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Salinity Induced Changes in hydrogen peroxide, Lipid Peroxidation, Antioxidant Enzymes Activities and Yield Attributes in Chickpea (*Cicer arietinum* L.) Cultivars

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A B S T R A C T

Salinity in soil or water is one of the major stresses and especially in arid and semi-arid regions, can severely limit crop production. Chickpea (*Cicer arietinum* L.)is sensitive to salinity that affects its yield and there is need to identify the tolerant genotypes. In order to evaluate the effect of soil salinity, a pot experiment with two chickpea genotypes was carried out under screen house conditions. The required amounts of chloride and sulphate salts of Na^{+}, Ca^{+2} and Mg^{+2} were added through NaCl, Na_2SO_4, $CaCl_2$, $MgCl_2$ and $MgSO_4$. Sodium and Ca^{+2} + Mg^{+2} were in the ratio of 1:1 where Ca^{+2} and Mg^{+2} were in the ratio of 1:3 to develop three (2.0, 4.0, 6.0 dS m^{-1}) levels of saline soil before sowing. The control plants were irrigated with distilled water. Sampling was done at 50-60 days after sowing. The hydrogen peroxide of leaves and roots and lipid peroxidation (MDA) of leaves and roots increased significantly in both the genotypes under different salinity levels. The H_2O_2 content increased significantly by 0.380 to 0.625 and 0.366 to 0.579 (moles g^{-1}DW) x 10^{-4} in leaves at 50-60 DAS in the genotypes CSG-8962 and HC-3, respectively with increasing the level of salinity. In roots, at 50-60 DAS, H_2O_2 content increased significantly from 0.260 to 0.470 in the genotype CSG-8962 and 0.217 to 0.401 (moles g^{-1}DW x 10_{-4}) in the HC-3, with increase in salinity levels from control to 6.0 dS m^{-1}. In leaves, at 50-60 DAS, a significant accumulation of MDA content was observed in both the genotypes under salinity levels. This increase was from 13.40 to 21.43 n moles g^{-1} dry weight in the genotypes CSG-8962 and 13.38 to 17.43 in HC-3 with increasing levels of salinity from control to 6.0 dS m^{-1}. In roots, an increase from 10.35 to 16.59 n moles g^{-1}dry weight in MDA content was observed in the genotype CSG-8962, however, in HC-3, this was from 10.55 to 13.83 with increasing levels of salinity from control to 6.0 dS m-1 at 50-60 DAS. The specific activities of ROS scavenging enzymes such as SOD, CAT, POX, APX, GPX, GR and GST increased in leaves of both the chickpea genotypes, upon increasing levels of salinity from control to 6.0 dS m^{-1} at 50-60 DAS. The increase was more in HC-3 as compared to CSG-8962. Despite the increase in the activity of these enzymes, AsA content decreased 42.3 and 36.1 % in the leaves of CSG-8962 and HC-3, respectively. Higher activities of antioxidant enzymes, lower accumulation H_2O_2, MDA and AsA content in HC-3 than in CSG-8962 indicated that these enzymes play a key role in removal of ROS better in HC-3 than CSG-8962, thus minimizing the cellular damage caused by ROS under salinity levels. The yield parameters like number of branches $plant^{-1}$, number of pods $plant^{-1}$, number of seeds $plant^{-1}$, 100 seed weight and seed yield $plant^{-1}$ decreased more in CSG-8962 than HC-3 with increasing salinity levels from control to 6.0 dS m^{-1}. The reduction is more in CSG-8962 as compared to HC-3. Hence, the mechanism of salt tolerance is relatively better in HC-3 than in CSG-8962 as found from physiological and yield attributes studied and could be used in crop improvement programme of chickpea for salinity tolerance.

