Root tissue (100 mg) was homogenised in 2 ml of 0.1 % (w/v) trichloroacetic acid (TCA). After centrifugation at 12000 rpm for 15 min at 4 °C the supernatant was collected. For MDA content, 1 ml of supernatant was added to 2 ml of 0.5 % TBA (2-Thiobarbituric acid) dissolved in 20 % TCA and incubated at 100 °C for 30 min in a boiling water bath. The reaction was stopped by placing the tubes at -20 °C for 15 min in deep fridge (Panasonic), followed by centrifugation at 10000 rpm for 5 min. Absorbance was measured at 532 nm and 600 nm on UV spectrophotometer (SPECORD 210 PLUS) using the supernatant against control and calculated the MDA content using extinction coefficient 155. The reaction containing 1 ml of supernatant, 1 ml of 10 mM potassium phosphate buffer (pH 7.0), and 2 ml of 1 M potassium iodide was used to read the absorbance at 390 nm using control as blank for hydrogen peroxide estimation. The standard curve was plotted using micromoles concentrations of hydrogen peroxide.

### 2.8. Assay for enzymatic antioxidants

Enzyme extract from fresh root tissue (300 mg) was prepared using 3 ml of 0.1 M potassium phosphate buffer (pH 7) in three biological replications. Centrifuged at 12000 rpm for 15 min and

collected the supernatant. An assay for superoxide dismutase (SOD: EC 1.15.1.1) activity was conducted following the [Beauchamp and Fridovich \(1971\)](#) method, and the absorbance was read at 560 nm on UV spectrophotometer (SPECORD 210). The formula given by [Giannopolitis and Ries \(1977\)](#) was applied and finally, extinction coefficient  $167 \text{ mM}^{-1}\text{cm}^{-1}$  was used to calculate the SOD activity in EU/g FW (Enzyme unit/gram fresh weight), and one EU is the enzyme required to inhibit the photoreduction of  $1 \mu\text{mol}$  of Nitro blue tetrazolium. Decomposition of  $\text{H}_2\text{O}_2$  by catalase (CAT: EC 1.11.1.6) was analyzed by the method of [Aebi \(1984\)](#). The decline in absorbance was observed for 3 min at 240 nm, an extinction coefficient of  $0.036 \text{ mM}^{-1}\text{cm}^{-1}$  was used to calculate the activity in EU/g FW that define one  $\text{mmol H}_2\text{O}_2 \text{ ml}^{-1}\text{min}^{-1}$ . A decrease in absorbance at 290 nm was observed for ascorbate peroxidase (APX:EC 1.11.1.11) as per the method of [Nakano and Asada \(1981\)](#). The activity was calculated using the extinction coefficient  $2.8 \text{ mM}^{-1}\text{cm}^{-1}$  and expressed as EU/g FW, which defines the enzyme required to oxidize one  $\text{nmol}$  of ascorbate  $\text{min}^{-1}$ . Glutathione reductase (GR:EC 1.6.4.2) activity assay was conducted using the reaction mixture mentioned by [Foyer and Halliwell \(1976\)](#). NADPH oxidation led to a decrease in the absorbance at 340 nm, and enzyme activity was calculated using the extinction coefficient  $6.22 \text{ mM}^{-1}\text{cm}^{-1}$  and expressed as EU/g FW. One enzyme unit is defined as the amount of enzyme required to oxidize one  $\text{nmol}$  of NADPH  $\text{min}^{-1}$ . Assay for guaiacol peroxidase (POX:EC 1.11.1.7) was performed at 470 nm where formation of tetra-guaiacol led to the increase in the absorbance [Castillo et al., \(1984\)](#). Extinction coefficient  $26.6 \text{ mM}^{-1}\text{cm}^{-1}$  was used to calculate the POX activity and expressed as EU/ g FW, where one unit of GPOX activity is defined as the amount of enzyme required to oxidize one  $\text{nmol}$  of guaiacol  $\text{min}^{-1}\text{ml}^{-1}$ .

## 2.9. Osmoprotectants quantification assay

The method [Bates et al., \(1973\)](#) was used to estimate proline in root tissue (200 mg) extracted in 5 ml of 3% sulphosalicylic acid in three biological replications. The supernatant was collected after centrifugation (10,000 rpm, 10 min, 4°C). A reaction mixture containing 2 ml supernatant + 2 ml of acid ninhydrin reagent (1.25g

ninhydrin added to 20 ml of 6 N o-phosphoric acid and 30 ml of glacial acetic acid) + 2 ml of glacial acetic acid was incubated at 100 °C for 1 h and after cooling the contents 4 ml of toluene was added. After vortexing upper phase was used to read the absorbance at 520 nm on a UV spectrophotometer (SPECORD 210 PLUS) using toluene as blank. A standard curve plotted using L-proline was used for calculation. Estimation of total soluble sugar (TSS) content was done according to the method given by [Yemm and Willis \(1954\)](#). Crushed 100 mg of sample in 2.5 ml of 80% ethanol and centrifuged (10,000 rpm, 10 min, 4°C). The supernatant (100 µl) was pipetted in a test tube and 5 ml of anthrone reagent (0.4% anthrone prepared in chilled concentrated sulphuric acid) was added. Incubated at 100 °C for 10 min, cooled the contents and read the absorbance at 620 nm on UV spectrophotometer using anthrone reagent as blank. The standard curve of D glucose was used for calculation.

## 2.10. Analysis of ionic composition

Dried and powdered root tissue digested with 80% nitric acid was used to estimate the presence of Na and  $\text{K}^+$  ions. A flame photometer (PFP7, Jenway, Bibby Scientific, UK) was used to estimate  $\text{Na}^+$  and  $\text{K}^+$  ions. The instrument was calibrated using the standard solutions of sodium chloride (NaCl) and potassium chloride (KCl) for  $\text{Na}^+$  and  $\text{K}^+$  respectively and the reading was represented in percentage after standard calculations.

## 2.11. Quantitative expression of the studied genes

Total RNA content was extracted using Trizol reagent (Hi-media) according to the manufacturer's protocol, and DNA was removed using DNase I (Thermo Scientific). The concentration and purity of RNA was checked on Nanodrop (Denovix® DS-11+ - Spectrophotometer). First-strand of cDNA was synthesized from DNase I treated RNA template with iScript™cDNA synthesis kit (Bio-Rad). qPCR was performed with 20 µl PCR reaction volume in three biological replications along with three technical replications using SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad). The gene sequences for Superoxide dismutase (SOD),

**Table 1**  
Genes and their corresponding primers with expected amplicon length (bp).

GENE	PRIMER SEQUENCE	EXPECTED PRODUCT SIZE (bp)	Annealing temperature (Tm)	ACCESION NUMBER
SOD	FP- GTG TGT TGT GGT TGT GCT AG RP- GCA AGC ATA CGA GGA TCT G	138	56	NM_001309708.1
CAT	FP-CTA CAA GCA CCG TCC ATC TAG RP-GAT CCA ACG GTT AGA GAG G	99	56	XM_004500820.3
APX	FP- ATG CGC TCC TCT WAT GCT CCG T RP-GCC TAA CAG CRA TRT CAA GAC C	145	54	XM_004505886.3
MDHAR	FP- GAC AAT GTT GGT GAC ACA GTG RP- GCC TTG TTC TCA TCA GGA G	137	54	NM_001309692.1
DHAR	FP- GGA CAA TAC CTG ATT CAC TTA CC RP- CGC CAA CCT TCAATG ACA TC	112	54	KF276974.1
GR	FP- GGT ATA ACA TCT GAC GAG G RP- GAG ATG GAC ATT GGA ACC	126	56	KF276973.1
POX	FP- GTG TGT TGT GGT TGT GCT AG RP- GCA AGC ATA CGA GGA TCT G	143	56	KJ808789.1
P5CR	FP- GCT TCT CAG ACT GTA TTA GGA G RP- CCT CCA GGA GAA GTT ACG TC	92	56	XM_004493357.3
P5CS	FP- CTCGGTCTTGTCATTTT RP- GTGCTAATCTTCCATCACTC	119	56	NM_001309676.2
PD	FP-GGT TGA ATC CTG TAG CCT AC RP-CAG GAA GGA GTT GGT TAG GAG	97	56	XM_004507197.3
HKT1	FP- CCA CAC CTC TGT TCT TCA ATT AC RP- GGA TAG AGA ACG AGA CAA GG	121	54	XM_004513052.3
ACT	FP-CCA GCA TTG TAG GTC GTC C RP- GCT TAG ACT GTG CCT CAT C	90	56	NM_001278957.1

