

Amelioration of Salinity stress in Chickpea (*Cicer arietinum* L.) variety PBG5 through foliar spray of Ascorbic acid

Project report submitted to Central University of Punjab

For the award of
M.Sc. Life Sciences Specialization in Plant
Sciences

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May 2018

DECLARATION

I declare that project entitled “**Amelioration of Salinity stress in Chickpea (*Cicer arietinum* L.) variety PBG5 through foliar spray of Ascorbic acid**” has been prepared by me under the guidance of, Dr.Krishna Kumar Choudhary, Centre for Plant Sciences, School of Basic and Applied Sciences, Central University of Punjab. No part of this project has formed the basis for the award of any degree or fellowship previously.

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CERTIFICATE

I certify that Rajneesh Kumar (Reg. no. 16mslsp15) has prepared his project report entitled “**Amelioration of Salinity stress in Chickpea (*Cicer arietinum* L.) variety PBG5 through foliar spray of Ascorbic acid**” for the award of M.Sc. degree of the Central University of Punjab, under guidance. He has carried out this work at the centre for Plant Sciences, School of Basic and Applied Sciences, Central University of Punjab, Bathinda.

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ACKNOWLEDGMENTS

I acknowledge my sincere thanks to my Supervisor, **Dr. K.K. Choudhary**, Assistant Professor, Department of Plant Sciences, Central University of Punjab for encouraging and guiding me throughout my project work.

I express my deep gratitude to all the faculty of Plant Sciences for providing me with all the necessary facilities. I am also very grateful to Ph.D. scholar **Sumandeep Juneja** for her valuable advices, frank criticism and whole hearted cooperation during this project work. I also thank my friend **Ajay PrakashUniyal** for his unequivocal support and encouragement throughout.

I am indebted to all my lab mates and seniors for their positive encouragement, sincere help and guidance at every stage of this work.

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ABSTRACT

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Key Words: Ascorbic acid, salt stress, Membrane damage, Water status, Chlorophyll content, SOD, CAT

Chickpea (*C. arietinum* L.) is the second most important legume crop sown as a winter crop in northern parts of India, and its productivity is constraint by several abiotic stress, and salt stress is one of the important factor for significant yield loss. Spraying the plants with ascorbic acid might trigger the signaling pathways that will prepare the plants to combat lethal salt stress. In this study, chickpea variety PBG5 was sprayed with ascorbic acid along with 50 mM of NaCl stress and further analyzed for various parameters. To see the effects of ascorbic acid spray various cell responses were monitored by measuring membrane damage (ELI & MDA content), water status (RLWC), Chlorophyll content and Antioxidant enzyme activity (SOD and CAT) along with growth and total biomass. Present study clearly reflected that foliar spray of ascorbic acid enhances the tolerance capacity of the plants and prevents noticeable damage in them. To conclude whole study, treatment with ascorbic acid has ability to improve the tolerance against salt stress in chickpea.

Rajneesh Kumar

Dr. K. K. Choudhary

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Abbreviations

Sr.No.	Full form	Abbreviation
1	Ascorbic acid	AsA
2	Catalase	CAT
3	Chlorophyll	Chl
4	Cytoplasm	Cyt
5	Dehydroascorbate	DHA
6	Dehydroascorbatereductase	DHAR
7	Electrolytic Leakage Index	ELI
8	Glutathione	GSSG
9	Glutathione peroxidase	GPOX
10	Glutathione reductase	GR
11	Gram	gm
12	Hydrogen Peroxide	H ₂ O ₂
13	Malondialdehyde assay	MDA
14	Milligram	mg
15	Mitochondria	Mit
16	Peroxidase	POX
17	Peroxisome	Per
18	Reactive Oxygen Species	ROS
19	Reduced Glutathione	GSH
20	Relative Leaf Water Content	RLWC
21	Superoxide Dismutase	SOD
22	Sodium Chloride	NaCl

Chapter 1

Introduction

World population is expected to reach upto 9.5 billion by 2050 (United Nations, Department of Economic and Social affair, 2013). With the increasing population there will be demand of increasing supply of food. To feed such large populations, the demand for sustainable and alternate agriculture should have to be increased. As pulses and cereals are the major nutritive crops, the strategy will be to increase the production of these plants. Chickpea is the second major food legume cultivated in winter season in the northern India. It is the major source of the nutrition- carbohydrates 59%, and proteins 13-31% (Gangola et al.,2013). But chickpea and other legumes are prone to several abiotic stresses (cold, heat, UV, ozone, drought, salinity etc.). Out of these stresses salinity is of major concern.