Introduction

Chickpea (*Cicer arietinum* Linnaeus), a member of family Fabaceae, is an ancient self pollinated leguminous crop, diploid annual (2N=16 chromosomes) grown since 7000 BC, in different area of the world (Tekeoglu *et al.*, 2000) but its cultivation is mainly concentrated in arid and semi-arid environments such South Asia, West Asia, North Africa, East Africa, Southern Europe, North and South America, and Australia (Arefian *et al.*, 2014; Flowers *et al.*, 2010). In India, Madhya Pradesh, Rajasthan, Maharashtra, Uttar Pradesh, Andhra Pradesh, Karnataka, Chhattisgarh, Bihar and Jharkhand are major chickpea producing states contributing more than 95% to the total chickpea production. Madhya Pradesh is the single largest producer in the country accounting for over 40 % of total production while Rajasthan, Maharashtra, Uttar Pradesh and Andhra Pradesh contribute about 14 %, 10%, 9% and 7%, respectively. The share of Andhra Pradesh and Karnataka has consistently been rising during the past one decade. Further, states like Jharkhand and Chhattisgarh are expanding their area and production of chickpea crop (AICRP, 2014-15).

The chickpea seed is a valuable source of carbohydrates and proteins, which together constitute 80 % of the total dry seed weight. The crude protein content of chickpea varies from 17 % to 24 % containing the essential amino acids like tryptophan, methionine and cysteine (Williams and Singh, 1987). Thus, chickpea serves as a main source of dietary protein for more than 80% of the Indian population which is vegetarian in nature. Chickpea acquires importance as it provides food for humans as well as for livestock. Furthermore, chickpea pod covers and seed coats can also be used as fodder. Chickpea nitrogen fixation plays an important role in maintenance of the soil fertility, particularly in the arid and low rainfall area (Roy *et al.*, 2010).

Soil salinity is known as a major inevitable problem, especially in arid and semi-arid regions of the world and affects about 80 million hectare of arable lands (Flowers *et al.*, 2010), 2.95 million hectare in India and 49.2 thousand hectare in Haryana and this area is expanding (Ali, 2009). Despite the high yield potential of chickpea of over 4000 kg per hectare (Singh, 1990). The chickpea suffer losses from salinity both in soil and water (Flowers, 2010).

Salinity stress induced production of H2O2 and may trigger genetically programmed cell suicide (Farouk, 2011). Salinity induced the generation of H_2O_2 (Sairam and Tyagi, 2004). The chief toxicity of H_2O_2 are production of hydroxyl radicals and other destructive species such as lipid peroxides lead to damage vital cellular macromolecules (Vaidyanathan *et al.*, 2003). Increased in H_2O_2 production under salinity has been reported in chickpea roots (Kukreja *et al.*, 2005), tomato leaves (He and Zhu, 2008) and cellular macromolecules (Vaidyanathan *et al.*, 2003). Increased in H2O2 production under salinity has been reported in chickpea roots (Kukreja *et al.*, 2005), tomato leaves (He and Zhu, 2008).

Lipid peroxidation is the symptom most easily ascribed to oxidative damage. It is an attack upon polyunsaturated fatty acids of the membrane (Heath and Packer, 1968) which lead to the breakdown of lipid and impairment of membrane function. Increased electrolyte leakage and lipid peroxidation were noticed with increased salinity in roots and shoot of green gram (Panda, 2001).

Superoxide dismutase (SOD) catalyzes the dismutation of superoxide into hydrogen peroxide and oxygen (Ashraf and Harris,

2004) which is in-turn metabolized by the action of peroxidases (Ahmad *et al.,* 2008). SOD originally discovered by McCord and Fridovich (1969) react with superoxide radicals at almost diffusion-limited rates to produce hydrogen peroxide.

CAT converts H2O2 to water and molecular oxygen. In plant, CAT is found predominantly in peroxisomes and glyoxysomes where it functions chiefly to remove the H_2O_2 form during the photorespiration (Geetanjali and Neera, 2008) and also during salt stress and other abiotic stress condition (Willekens *et al.,* 1995).

POX localized in almost all compartments of the plant cell, it plays role in stability the level of H_2O_2. Peroxidases, besides their main function in H_2O_2 elimination, can also catalyse O_2 and H_2O_2 formation by a complex reaction in which NADH is oxidized using trace amounts of H_2O_2 (Blokhina *et al.,* 2003). Peroxidases are also involved in biosynthesis of cell wall lignifications and suberization (Passardi *et al.,* 2004).