**Table 2**  
Effect of saline irrigation on root morphological traits of chickpea genotypes.

Treatment Traits	Length (cm)			Fresh weight (g)			Dry weight (g)		
	Control	EC <sub>iw</sub> 6 dS/m	EC <sub>iw</sub> 9 dS/m	Control	EC <sub>iw</sub> 6 dS/m	EC <sub>iw</sub> 9 dS/m	Control	EC <sub>iw</sub> 6 dS/m	EC <sub>iw</sub> 9 dS/m
CSG 8962	16.67 ± 0.14 <sup>cd</sup>	14.92 ± 0.38 <sup>bc</sup>	12.33 ± 0.14 <sup>cd</sup>	4.76 ± 0.23 <sup>cd</sup>	3.83 ± 0.05 <sup>bc</sup>	3.08 ± 0.13 <sup>bc</sup>	1.98 ± 0.22 <sup>b</sup>	1.64 ± 0.07 <sup>bc</sup>	1.29 ± 0.08 <sup>bc</sup>
BG 1103	19.00 ± 1.32 <sup>abc</sup>	15.92 ± 1.13 <sup>abc</sup>	12.58 ± 0.63 <sup>bcd</sup>	5.32 ± 0.12 <sup>b</sup>	3.89 ± 0.11 <sup>b</sup>	2.74 ± 0.10 <sup>cd</sup>	2.15 ± 0.11 <sup>b</sup>	1.62 ± 0.07 <sup>bcd</sup>	1.10 ± 0.08 <sup>bcd</sup>
S7	20.42 ± 1.13 <sup>ab</sup>	17.58 ± 0.29 <sup>a</sup>	14.92 ± 0.38 <sup>ab</sup>	6.84 ± 0.39 <sup>a</sup>	5.34 ± 0.17 <sup>a</sup>	4.30 ± 0.23 <sup>a</sup>	3.04 ± 0.28 <sup>a</sup>	2.31 ± 0.10 <sup>a</sup>	1.92 ± 0.18 <sup>a</sup>
DCP 92-3	17.92 ± 1.01 <sup>cd</sup>	14.75 ± 0.75 <sup>c</sup>	11.92 ± 0.29 <sup>d</sup>	4.72 ± 0.42 <sup>cde</sup>	3.24 ± 0.09 <sup>de</sup>	1.86 ± 0.09 <sup>f</sup>	2.03 ± 0.30 <sup>b</sup>	1.32 ± 0.03 <sup>cd</sup>	0.86 ± 0.04 <sup>de</sup>
ICCV 10	16.50 ± 0.50 <sup>d</sup>	15.83 ± 0.58 <sup>abc</sup>	14.17 ± 0.52 <sup>abcd</sup>	4.27 ± 0.22 <sup>e</sup>	3.48 ± 0.09 <sup>bcd</sup>	2.85 ± 0.11 <sup>bc</sup>	1.79 ± 0.18 <sup>b</sup>	1.43 ± 0.11 <sup>bcd</sup>	1.18 ± 0.06 <sup>bed</sup>
KWR 108	18.58 ± 1.01 <sup>abcd</sup>	17.17 ± 0.72 <sup>ab</sup>	15.50 ± 1.15 <sup>a</sup>	4.68 ± 0.23 <sup>cde</sup>	3.90 ± 0.07 <sup>b</sup>	3.26 ± 0.09 <sup>b</sup>	2.12 ± 0.20 <sup>b</sup>	1.74 ± 0.09 <sup>b</sup>	1.44 ± 0.10 <sup>b</sup>
BG 256	20.92 ± 0.63 <sup>a</sup>	16.83 ± 1.13 <sup>abc</sup>	13.50 ± 1.15 <sup>abcd</sup>	5.47 ± 0.15 <sup>b</sup>	3.53 ± 0.24 <sup>bcd</sup>	2.04 ± 0.17 <sup>ef</sup>	2.12 ± 0.14 <sup>b</sup>	1.25 ± 0.17 <sup>d</sup>	0.85 ± 0.12 <sup>de</sup>
K 850	20.42 ± 0.76 <sup>ab</sup>	17.17 ± 0.38 <sup>ab</sup>	15.83 ± 1.13 <sup>a</sup>	5.02 ± 0.10 <sup>bc</sup>	3.41 ± 0.02 <sup>cde</sup>	2.82 ± 0.08 <sup>bcd</sup>	2.10 ± 0.08 <sup>b</sup>	1.45 ± 0.05 <sup>bcd</sup>	1.13 ± 0.15 <sup>bcd</sup>
JG 16	18.17 ± 1.76 <sup>bcd</sup>	16.17 ± 1.01 <sup>abc</sup>	14.50 ± 1.15 <sup>abc</sup>	4.35 ± 0.21 <sup>de</sup>	3.47 ± 0.13 <sup>bcd</sup>	2.36 ± 0.18 <sup>de</sup>	1.95 ± 0.25 <sup>b</sup>	1.50 ± 0.11 <sup>bcd</sup>	1.03 ± 0.18 <sup>cd</sup>
ICC 4463	18.00 ± 0.86 <sup>cd</sup>	15.00 ± 0.75 <sup>bc</sup>	13.00 ± 0.87 <sup>bcd</sup>	4.36 ± 0.17 <sup>de</sup>	2.94 ± 0.05 <sup>e</sup>	1.19 ± 0.09 <sup>g</sup>	2.02 ± 0.11 <sup>b</sup>	1.27 ± 0.04 <sup>cd</sup>	0.49 ± 0.04 <sup>e</sup>
<b>General Mean</b>	<b>18.66<sup>A</sup></b>	<b>16.13<sup>B</sup></b>	<b>13.83<sup>C</sup></b>	<b>4.98<sup>A</sup></b>	<b>3.7<sup>B</sup></b>	<b>2.65<sup>C</sup></b>	<b>2.13<sup>A</sup></b>	<b>1.55<sup>B</sup></b>	<b>1.13<sup>C</sup></b>
CV	T-5.74, G-4.50			T-4.94, G-4.25			T-9.37, G-7.35		
LSD	T-0.58, G-1.23, T × G 1.85			T-0.12, G-0.27, T × G 0.37			T-0.09, G-0.20, T × G 0.30		

Different letters represent level of significance, ( $p < 0.05$ ) using Tukeys test, among the genotypes facing same treatment, values with at least one letter common are non-significant. CV: coefficient of variance, LSD: Least significant difference, T: Treatment, G: Genotype, T × G: Treatment × Genotype.

Catalase (CAT), Ascorbate peroxidase (APX), Monodehydroascorbate reductase (MDHAR), Dehydroascorbate reductase (DHAR), Glutathione reductase (GR), Peroxidase (POX), Pyrroline-5-carboxylate synthetase (P5CS), Pyrroline-5-carboxylate reductase (P5CR), Proline dehydrogenase (PD), Sodium transporter (HKT1) and Actin (ACT) were selected from National Centre for Biotechnology Information (NCBI, <https://www.ncbi.nlm.nih.gov/>) and corresponding primers (details mentioned in Table 1) were designed using Primer Quest tool from Integrated DNA Technologies (<https://sg.idtdna.com/PrimerQuest/Home/Index>). Actin was used for normalization. Data obtained was calculated by applying the  $2^{-\Delta\Delta Ct}$  method (Livak and Schmittgen, 2001).

## 2.12. Statistical analysis

Data in triplicates was analysed following the two-way factorial analysis of variance (ANOVA) using SAS software (Version 9.3, SAS Institute Inc., USA). Mean differences were compared at a 5 % probability level using TUKEY'S Honest Significant Test.

## 3. Results

### 3.1. Saline irrigation affected the root growth

Significant negative effects of saline irrigation were seen on the root morphology with genotypic variations for reductions in root length, root fresh, and dry weight (Table 2) (percent reduction mentioned in supplementary table 2). Root length serves as a criterion to evaluate the stress responses of genotypes and found a mean reduction of 13.56 % and 25.88 % under EC<sub>iw</sub> 6 dS m<sup>-1</sup> and EC<sub>iw</sub> 9 dS m<sup>-1</sup>, respectively. Genotypes ICCV 10 (4.0 and 14.12 %) and KWR 108 (7.6 and 16.58 %) showed minimum reduction at EC<sub>iw</sub> 6 dS m<sup>-1</sup> and 9 dS m<sup>-1</sup> respectively compared to control. At higher salinity of EC<sub>iw</sub> 9 dS m<sup>-1</sup>, more than a 30 % reduction in root length was observed in genotypes BG 256 (35.46 %), BG 1103 (33.77 %), and DCP 92-3 (33.49 %).

A consistent decrease was noted in root fresh and dry weight in all the genotypes, but genotypes KWR 108, ICCV 10, CSG 8962, JG 16, and S7 showed <25 % reduction in fresh root weight at EC<sub>iw</sub> 6 dS m<sup>-1</sup> and <40 % reduction at EC<sub>iw</sub> 9 dS m<sup>-1</sup>. Dry weight was also reduced with increasing salt load in irrigation water, and more than 30 % reduction was observed in genotypes K 850 (30.68 %), DCP 92-3 (35.14 %), ICC 4463 (37.07 %), and BG 256 (41.10 %) at EC<sub>iw</sub> 6 dS m<sup>-1</sup> and more than 50 % reduction was observed in genotypes DCP 92-3 (57.80 %), BG 256 (59.84 %) and ICC 4463 (75.78 %) at EC<sub>iw</sub> 9 dS m<sup>-1</sup>.

### 3.2. Root water relations

Plant water attributes played an essential role under stress conditions and it was seen that salinity reduced the water uptake because a lowering in water potential ( $\psi_w$ ) and Osmotic Potential ( $\psi_s$ ) was observed (Fig. 1). Water potential under control conditions was observed between -0.66 MPa (BG 1103) to -0.87 MPa (JG 16), but reduction due to salinity was observed and at EC<sub>iw</sub> 6 dS m<sup>-1</sup>, the maximum value was displayed by the genotype KWR 108 (-1.23) followed by CSG 8962 (-1.32) and the minimum value was displayed by the genotype ICC 4463 (-1.72) followed by BG 256 (-1.65). At EC<sub>iw</sub> 9 dS m<sup>-1</sup>, the maximum value was displayed by the genotype KWR 108 (-1.67) and minimum by the genotype BG 256 (-2.01). Similarly, osmotic potential reduced with salinity, and at EC<sub>iw</sub> 6 dS m<sup>-1</sup> maximum osmotic potential was observed in KWR 108 (-1.47), followed by CSG 8962 (-1.51), and minimum osmotic potential was observed in ICC 4463 (-1.84) followed by DCP 92-3 (-1.81). At EC<sub>iw</sub> 9 dS m<sup>-1</sup> level, the maximum osmotic

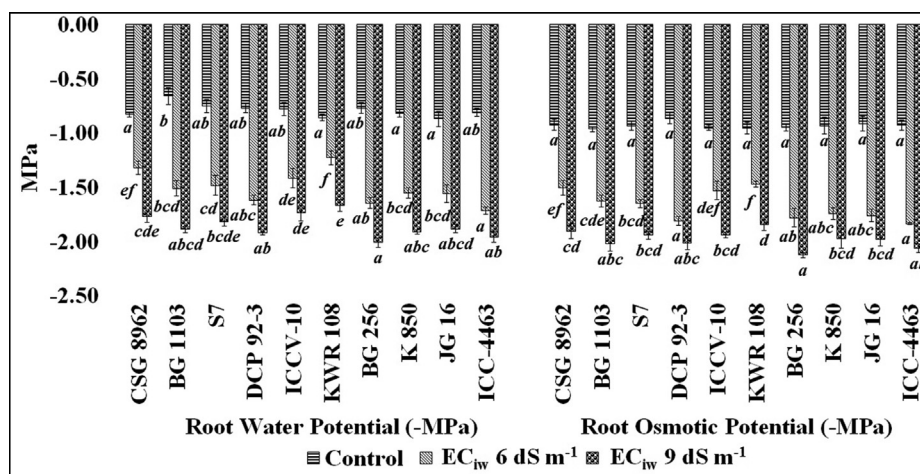


Fig. 1. Effect of saline irrigation on water potential ( $\psi_w$ ) and osmotic potential ( $\psi_o$ ) in chickpea roots. Different letters represent level of significance, ( $p < 0.05$ ) using Tukeys test, among the genotypes facing same treatment, values with at least one letter common are non-significant.