GSTs are a family of multifunctional enzymes that play important roles in oxidative stress resistance (Joseph and Jini, 2011). These dimeric enzymes catalyze the conjugation of GSH to avariety of electrophylic, hydropobic, and often toxic substrates, thereby reducing their toxicity (Hossain and Fujita, 2010).

GPX is ubiquitously occurring enzymes in plant cells that involved in scavenging of H2O2 and sever to detoxify products of lipid peroxidation formed due to activity of ROS. GPX activity was reported to decrease upon salinization of rice (Lee *et al.,* 2001). However, GPX activity was reported increased in leaves of tomato plant grown with 100 mM NaCl (He and Zhu, 2008).

GR catalyses the rate limiting last step of AsA-GSH pathway (Ahmad *et al.,* 2008). GR catalases the NADPH dependent reaction of disulphide bond of GSSG and is thus important in providing protection against oxidative damage in plants by maintaining the reduced form of glutathione (Foyer *et al.,* 1991). GR activity has been reported to increase in *B.juncea* seedlings with increase in salinity level (Verma and Mishra, 2005).

AsA is the most abundant antioxidant and serves as a major contributor to the cellular redox state and protects plants against oxidative damage resulting from aerobic metabolism and a range of biotic and abiotic stresses (Smirnoff, 2000). It is substrate of cAPX and the corresponding organellar iso-forms, which are critical components of AsA-GSH cycle for H_2O_2 detoxification (Nakano and Asada, 1981). Salt stress caused a decreased in total AsA in tomato (He and Zhu, 2008) and wheat (Farouk, 2011).

Materials and Methods

Two chickpea (*Cicer arietinum* L.) genotypes CSG-8962 (salt tolerant) and HC-3 (released variety) were raised in pots filled with dune sand [93.3% sand + 3.0 % slit + 3.7 % clay, saturation capacity 25 %, pH 8.2, ECe_2 0.8 dS m^{-1} at 25 ºC, 10.3 mg (N) kg^{-1}, 2.5 mg (P) kg^{-1}, 180 mg (K) kg^{-1}] under screen house conditions in the Department of Botany and Plant Physiology, CCS Haryana Agricultural University, Hisar-125 004, India. The seeds before sowing were surface sterilized and inoculated with effective *Rhizobium culture* (Ca 181). The desired salinity was developed before sowing and maintains four levels (control, 2.0, 4.0 and 6.0 dS m^{-1}) of chloride dominated salinity. The crop was supplied with an equal quality of nitrogen free nutrient solution with at regular interval of 15 d. The chloride (Cl^-) dominated salinity was prepared by using a mixture of different salts such as NaCl, $MgCl_2$, $MgSO_4$ and$CaCl_2$ where Na:Ca+Mg was in the ratio of 1:1 and Ca:Mg in the ratio of 1:3, the Cl:SO_4 ratio was 7:3 on

a meq basis. Sampling was done at 50-60 days after sowing (DAS).

H_2O_2 content of the leaves and roots was determined by a modified Patterson *et al.*, (1984) method. MDA is a product of lipid peroxidation and was measured by thiobarbituric acid (TBA) reaction with minor modifications of the method of Heath and Packer (1968). The activity of SOD was assayed by measuring its ability to inhibit the photochemical reduction of NBT according to Beauchamp and Fridovich (1971). The CAT activity was estimated according to the procedure described by Aebi (1984). GR activity was analysed by the method of Halliwell and Foyer (1978). The APX enzyme activity was determined according to the method described by Nakano and Asada (1981). The procedure of Siegel and Siegel (1969) was followed for estimating peroxidase activity. Ascorbic acid content was determined with a modification of the procedure of Takahama and Oniki (1992). The GPX activity was estimated according to the procedure described by Hossain and Fujita (2010).

Data were subjected to analysis of variance (ANOVA) using online Statistical Analysis Package (OPSTAT, Computer Section, CCS Haryana Agricultural University, Hisar, Haryana, India) and treatment means were compared by the least significant differences (LSD) ($p < 0.05$).