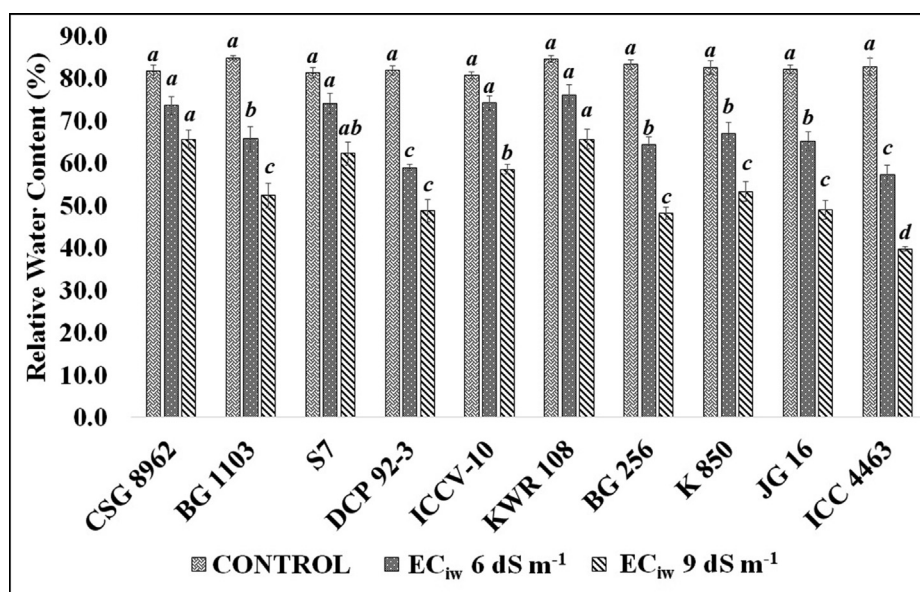


Fig. 2. Effect of saline irrigation on relative water content. Different letters represent level of significance, ( $p < 0.05$ ) using Tukeys test, among the genotypes facing same treatment, values with at least one letter common are non-significant.

potential was observed in KWR 108 (-1.85) followed by CSG 8962 (-1.91) and minimum in BG 256 (-2.13) followed by ICC 4463 (-2.06).

Root RWC also showed significant reduction with variable genotypic responses and at  $EC_{iw}$  6  $dS\ m^{-1}$  more than 15 % reduction was observed in the genotypes K 850 (18.94 %), JG 16 (20.67 %), BG 1103 (22.61 %), BG 256 (22.69 %), DCP 92-3 (27.95 %) and ICC 4463 (30.79 %) with respect to control. The salinity level of  $EC_{iw}$  9  $dS\ m^{-1}$  further reduced RWC, but genotypes CSG 8962, KWR 108, S7, and ICCV 10 showed <30 % reduction with respect to control (Fig. 2).

### 3.2.1. Reactive oxygen species components and their scavenging

Membrane stability under salinity stress is inversely related to MDA content and  $H_2O_2$  (Table 3) (percent reduction/increment mentioned in supplementary table 3). Under control conditions, membrane stability was observed between 80.51 % and 83.10 % decreasing by 16.18 % and 28.3 % at  $EC_{iw}$  6  $dS\ m^{-1}$  and 9  $dS\ m^{-1}$  respectively. At  $EC_{iw}$  6  $dS/m$ , the minimum reduction was observed in genotype KWR 108 (12.17 %) followed by S7 (14.63 %), and max-

imum reduction was observed in genotype ICC 4463 (23.36 %) followed by DCP 92-3 (22.01 %).

At  $EC_{iw}$  9  $dS\ m^{-1}$ , <30 % reduction in membrane stability was observed in genotypes JG 16 (25.14 %), KWR 108 (25.73 %), S7 (27.50 %), ICCV 10 (28.11 %) and CSG 8962 (28.30 %) w.r.t control. MDA content is a product of membrane lipid peroxidation of lipids which increases with salinity. At  $EC_{iw}$  6  $dS\ m^{-1}$ , <35 % increment w.r.t control was reported in genotypes KWR 108 (25.05 %), CSG 8962 (26.48 %), S7 (29.67 %) and ICCV 10 (34.36 %) and at  $EC_{iw}$  9  $dS\ m^{-1}$  these genotypes showed approximately 55 % increment w.r.t control. Similarly,  $H_2O_2$  content enhanced with increasing salinity and at  $EC_{iw}$  6  $dS\ m^{-1}$ , maximum accumulation was recorded in genotype ICC 4463 (3.13  $\mu moles/g\ FW$ ) and minimum in ICCV 10 (1.73  $\mu moles/g\ FW$ ). At  $EC_{iw}$  9  $dS\ m^{-1}$ ,  $H_2O_2$  content increased with maximum accumulation in ICC 4463 (4.90  $\mu moles/g\ FW$ ) and minimum in KWR 108 (2.80  $\mu moles/g\ FW$ ).

Generally, plants enhance the synthesis of antioxidative enzymes to balance the level of ROS generated due to abiotic stress conditions. The present study also observed enhancement in the activity of SOD, CAT, APX, GR and POX (Fig. 3).