Results and Discussion

The H_2O_2 content increased significantly by 0.380 to 0.625 and 0.366 to 0.579 (moles g^{-1} DW) x 10_{-4}) in leaves at 50-60 DAS in the genotypes CSG-8962 and HC-3, respectively with increasing the level of salinity. In roots, H2O2 content increased significantly from 0.260 to 0.470 in the genotype CSG-8962 and 0.217 to 0.401 in the HC-3 (Figure 1 a). Increased H_2O_2 production under salinity has been reported in chickpea roots (Kukreja *et al.,* 2005), tomato leaves (He and Zhu, 2008). A progressive increase in H_2O_2 content with increasing NaCl concentration was observed in *Brassica juncea* (Verma and Mishra, 2005).

In leaves, a significant accumulation of MDA content was observed in both the genotypes under salinity levels (Figure 1 b). This increase was from 13.40 to 21.43 n moles g^{-1}dry weight in the genotypes CSG-8962 and 13.38 to 17.43 in HC-3. In roots, an increase from 10.35 to 16.59 n moles g^{-1} dry weight in MDA content was observed in the genotype CSG-8962, however, in HC-3, this was from 10.55 to 13.83 with increasing levels of salinity. Increase in electrolyte leakage with increasing saline stress has been reported in wheat leaf senescence (Farouk, 2011) and in wheat young leaf (Farooq and Azam, 2006). Similar results have been observed in green gram (Panda, 2001) and chickpea (Kukreja *et al.,* 2006; Sheokand *et al.,* 2008).

The specific activity of SOD in leaves increased from 1.20 to 10.70 and 1.36 to 11.67 Units mg^{-1} protein at 6.0 dS m^{-1} over their control in genotypes CSG-8962 and HC-3 (Figure 2 a). In roots the specific activity of SOD increased from 1.83 to 10.46 and 1.76 to 12.65 Units mg^{-1}protein in both the genotypes CSG-8962 and HC-3, respectively. Kukreja *et al.,* (2006) reported two-fold increase in the specific activity of SOD activity in chickpea roots under short term salinization.

The specific activity of CAT in leaves increased from 2.23 to 6.93 and 2.60 to 8.50 Units mg^{-1}protein in both genotypes CSG-8962 and HC-3, respectively. In roots, 3.04 to 6.05 in CSG-8962 and 3.35 to 7.36 in HC-3 increase the specific activity of CAT was observed (Figure 2 b). Catalase (CAT) is a key antioxidant enzyme which converts H_2O_2 to water and molecular oxygen.

Table.1 Changes in yield and its attributes of chickpea genotypes under different salinity levels

Parameters	Genotypes	Salinity levels (dS m^{-1})				
		0	2	4	6	M
Branches $plant^{-1}$	HC-3	9.00	8.33	8.00	6.33	7.91
	CSG-8962	8.00	7.00	6.6	5.00	6.91
	Mean	8.50	8.16	7.33	5.66	
	CD at 5 %	Genotype = 0.30; Salinity = 0.43; G x S = NS				
Pods $plant^{-1}$	HC-3	13.66	13.33	11.33	8.66	11.75
	CSG-8962	12.66	11.33	8.33	7.66	10.00
	Mean	13.16	12.33	9.83	8.16	
	CD at 5 %	Genotype = 0.50; Salinity = 0.71; G x S = 1.00				
Seeds pod^{-1}	HC-3	1.66	1.33	1.33	1.33	1.41
	CSG-8962	1.66	1.30	1.00	1.00	1.24
	Mean	1.66	1.31	1.16	1.16	
	CD at 5 %	Genotype = 0.04; Salinity = 0.05; G x S = 0.08				
100 seed weight (g)	HC-3	29.36	24.72	15.75	10.97	20.20
	CSG-8962	13.68	10.82	8.83	7.47	
	Mean	21.52	17.77	12.29	9.22	
	CD at 5 %	Genotype = 0.51; Salinity = 0.72; G x S = 1.02				
Seed yield $plant^{-1}$ (g)	HC-3	26.00	25.00	23.00	19.00	23.25
	CSG-8962	25.00	23.00	22.33	16.00	21.58
	Mean	25.50	24.00	22.66	17.50	
	CD at 5 %	Genotype = 0.59; Salinity = 0.84; G x S = 1.19				

Figure.1 Changes in hydrogen peroxides (H_2O_2) content (a) and lipid peroxidation (MDA) content (b) of chickpea genotypes under different salinity levels

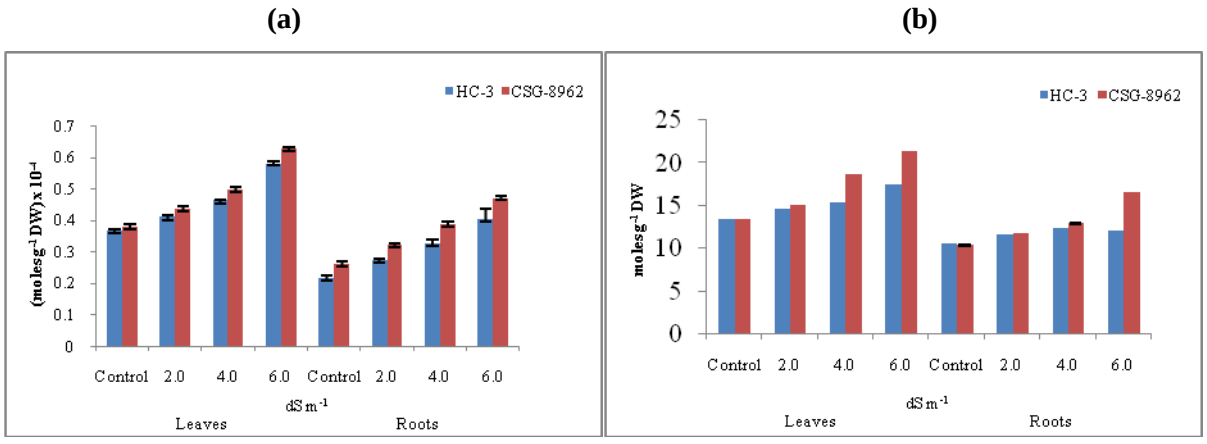
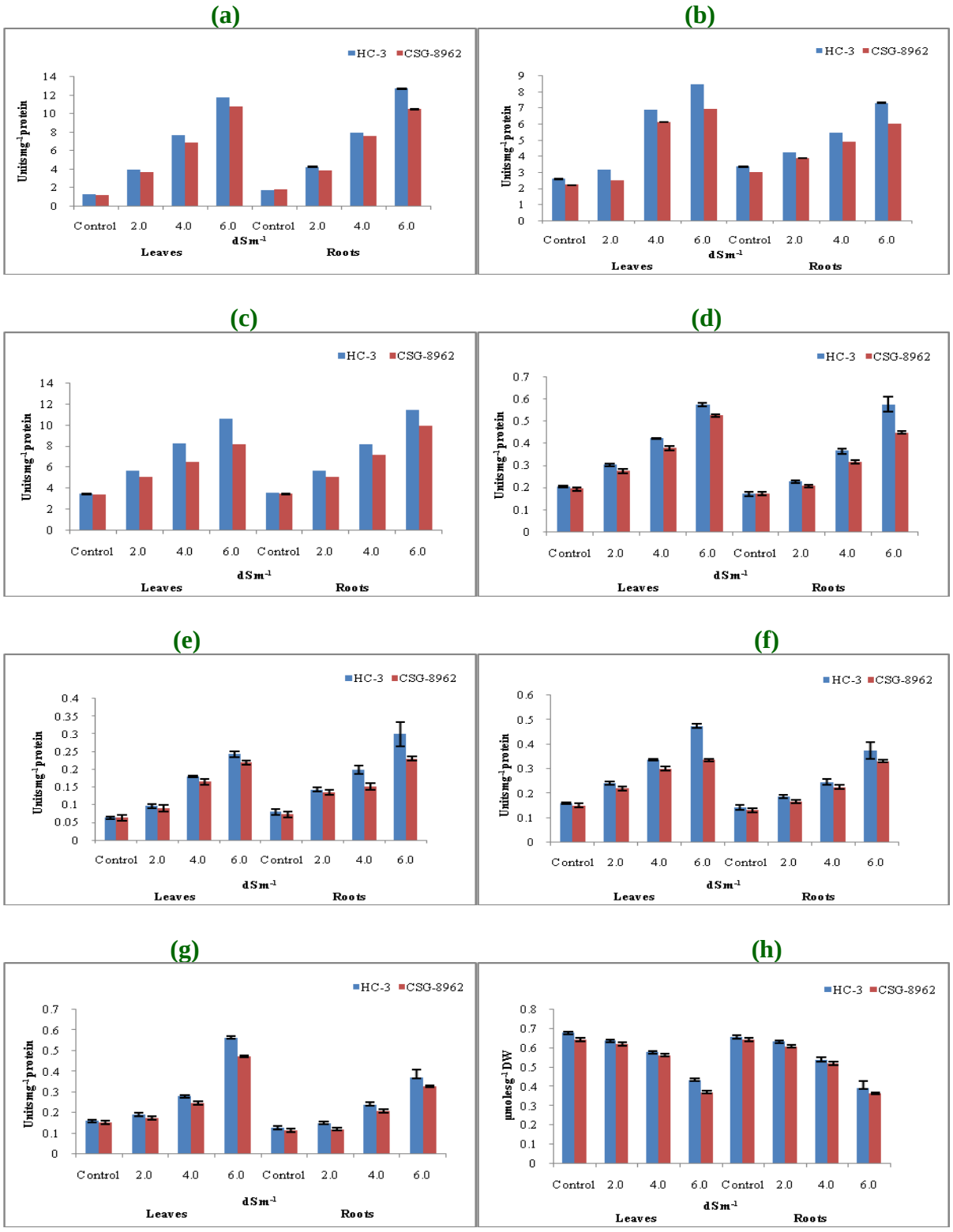