**Table 3**  
Effect of saline irrigation on membrane stability, MDA and H<sub>2</sub>O<sub>2</sub> content.

Treatment Traits	Membrane Stability (%)			MDA content (nmol/g FW)			Hydrogen Peroxide (μmoles/g FW)					
	C	EC <sub>iw</sub> 6 dS/m	EC <sub>iw</sub> 9 dS/m	Mean	C	EC <sub>iw</sub> 6 dS/m	EC <sub>iw</sub> 9 dS/m	Mean	C	EC <sub>iw</sub> 6 dS/m	EC <sub>iw</sub> 9 dS/m	Mean
CSG 8962	82.70 ± 1.02 <sup>a</sup>	69.32 ± 0.64 <sup>abc</sup>	59.30 ± 0.25 <sup>ab</sup>	70.44 <sup>a</sup>	2.53 ± 0.07 <sup>a</sup>	3.45 ± 0.02 <sup>f</sup>	5.57 ± 0.01 <sup>f</sup>	3.85 <sup>f</sup>	0.82 ± 0.06 <sup>a</sup>	1.79 ± 0.15 <sup>f</sup>	2.87 ± 0.12 <sup>de</sup>	1.83 <sup>d</sup>
BG 1103	80.51 ± 0.06 <sup>b</sup>	64.37 ± 1.59 <sup>def</sup>	53.75 ± 0.86 <sup>cd</sup>	66.21 <sup>cd</sup>	2.51 ± 0.03 <sup>a</sup>	4.29 ± 0.02 <sup>cd</sup>	6.43 ± 0.02 <sup>e</sup>	4.41 <sup>e</sup>	0.76 ± 0.14 <sup>a</sup>	2.17 ± 0.15 <sup>e</sup>	3.95 ± 0.25 <sup>b</sup>	2.29 <sup>bc</sup>
S7	83.10 ± 1.33 <sup>a</sup>	70.94 ± 1.66 <sup>ab</sup>	60.25 ± 1.26 <sup>a</sup>	71.43 <sup>a</sup>	2.51 ± 0.04 <sup>a</sup>	3.57 ± 0.04 <sup>f</sup>	5.55 ± 0.12 <sup>f</sup>	3.88 <sup>f</sup>	0.81 ± 0.10 <sup>a</sup>	1.75 ± 0.23 <sup>f</sup>	2.83 ± 0.17 <sup>de</sup>	1.8 <sup>d</sup>
DCP 92-3	81.91 ± 0.49 <sup>a</sup>	63.88 ± 1.63 <sup>ef</sup>	51.47 ± 1.66 <sup>de</sup>	65.75 <sup>cd</sup>	2.61 ± 0.08 <sup>a</sup>	5.12 ± 0.04 <sup>b</sup>	7.81 ± 0.21 <sup>c</sup>	5.18 <sup>c</sup>	0.72 ± 0.02 <sup>a</sup>	2.77 ± 0.17 <sup>bc</sup>	3.15 ± 0.13 <sup>cd</sup>	2.22 <sup>c</sup>
ICCV 10	82.29 ± 1.53 <sup>a</sup>	67.80 ± 1.26 <sup>bcd</sup>	59.16 ± 2.17 <sup>ab</sup>	69.7 <sup>ab</sup>	2.55 ± 0.08 <sup>a</sup>	3.89 ± 0.17 <sup>e</sup>	5.66 ± 0.11 <sup>f</sup>	4.03 <sup>f</sup>	0.84 ± 0.03 <sup>a</sup>	1.73 ± 0.06 <sup>f</sup>	2.95 ± 0.05 <sup>cde</sup>	1.84 <sup>d</sup>
KWR 108	81.95 ± 0.48 <sup>a</sup>	71.98 ± 1.33 <sup>a</sup>	60.87 ± 0.92 <sup>a</sup>	71.6 <sup>a</sup>	2.74 ± 0.10 <sup>a</sup>	3.65 ± 0.11 <sup>ef</sup>	5.35 ± 0.04 <sup>f</sup>	3.91 <sup>f</sup>	0.82 ± 0.15 <sup>a</sup>	1.79 ± 0.14 <sup>f</sup>	2.80 ± 0.07 <sup>e</sup>	1.8 <sup>d</sup>
BG 256	81.13 ± 0.79 <sup>a</sup>	63.65 ± 0.76 <sup>ef</sup>	50.05 ± 0.23 <sup>e</sup>	64.94 <sup>d</sup>	2.52 ± 0.04 <sup>a</sup>	5.78 ± 0.04 <sup>a</sup>	8.49 ± 0.14 <sup>b</sup>	5.6 <sup>b</sup>	0.71 ± 0.14 <sup>a</sup>	3.09 ± 0.15 <sup>ab</sup>	4.60 ± 0.17 <sup>a</sup>	2.8 <sup>a</sup>
K 850	81.76 ± 1.35 <sup>a</sup>	65.29 ± 1.10 <sup>def</sup>	56.18 ± 1.12 <sup>bc</sup>	67.74 <sup>bc</sup>	2.78 ± 0.10 <sup>a</sup>	4.59 ± 0.19 <sup>c</sup>	7.36 ± 0.32 <sup>d</sup>	4.91 <sup>d</sup>	0.85 ± 0.02 <sup>a</sup>	2.32 ± 0.07 <sup>de</sup>	3.25 ± 0.14 <sup>c</sup>	2.14 <sup>c</sup>
JG 16	82.65 ± 0.41 <sup>a</sup>	66.07 ± 0.98 <sup>cde</sup>	61.87 ± 1.16 <sup>a</sup>	70.2 <sup>ab</sup>	2.53 ± 0.02 <sup>a</sup>	4.23 ± 0.16 <sup>d</sup>	6.35 ± 0.02 <sup>e</sup>	4.37 <sup>e</sup>	0.82 ± 0.03 <sup>a</sup>	2.58 ± 0.03 <sup>cd</sup>	3.95 ± 0.10 <sup>b</sup>	2.45 <sup>b</sup>
ICC 4463	81.28 ± 0.42 <sup>a</sup>	62.29 ± 3.34 <sup>f</sup>	50.07 ± 0.10 <sup>e</sup>	64.54 <sup>d</sup>	2.57 ± 0.10 <sup>a</sup>	5.96 ± 0.16 <sup>a</sup>	9.62 ± 0.15 <sup>a</sup>	6.05 <sup>a</sup>	0.82 ± 0.11 <sup>a</sup>	3.13 ± 0.16 <sup>a</sup>	4.90 ± 0.06 <sup>a</sup>	2.95 <sup>a</sup>
<b>General Mean</b>	<b>81.93<sup>A</sup></b>	<b>66.56<sup>B</sup></b>	<b>56.3<sup>C</sup></b>	<b>64.5<sup>B</sup></b>	<b>2.59<sup>C</sup></b>	<b>4.45<sup>B</sup></b>	<b>6.82<sup>A</sup></b>	<b>6.05<sup>A</sup></b>	<b>0.8<sup>C</sup></b>	<b>2.31<sup>B</sup></b>	<b>3.53<sup>A</sup></b>	<b>2.31<sup>B</sup></b>
CV	T-1.65, G-2.28	T-2.5, G-2.63	T-2.5, G-2.63	T-2.5, G-2.63	T-2.5, G-2.63	T-2.5, G-2.63	T-2.5, G-2.63	T-2.5, G-2.63	T-6.39, G-4.35	T-6.39, G-4.35	T-6.39, G-4.35	T-6.39, G-4.35
LSD	T-0.70, G-2.63, T × G- 2.23	T-0.07, G-0.21, T × G- 0.23	T-0.07, G-0.21, T × G- 0.23	T-0.07, G-0.21, T × G- 0.23	T-0.07, G-0.21, T × G- 0.23	T-0.07, G-0.21, T × G- 0.23	T-0.07, G-0.21, T × G- 0.23	T-0.07, G-0.21, T × G- 0.23	T-0.09, G-0.16, T × G- 0.28	T-0.09, G-0.16, T × G- 0.28	T-0.09, G-0.16, T × G- 0.28	T-0.09, G-0.16, T × G- 0.28

Different letters represent level of significance, ( $p < 0.05$ ) using Tukeys test, among the genotypes facing same treatment, values with at least one letter common are non-significant. CV: coefficient of variance, LSD: Least significant difference, T: Treatment, G: Genotype, T × G: Treatment × Genotype.

Activity of SOD in genotypes CSG 8962, ICCV 10, S7 and KWR 108 was more than 45 % w.r.t control at EC<sub>iw</sub> 6 dS m<sup>-1</sup>. At, EC<sub>iw</sub> 9 dS m<sup>-1</sup>, further enhancement in SOD activity was observed and more than 55 % increment was observed in the genotypes JG 16 (55.21 %), K 850 (56.01 %), ICCV 10 (62.99 %), S7 (63.17 %) and KWR 108 (63.73 %) w.r.t control. CAT activity enhanced more than 20 % w.r.t control at EC<sub>iw</sub> 6 dS m<sup>-1</sup>, and maximum increment was observed in KWR 108 (47.06 %) and minimum in BG 256 (24.50 %). At EC<sub>iw</sub> 9 dS m<sup>-1</sup>, the maximum increment was observed in genotype ICCV 10 (58.24 %) and minimum in DCP 92-3 (36.50 %). Saline water irrigation also enhanced the activity of APX and GR enzymes, and maximum enhancement was observed in genotype KWR 108 and minimum in genotype ICC 4463 at both the salinity levels w.r.t control. POX enzyme activity also enhanced with salinity, and at EC<sub>iw</sub> 6 dS m<sup>-1</sup> maximum enhancement was observed in KWR 108 (42.40 %) and minimum in DCP 92-3 (17.78 %). At EC<sub>iw</sub> 9 dS m<sup>-1</sup>, the maximum increment was observed in genotype CSG 8962 (52.26 %) and the minimum in BG 256 (17.85 %).

### 3.2.2. Verification of enzymatic antioxidants biochemical data with gene expression

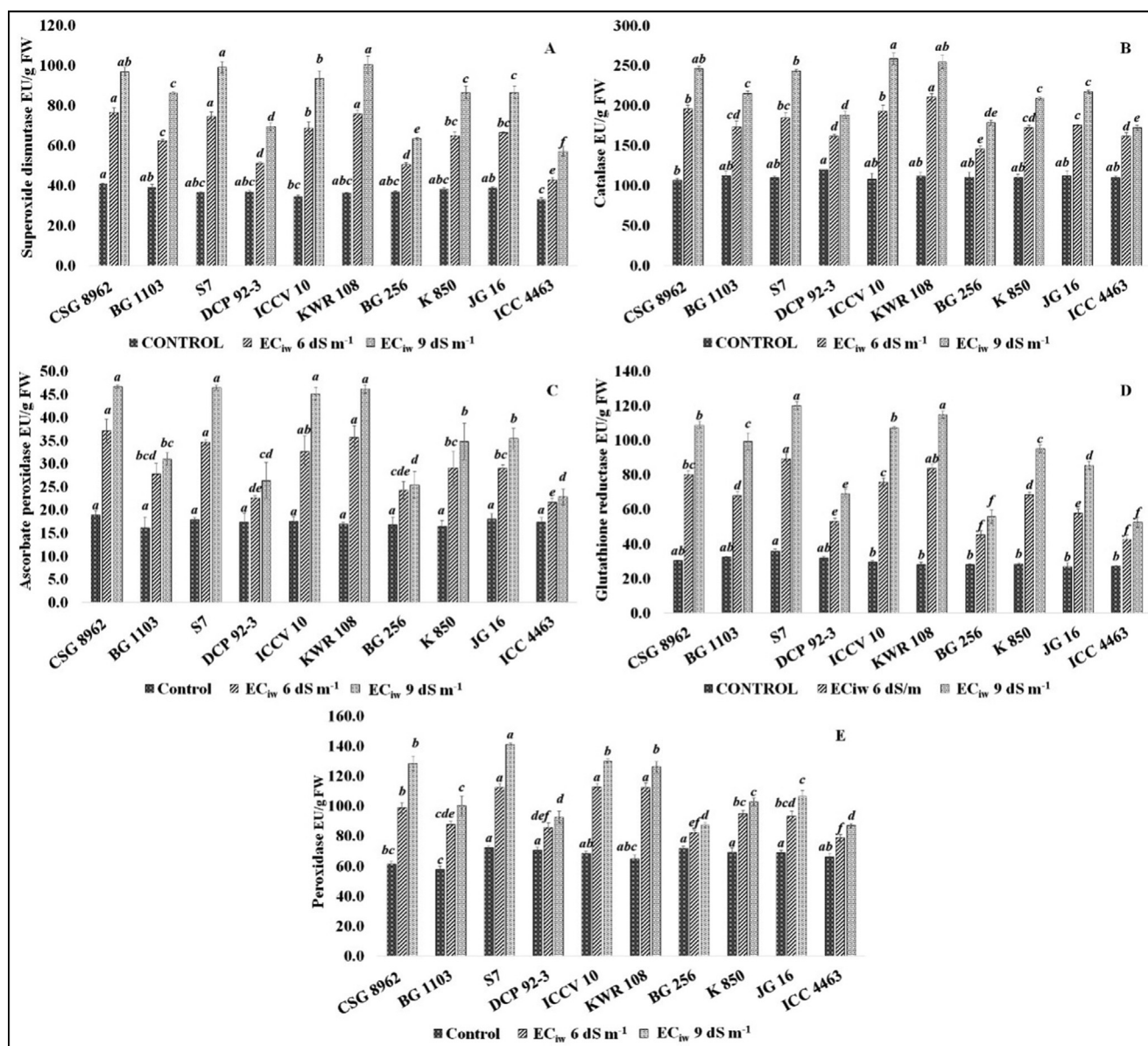
Based upon the present and our previous studies (Kaur et al., 2021) salinity contrasting chickpea genotypes (tolerant KWR 108 and sensitive ICC 4463) were selected and along with the salt-tolerant check CSG 8962, the quantitative real-time expression of various genes related to antioxidant defense system (SOD, CAT, APX, MDHAR, DHAR, GR, and POX) was monitored (Fig. 4). Log<sub>2</sub> fold change of 1.67, 1.78, 1.19 and 2.13, 2.29, 1.37 was observed in CSG 8962, KWR 108 and ICC 4463, respectively at EC<sub>iw</sub> 6 dS m<sup>-1</sup> and 9 dS m<sup>-1</sup> w.r.t control. Log<sub>2</sub> fold change in the expression of CAT enzyme at EC<sub>iw</sub> 6 dS m<sup>-1</sup> was 1.82 (CSG 8962), 2.03 (KWR 108) and 1.39 (ICC 4463) and at EC<sub>iw</sub> 9 dS m<sup>-1</sup> it was 2.34 (CSG 8962), 2.59 (KWR 108) and 1.46 (ICC 4463) w.r.t control. More than two log<sub>2</sub> fold change in the expression of genes encoding the enzymes APX, MDHAR, DHAR and GR, involved in the ascorbate-glutathione (ASH-GSH) cycle, was observed in genotypes CSG 8962 and KWR 108 at both the salinity levels w.r.t control. The gene encoding POX enzyme showed a log<sub>2</sub> fold change of 1.79 (CSG 8962), 2.19 (KWR 108) and 1.21 (ICC 4463) at EC<sub>iw</sub> 6 dS m<sup>-1</sup> and EC<sub>iw</sub> 9 dS m<sup>-1</sup> it was 2.24 (CSG 8962), 2.29 (KWR 108) and 1.39 (ICC 4463) w.r.t control.

### 3.2.3. Osmoregulation

Proline and sugars functions in osmoregulation, and hike in their content with salinity was also observed in the present study. Accumulation of proline with salinity is well reported and more than 70 % increment in the proline content was observed in the roots of genotypes K 850 (70.58 %), ICCV 10 (75.25 %), CSG 8962 (75.52 %), and KWR 108 (77.14 %) at EC<sub>iw</sub> 6 dS m<sup>-1</sup> w.r.t control. Proline content further increased at EC<sub>iw</sub> 9 dS m<sup>-1</sup> and more than 75 % increment was reported in genotypes CSG 8962 (79.54 %), ICCV 10 (81.85 %), KWR 108 (82.16 %) and S7 (82.76 %). Similarly, TSS content enhanced with salinity and more than 35 % increment was observed in genotypes JG 16 (36.12 %), S7 (39.19 %), ICCV 10 (39.32 %), CSG 8962 (41.64 %), and KWR 108 (44.25 %) at EC<sub>iw</sub> 6 dS m<sup>-1</sup> and in these genotypes, TSS further enhanced with an increment of 50 % at EC<sub>iw</sub> 9 dS m<sup>-1</sup> w.r.t control. Overall, KWR 108 and S7 accumulated more osmolytes than the salt tolerant check CSG 8962 (Table 4) (percent increment mentioned in supplementary table 4).

### 3.2.4. Proline biosynthesis at molecular level in salinity contrasting chickpea genotypes

Expression of the genes encoding the enzymes P5CS and P5CR of the glutamate pathway of proline biosynthesis and proline degradation enzyme proline dehydrogenase (PD) was monitored (Fig. 5).



**Fig. 3.** Influence of saline irrigation on the antioxidant enzyme activities (A) superoxide dismutase (B) catalase (C) ascorbate peroxidase (D) glutathione reductase (E) peroxidase. Different letters represent level of significance, ( $p < 0.05$ ) using Tukeys test, among the genotypes facing same treatment, values with at least one letter common are non-significant.

Upregulation in the *P5CS* and *P5CR* genes was noticed. At moderate salinity level of EC<sub>iw</sub> 6 dS m<sup>-1</sup>, *P5CS* gene showed a log<sub>2</sub> fold change of 3.26 (CSG 8962), 3.85 (KWR 108) and 1.85 (ICC 4463) w.r.t control and at extreme salinity level (EC<sub>iw</sub> 9 dS m<sup>-1</sup>), log<sub>2</sub> fold change of 4.81 (CSG 8962), 5.24 (KWR 108) and 2.13 (ICC 4463) was observed w.r.t control. Similarly, *P5CR* gene showed a log<sub>2</sub> fold change of 3.34 (CSG 8962), 4.05 (KWR 108) and 2.02 (ICC 4463) at EC<sub>iw</sub> 6 dS m<sup>-1</sup> and at extreme salinity (EC<sub>iw</sub> 9 dS m<sup>-1</sup>), log<sub>2</sub> fold change of 5.02 (CSG 8962), 5.48 (KWR 108) and 2.24 (ICC 4463) was observed w.r.t control. Downregulation of *PD* gene was noticed but significant reduction was observed in genotypes CSG 8962 and KWR 108 at both salinity levels.

### 3.3. Ionic relations and maintenance of ionic homeostasis with *HKT1* gene

Raising salinity level enhanced the Na<sup>+</sup>/K<sup>+</sup> ratio in all the genotypes, the maximum ratio being observed in ICC 4463 with (2.41 %) and (3.05 %) at EC<sub>iw</sub> 6 dS m<sup>-1</sup> and 9 dS m<sup>-1</sup> respectively and min-

imum in CSG 8962 with 0.73 % and 1.21 % at EC<sub>iw</sub> 6 dS m<sup>-1</sup> and EC<sub>iw</sub> 9 dS m<sup>-1</sup> respectively w.r.t control (Table 4) (percent increment mentioned in supplementary table 4). The expression of *HKT1* gene was enhanced with salinity and a log<sub>2</sub> fold change of 1.86 (CSG 8962), 1.71 (KWR 108), and 1.18 (ICC 4463) at EC<sub>iw</sub> 6 dS m<sup>-1</sup> was observed. At EC<sub>iw</sub> 9 dS m<sup>-1</sup>, log<sub>2</sub> fold change of 2.13 (CSG 8962), 1.95 (KWR 108) and 1.48 (ICC 4463) was observed w.r.t control (Fig. 5).

### 3.4. Correlation analysis

Correlation analysis was performed to understand the association between the morphophysiological and biochemical traits (Fig. 6). A strong positive correlation of RWC with water potential (0.928), osmotic potential (0.913), membrane stability (0.939), and negative correlation with MDA (-0.961), H<sub>2</sub>O<sub>2</sub> (-0.965), and Na<sup>+</sup>/K<sup>+</sup> (-0.937) was observed. All the enzymatic antioxidants were strongly positively correlated with proline and TSS. Na<sup>+</sup>/K<sup>+</sup> ratio was positively correlated with MDA (0.953) and H<sub>2</sub>O<sub>2</sub> (0.927)

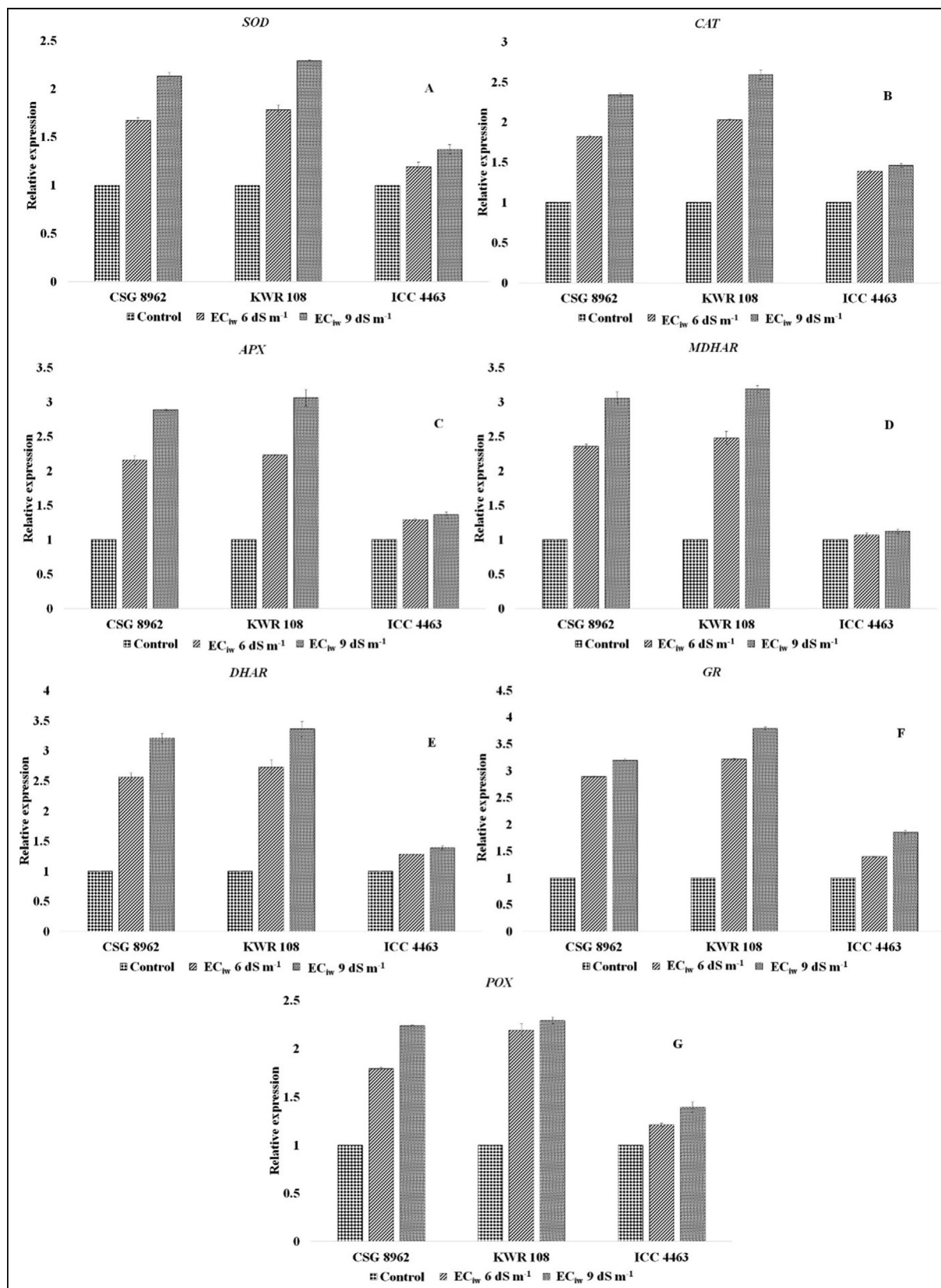


Fig. 4. Relative expression of the genes encoding antioxidative enzymes (A) SOD (B) CAT (C) APX (D) MDHAR (E) DHAR (F) GR (G) POX.