Figure.2 Changes in specific activity of Superoxide dismutase (a), Catalase (b), Peroxidase (c), Ascorbate peroxidase (d), Glutathione peroxidase (e), Glutathione reductase (f), Glutathione S-transferase (g) and Ascorbate (h) of chickpea genotypes under different salinity levels



An increased in the specific activities of the CAT is related to an increase in stress tolerance (Sairam and Srivastava, 2001).

In leaves, Specific activity of POX increased was from 3.41 to 8.20 and 3.46 to 10.59 Units mg^{-1}protein in the genotypes CSG-8962 and HC-3, respectively. In roots, increase in the specific activity of POX from 3.46 to 9.93 and 3.61 to 11.41 Units mg^{-1}protein was observed in genotypes CSG-8962 and HC-3, respectively with increasing levels of salinity from control to 6.0 dS m^{-1} (Figure 2 c). Experimental evidence also showed that salinity causes increases in POX activity in *Cassia angustifolia* (Agarwal and Pandey, 2004), *Brassia napus* (Dolatabadion *et al.*, 2008) and chickpea plants (Sheokand *et al.*, 2008).

The specific activity of APX increased from 0.193 to 0.525 and 0.204 to 0.574 Units mg^{-1}protein in genotypes CSG-8962 and HC-3, respectively at 50-60 DAS in leaves. The specific activity of APX is 0.173 to 0.506 in CSG-8962 and 0.170 to 0.447 Units mg^{-1}protein in HC-3 in roots (Figure 2 d). Hernandez *et al.*, (2000) reported that tolerant *Pisum sativum* response to long term NaCl treatment increased Ascorbate peroxidase 3 fold.

The GPX specific activity increased from 0.063 to 0.220 in CSG-8963 and 0.063 to 0.243 in HC-3 Units mg^{-1} protein in leaves. An increase from 0.073 to 0.230 and 0.080 to 0.300 Units mg^{-1} protein in GPX specific activity was observed in both genotypes CSG-8962 and HC-3, respectively in roots (Figure 2 e). GPX activity was reported to decrease upon salinization of rice (Lee *et al.*, 2001). However, GPX activity was reported increased in leaves of tomato plant grown with 100 mM NaCl (He and Zhu, 2008).