**Table 4**  
Effect of saline irrigation on proline, total soluble sugars and Na<sup>+</sup>/K<sup>+</sup>.

Treatment Traits	Proline (µg/g FW)			Total Soluble Sugars (mg/g FW)			Na <sup>+</sup> /K <sup>+</sup>					
	C	EC <sub>iw</sub> 6 dS/m	EC <sub>iw</sub> 9 dS/m	Mean	C	EC <sub>iw</sub> 6 dS/m	EC <sub>iw</sub> 9 dS/m	Mean	C	EC <sub>iw</sub> 6 dS/m	EC <sub>iw</sub> 9 dS/m	Mean
CSG 8962	13.03 ± 0.10 <sup>a</sup>	53.22 ± 0.47 <sup>a</sup>	63.66 ± 3.16 <sup>b</sup>	43.3 <sup>b</sup>	58.21 ± 2.8 <sup>a</sup>	99.74 ± 1.9 <sup>a</sup>	121.41 ± 8.5 <sup>ab</sup>	93.12 <sup>ab</sup>	0.29 ± 0.03 <sup>a</sup>	0.73 ± 0.04 <sup>d</sup>	1.21 ± 0.04 <sup>f</sup>	0.75 <sup>f</sup>
BG 1103	11.75 ± 0.74 <sup>a</sup>	33.11 ± 0.10 <sup>cd</sup>	51.33 ± 2.34 <sup>c</sup>	32.06 <sup>d</sup>	58.72 ± 4.0 <sup>a</sup>	85.41 ± 8.7 <sup>bc</sup>	91.70 ± 6.2 <sup>de</sup>	79.61 <sup>cd</sup>	0.30 ± 0.03 <sup>a</sup>	0.91 ± 0.01 <sup>cd</sup>	1.94 ± 0.03 <sup>de</sup>	1.05 <sup>cd</sup>
S7	12.44 ± 1.25 <sup>a</sup>	53.05 ± 0.47 <sup>a</sup>	72.15 ± 3.32 <sup>a</sup>	45.88 <sup>a</sup>	59.19 ± 5.3 <sup>a</sup>	97.34 ± 4.4 <sup>ab</sup>	119.59 ± 4.8 <sup>b</sup>	92.04 <sup>ab</sup>	0.30 ± 0.01 <sup>a</sup>	0.87 ± 0.06 <sup>cd</sup>	1.40 ± 0.12 <sup>f</sup>	0.86 <sup>ef</sup>
DCP 92-3	12.51 ± 1.01 <sup>a</sup>	30.41 ± 0.88 <sup>d</sup>	34.24 ± 1.09 <sup>ef</sup>	25.72 <sup>e</sup>	58.37 ± 5.8 <sup>a</sup>	68.55 ± 6.7 <sup>de</sup>	90.51 ± 3.0 <sup>f</sup>	72.47 <sup>de</sup>	0.40 ± 0.06 <sup>a</sup>	1.01 ± 0.06 <sup>bc</sup>	2.21 ± 0.15 <sup>bc</sup>	1.21 <sup>bc</sup>
ICCV 10	12.82 ± 1.00 <sup>a</sup>	51.80 ± 2.68 <sup>a</sup>	70.63 ± 2.44 <sup>a</sup>	45.08 <sup>ab</sup>	53.95 ± 0.9 <sup>a</sup>	88.92 ± 8.9 <sup>abc</sup>	113.34 ± 9.8 <sup>bc</sup>	85.4 <sup>bc</sup>	0.35 ± 0.03 <sup>a</sup>	0.77 ± 0.02 <sup>cd</sup>	1.86 ± 0.04 <sup>e</sup>	0.99 <sup>de</sup>
KWR 108	12.16 ± 0.76 <sup>a</sup>	53.19 ± 2.11 <sup>a</sup>	68.16 ± 0.98 <sup>a</sup>	44.5 <sup>ab</sup>	57.03 ± 2.7 <sup>a</sup>	102.29 ± 5.8 <sup>a</sup>	134.06 ± 6.4 <sup>a</sup>	97.79 <sup>a</sup>	0.35 ± 0.03 <sup>a</sup>	0.83 ± 0.01 <sup>cd</sup>	1.79 ± 0.05 <sup>e</sup>	0.99 <sup>de</sup>
BG 256	12.83 ± 0.99 <sup>a</sup>	31.14 ± 0.96 <sup>cd</sup>	37.46 ± 0.61 <sup>e</sup>	27.14 <sup>e</sup>	57.41 ± 3.2 <sup>a</sup>	69.00 ± 4.2 <sup>de</sup>	73.59 ± 7.4 <sup>d</sup>	66.67 <sup>de</sup>	0.28 ± 0.03 <sup>a</sup>	1.16 ± 0.09 <sup>b</sup>	2.41 ± 0.32 <sup>b</sup>	1.28 <sup>b</sup>
K 850	13.40 ± 1.08 <sup>a</sup>	45.57 ± 0.83 <sup>b</sup>	47.21 ± 1.76 <sup>d</sup>	35.39 <sup>c</sup>	52.71 ± 1.6 <sup>a</sup>	77.49 ± 3.1 <sup>cd</sup>	92.84 ± 4.5 <sup>de</sup>	74.35 <sup>de</sup>	0.40 ± 0.01 <sup>a</sup>	0.89 ± 0.03 <sup>cd</sup>	2.12 ± 0.10 <sup>cd</sup>	1.14 <sup>bcd</sup>
JG 16	12.76 ± 0.36 <sup>a</sup>	34.63 ± 2.42 <sup>c</sup>	44.57 ± 0.82 <sup>d</sup>	30.65 <sup>d</sup>	52.33 ± 3.3 <sup>a</sup>	81.91 ± 2.8 <sup>cd</sup>	104.66 ± 5.4 <sup>cd</sup>	79.63 <sup>cd</sup>	0.44 ± 0.03 <sup>a</sup>	0.96 ± 0.09 <sup>bcd</sup>	1.97 ± 0.22 <sup>cde</sup>	1.13 <sup>bcd</sup>
ICC 4463	12.70 ± 0.52 <sup>a</sup>	25.93 ± 0.40 <sup>c</sup>	30.82 ± 1.31 <sup>f</sup>	23.15 <sup>f</sup>	56.39 ± 2.3 <sup>a</sup>	62.62 ± 2.6 <sup>e</sup>	72.68 ± 3.0 <sup>f</sup>	63.9 <sup>f</sup>	0.39 ± 0.04 <sup>a</sup>	2.41 ± 0.08 <sup>a</sup>	3.06 ± 0.11 <sup>a</sup>	1.95 <sup>a</sup>
<b>General Mean</b>	<b>12.64<sup>c</sup></b>	<b>41.2<sup>b</sup></b>	<b>52.02<sup>a</sup></b>	<b>36.43<sup>c</sup></b>	<b>56.43<sup>c</sup></b>	<b>83.33<sup>b</sup></b>	<b>101.74<sup>a</sup></b>	<b>63.9<sup>f</sup></b>	<b>0.35<sup>c</sup></b>	<b>1.05<sup>b</sup></b>	<b>2<sup>a</sup></b>	<b>1.05<sup>a</sup></b>
CV	T-4.58, G-3.74	T-6.67, G-5.87	T-6.67, G-5.87	T-6.67, G-5.87	T-6.67, G-5.87	T-6.67, G-5.87	T-6.67, G-5.87	T-6.67, G-5.87	T-8.05, G-8.41	T-8.05, G-8.41	T-8.05, G-8.41	T-8.05, G-8.41
LSD	T-1.02, G-2.23, T × G 3.21	T-3.38, G-7.99, T × G-10.67	T-3.38, G-7.99, T × G-10.67	T-3.38, G-7.99, T × G-10.67	T-3.38, G-7.99, T × G-10.67	T-3.38, G-7.99, T × G-10.67	T-3.38, G-7.99, T × G-10.67	T-3.38, G-7.99, T × G-10.67	T-0.06, G-0.16, T × G-0.18	T-0.06, G-0.16, T × G-0.18	T-0.06, G-0.16, T × G-0.18	T-0.06, G-0.16, T × G-0.18

Different letters represent level of significance, (p < 0.05) using Tukeys test, among the genotypes facing same treatment, values with at least one letter common are non-significant. CV: coefficient of variance, LSD: Least significant difference, T: Treatment, G: Genotype, T × G: Treatment × Genotype.

and negatively correlated with RWC (-0.937) and membrane stability (-0.900).

### 3.5. Clustering analysis

The heatmaps were generated using percentage reduction or increment in various biochemical parameters at EC<sub>iw</sub> 6 dS m<sup>-1</sup> and 9 dS m<sup>-1</sup> w.r.t control (Fig. 7A and B). Distribution patterns of various traits grouped the genotypes into three main clusters (Cluster-I, -II and -III). The same distribution pattern of all the genotypes was obtained at both the salinity levels. Three genotypes, S7, ICCV 10 and KWR 108, were found in Cluster-I along with the salt-tolerant check CSG 8962. Cluster-II contained three genotypes (K 850, JG 16, and BG 1103) and three genotypes were found in Cluster-III (BG 256, ICC 4463 and DCP 92-3).

## 4. Discussion

### 4.1. Salinity and root growth

Root is the primary plant organ defining whole plant responses to salinity because it is directly connected with the soil and hence suffers initial damages caused by salinity affecting plant growth and development. A reduction in the root length in response to saline irrigation was observed and has earlier been reported in chickpea (Buttar et al., 2021; Mann et al., 2019). Elongation of the root results from cell division and cell expansion and salinity alters both these processes (Arif et al., 2019). Weight of the root organ is more important while studying salinity rather than the length. The results obtained in the present study revealed that both fresh and dry weight of root reduced, and this reduction was due to a lesser number of secondary roots that decreased that root volume. Hindrance in the water uptake leads to a loss in turgor under salinity that might affect the fresh and dry weight of the root (Chang et al., 2019; Mann et al., 2019).

### 4.2. Root water relations

The initial phase of salinity i.e., the osmotic phase reduces the efficiency of the root to uptake water and the traits related to water are affected. The water potential of the soil is lowered due to the presence of high salts in the rhizosphere which lowers the efficiency of the root to uptake water and hence RWC is lowered (Soni et al., 2021). Salinity drops the water content and induces the accumulation of excess ions in the plant, decreasing the osmotic potential (Gandonou et al., 2018). In our results also, we observed decreased RWC with increasing saline levels in chickpea roots along with more negative water and osmotic potential.

### 4.3. Membrane stability and ROS generation and their scavenging by enzymatic antioxidants

Free radical generation due to salinity alters the membrane charge and composition and decreases the membrane stability, and this reduction was also observed in the present study. Salinity enhances the production of ROS which was reflected due to the overproduction of H<sub>2</sub>O<sub>2</sub> and MDA in the present study. ROS causes peroxidation of the lipids present in the membranes, and MDA is one of the products of this reaction. Sharp increment in MDA content with salinity has been reported in the salt sensitive chickpea cultivars, but the tolerant genotypes showed non-significant change up to seven days of applied treatment and only a marginal increment was reported in later stages of saline stress (Arefian et al., 2014, 2018; Arefian and Shafaroudi, 2015). In the sensitive

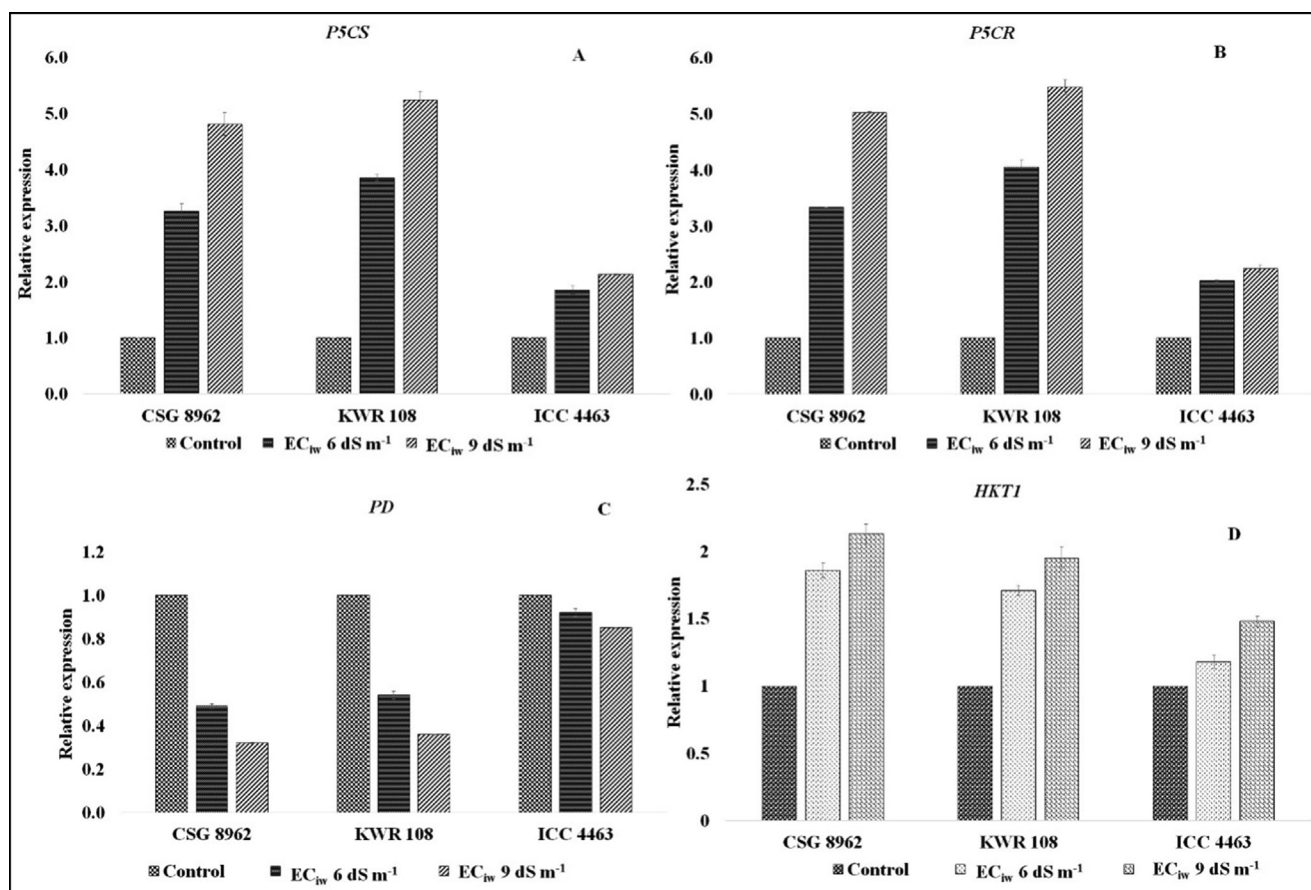


Fig. 5. Relative expression of the genes involved in proline biosynthesis (A) *P5CS* and (B) *P5CR* and degradation (C) *PD* and sodium transporter (D) *HKT1*.

cultivar of *Vicia faba*, more enhancement in the electrolyte leakage, MDA, and  $H_2O_2$  in response to salinity has been reported due to the less activity of SOD, CAT, GR, and ascorbic acid as compared to the tolerant cultivar (Alzahrani et al., 2019; Sharma et al., 2013). Oxidative stress results from the imbalance between ROS production and its scavenging by antioxidants. Plants enhance the biosynthesis of various antioxidants to balance the ROS when their production crosses a threshold level. SOD, by dismutation reaction, converts superoxide ions into molecular oxygen and  $H_2O_2$ .  $H_2O_2$  generated by this reaction or other cellular reactions is detoxified by the enzymes CAT, POX and the enzymes of ascorbate-gluthione (ASH-GSH) pathway (Abd-Alla et al., 2019). Significant enhancement in the activities of SOD, POD, CAT, APX and GR have been reported in both leaves and root of wheat cultivars (Zeeshan et al., 2020) and the same trend has been observed in chickpea roots in the present study. Significant enhancement in the relative expression of SOD, CAT and APX, in salt-tolerant chickpea cultivars have been reported (Rasool et al., 2013). In the present study, greater the salinity level higher is the activity of the antioxidative enzymes in the tolerant genotypes and same trend has been reported in chickpea (Khamesi et al., 2020). Moreover, higher activity of antioxidative enzymes due to salt stress was also correlated with the ability of the plant to maintain ion homeostasis as it was reflected by smaller value of  $Na^+/K^+$  in the tolerant genotype (Khamesi et al., 2020).

#### 4.4. Osmoregulation

Salt stress-induced accumulation of proline and TSS aids in osmotic adjustment and enhanced biosynthesis of these osmopro-

tectants (proline and TSS) was also observed, with salinity more accumulated in the roots of tolerant cultivars in the present study. Osmoprotectants are critical for sustaining cell turgor which maintains plant growth, productivity, and yield (Arif et al., 2020). Proline is not only an osmoprotectant, but it also possesses antioxidative properties, moreover the amino acid act as a cytosolic pH regulator and protein stabilizer, and in any one of the property, the amino acid might have helped the plant mitigate the effects of salt stress (Arif et al., 2020; Mansour and Ali, 2017). The enhanced biosynthesis of proline in the present study was due to the upregulation of *P5CS* and *P5CR* genes and downregulation of the gene encoding enzyme proline dehydrogenase and similar expression of genes encoding these enzymes have been reported earlier in wheat under salinity (Tavakoli et al., 2016). It has been reported in literature that when plants are exposed to high salinity a large number of reduced sugars such as fructose, glucose and sucrose are produced with in the plant that providing tolerance against salinity. Sugars stabilize the membrane integrity thus protecting the proteins from aggregation and denaturation (Arif et al., 2020). Enhanced production of TSS has been reported in both root and shoot of barley under saline stress (Narimani et al., 2020), but TSS content decreased in root and increased in the shoot in wheat under salinity (Soni et al., 2021).