In leaves the specific activity of GR increased from 0.150 to 0.366 and 0.159 to 0.475 Units mg^{-1} protein in genotype CSG-8962 and HC-3, respectively with increasing levels of salinity from control to 6.0 dS m^{-1} at 50-60 DAS. In roots, the specific activity of GR increased was from 0.132 to 0.302 and 0.143 to 0.375 Units mg^{-1} protein in genotypes CSG-8962 and HC-3, respectively (Figure 2 f). The increase in GR activity has also been observed in salt tolerant varieties of rice (Dionisio and Tobita, 1998), pea (Hernandez *et al.*, 2000), in *Brassica juncea* (Verma and Mishra, 2005) and wheat (Sairam *et al.*, 2002) than their respective sensitive varieties.

The specific activity of GST increase from 0.153 to 0.473 in CSG-8962 and 0.162 to 0.564 Units mg^{-1} protein in HC-3 was observed in leaves. In roots increase from 0.115 to 0.327 and 0.128 to 0.373 Units mg^{-1}protein specific activity of GST were observed in genotypes CSG-8962 and HC-3, respectively (Figure 2 g). GST increased by 95% was reported at 10 dS m^{-1} salinity level in chickpea roots (Kukreja *et al.*, 2005).

In contrast to antioxidant enzyme activities, the AsA content decreased in both leaves and roots with increasing salinity levels from control to 6.0 dS m^{-1}. In leaves the AsA content decreased from 0.645 to 0.372 and 0.681 to 0.435 µ moles g^{-1} dry weight were observed in genotypes CSG-8962 and HC-3, respectively. In roots, a decreased from 0.645 to 0.365 in CSG-8962 and 0.659 to 0.395 HC-3 µ moles g^{-1}dry weight were observed (Figure 2 h). The decline in Ascorbate content was also observed with increasing salinity level in chickpea (Kukreja *et al.*, 2006), tomato (He and Zhu, 2008) and wheat (Farouk, 2011). Hernandez *et al.*, (1999; 2000) reported NaCl concentration at 70 mM decreased AsA in both NaCl tolerant and NaCl sensitive pea cultivars.

Number of branches $plant^{-1}$ reduced to 37.5 % and 29.6 % in the genotypes CSG-8962 and HC-3, respectively, at 6.0 dS m^{-1} salinity

level. The number of pods plant^{-1} reduced to 39.5 % and 36.6 % in the genotypes CSG-8962 and HC-3, respectively. The percent reduction in number of seeds pod^{-1} was 39.7 % in CSG-8962 and 19.8 % in HC-3. The percent reduction in test weight was 7.4 % in CSG-8962 and 10.9 % in HC-3 at 6.0 dS m^{-1}. The percent reduction in seed yield plant^{-1} was 27.3, 43.8 and 58.0 % and 19.0, 31.5 and 52.0 % in the genotypes CSG-8962 and HC-3, respectively at 2.0, 4.0 and 6.0 dS m^{-1} salinity level with respect to their control (Table 1). Turner *et al.,* (2013) also observed that saline treatment (40mM NaCl) significantly decreased the seed yield in chickpea genotypes and genotypic variation for salinity tolerance exists in chickpea.

In conclusion, HC-3 showed comparative better perform than CSG-8962 on the basis of various biochemical traits related to hydrogen peroxide, lipid peroxidation, antioxidant defense system and yield attributes under saline conditions.

Abbreviations: *APX – Ascorbate peroxidse, CAT - Catalase, dS m^{-1} – DeciSiemens per metre, DAS – Days after sowing, DW - Dry weight, GPX – Glutathione peroxidase, GR – Glutathione reductase and GST – Glutathione –S- transferase, H_2O_2 – Hydrogen peroxide, MDA – Malondialdehyde, POX – Peroxidase, SOD – Superoxide dismutase.*

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