#### 4.5. *HKT1* gene plays an important role in maintaining ion homeostasis in chickpea

The root is the first line of defense that directly limits/excludes the uptake of toxic ions (especially  $Na^+$ ) responsible for the ionic phase of salinity.  $Na^+/K^+$  ratio was enhanced in the present study

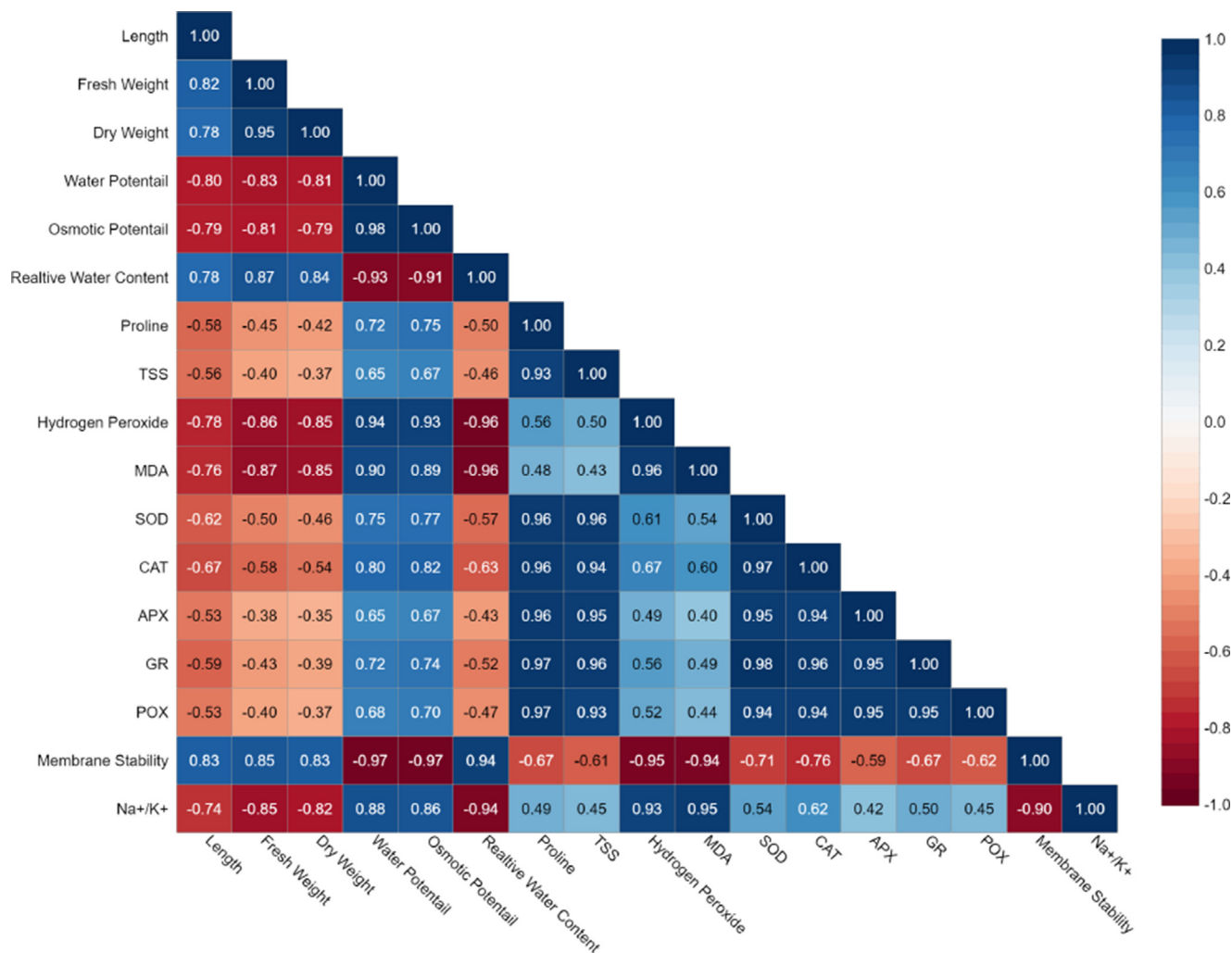


Fig. 6. Correlation analysis of root morpho-physiological and biochemical traits of chickpea genotypes exposed to salinity.

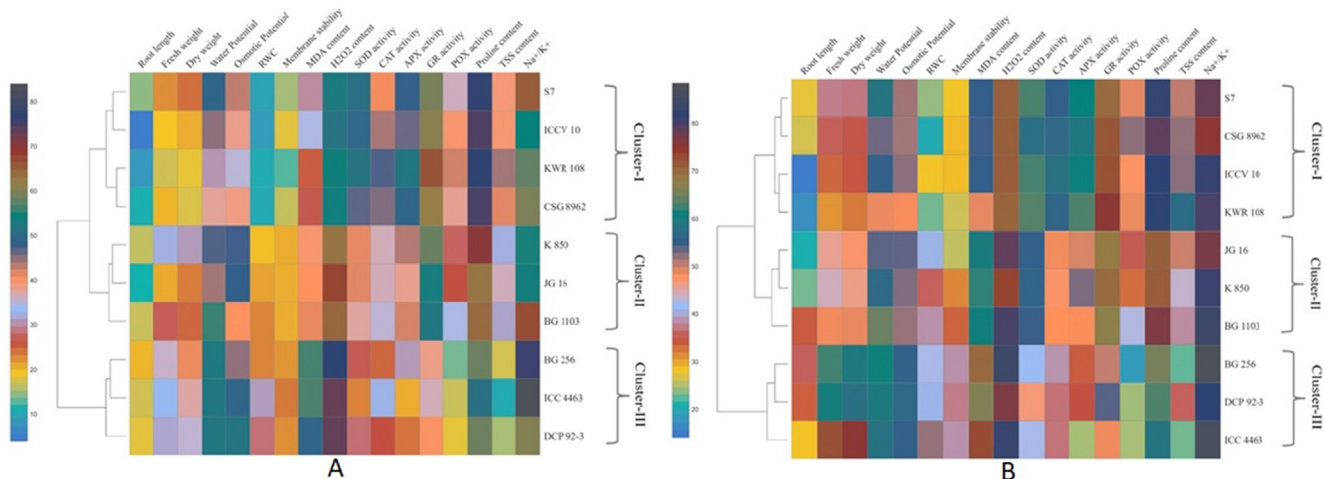


Fig. 7. Clustering of genotypes on the basis of percentage reduction or increment in biochemical traits at (A)  $EC_{iw}$  6  $dS\ m^{-1}$  and (B)  $EC_{iw}$  9  $dS\ m^{-1}$  w.r.t Control.

in all genotypes, but more than one ratio was observed only in ICC 4463, BG 256, and DCP 92–3 at moderate saline level ( $EC_{iw}$  6  $dS\ m^{-1}$ ), and it has been reported in chickpea that more the ratio more is the susceptibility of the genotype towards salinity

(Kumar et al., 2020). Salinity enhanced the  $Na^+/K^+$  ratio in both root and shoot, but tolerant genotypes of wheat were able to maintain a  $Na^+/K^+$  ratio below one both in root and shoot at  $EC_{iw}$  10  $dS\ m^{-1}$ , and authors suggested that due to the same ionic radii,  $Na^+$  and

K<sup>+</sup> ions compete with each other for entry into the plant root cells, and Na<sup>+</sup> replaces K<sup>+</sup> that enhanced the Na<sup>+</sup>/K<sup>+</sup> ratio (Soni et al., 2021). Similarly, in chickpea roots, the expression of the *HKT1* gene enhanced with salinity in our study. Transport of Na<sup>+</sup> ions from root to shoot is through transpiration stream, and Na<sup>+</sup> transporter *HKT1* selectively unloads Na<sup>+</sup> ions from xylem stream into xylem parenchyma cells. Silencing of *TaHKT1;5-D* located on plasma membrane in wheat root stele, increased the concentration of Na<sup>+</sup> ions in the leaves and this is an indication that this transporter retrieves Na<sup>+</sup> ions from xylem stream and restricts them to the root and helps in maintaining low Na<sup>+</sup>/K<sup>+</sup> ratio in the leaves (Byrt et al., 2014). Up-regulation of the *HKT* gene has also been reported in the root of chickpea and in the tolerant genotype it was 1.55 times higher compared to the sensitive genotype at the reproductive stage (Kaashyap et al., 2018).

Correlation of various salinity-affected traits has been reported in the literature on various crops. High proline content was reported in the root of the model legume plant *Medicago truncatula* and it was positively correlated with salt tolerance (Kang et al., 2019). In the present work, all the studied antioxidative enzymes were found to be positively correlated with each other and similar positive correlation between SOD, CAT, APX and POX in salinity-treated wheat have been reported (Soni et al., 2021).

## 5. Conclusion

Briefly from these findings, we can summarize that chickpea genotypes can tolerate salinity by maintaining plant water-related traits, higher osmolyte accumulation and lower Na<sup>+</sup>/K<sup>+</sup>. In addition, genotypes capable of synthesizing higher amount of antioxidant enzymes helps the plant to minimize the effects of salinity-generated ROS. Maintaining ionic homeostasis is important to mitigate the harmful effects of ionic stress, and in the present study, it was found that the *HKT1* gene plays an important role in maintaining ion homeostasis in chickpea roots. Genotypes CSG 8962, S7, ICCV 10 and KWR 108 showed these activities in their roots that enabled the entire plant to survive in the saline environment and hence can be taken for further advanced studies. The selection of root traits is generally ignored due to more laborious studies but root being the first plant organ, decides the fate of the plant in growing environments. Hence, the various root traits explored in the present study can be taken up for further breeding of salt-tolerant crops, and their positive correlation with enhanced yield under salinity could be identified as selection traits.

## 6. Authors' contributions

GK performed the experiments. GK, SKS, NS and AM designed the experiments and wrote the manuscript. AK helped in data analysis. AK and NK provided technical guidance. All authors contributed in the revision of the manuscript. All authors read and approved the manuscript.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.sjbs.2022.103464>.

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