Salinity had affected over 45 million hectares of land accounting for over 20% of total land (Munns and Tester, 2008). Saline soils contain salts soluble in water in the quantities that can affect the various stages of plants and crops yield (Saxena, 1990). It impairs germination, retard plant development and cause reduction in nodule formation (Greenway and Munns, 1980). Saline soils create osmotic potential which prevent the uptake of water or cause toxicity effects that can have drastic impact on the viability of embryo. The shoot growth is also affected by imposition of ionic inhibitory effect on the cell division and its enlargement. Some soils are naturally saline but secondary salinization is also caused due to irrigation system. It imposes largest threat to sustainability of legume in arid region in limited water supply. Salinity is affecting many pulses especially the chickpea affected mostly by chloride ions (Manchandra et al.,1990). The effect of salinity on chickpea is very wide varying from germination to vegetative stages. Crop is salt sensitive particularly at the early stages of its growth and development (Zawude et al.,2017). Seedling is the most critical stage. At the same time, the effect of salt stress on chickpea also varied among cultivars. Some varieties

germinate well in saline soils while other have poor seedling growth. There is a report in which various genotypes fail to grow over 25mM NaCl but the tolerant one survives upto 100mM NaCl (Habtamu et al.,2013).

Chickpea is the third largest legume crops in world growing over 54 countries after bean and pea (FAO, 2016). The crop has also great role in maintaining soil fertility, especially in arid regions. Chickpea seed is nutritive with high protein content. Salinity affects hydrogen peroxide scavenging system, plant water status and membrane integrity of chickpea at vegetative stages (Kukreja et al. 2005). It was also noticed that at 30 mMNaCl cause reduction in plant height and weight of leaves, stem and roots. Osmotic stress is the major salt stress and salt tolerance is majorly determined by the osmotic tolerance. Chickpea can maintain osmotic tolerance by maintaining water relations, stomatal conductance and photosynthesis and other metabolisms. This will increase the photoassimilates availability to the reproductive structures and hence increase in seed growth (Tibbrook and Roy, 2014). In chickpea salinity has degraded leaf chlorophyll upto 78% and reduced leaf photosynthesis upto 60% (Datta and sharma, 1990). On the other hand crop can also exclude the ions accumulation and provide tissue tolerance. The crop possess its own defense mechanism to counter. Selecting the tolerant crops would be an appropriate strategy for alleviation of adverse effect of salinity (Hasegawa et al.,2000).

Plants are equipped with the system for scavenging the free radicals such as ascorbic acid (Beltagi 2008).Ascorbic acid increase the cell divisions and have antioxidant property preventing the damage from the oxygen and other free radicals generated during salt stress. AA is the major substance in the antioxidant network that include ascorbate, glutathione, tocopherol and several antioxidant enzymes. It is majorly found in the cytosol, vacuoles, mitochondria and chloroplasts in plant cells. It quenches ROS directly or by Asada–Halliwell–Foyer pathway (Gest et al.,2013). Seed treatment with ascorbic acid had increased resistance to salinity in the tomato

by decreasing the active oxygen production (Sayed et al.,2016) and had also improved tolerance in potato (Sajib and Aftab 2016). Positive effects of ascorbic acid involvement in growth of wheat were also reported by Rafique et al. 2011.

Foliar spray of Ascorbic Acid

Ascorbic acid is known to play multiple roles in plant growth, such as cell division, cell wall expansion and other processes as developmental pathways (Pignocchi and Foyer, 2003).The fundamental role of is to protect the metabolic process of plants against hydrogen peroxides and other reactive oxygen derivatives. It reacts non enzymatically with the superoxide, singlet oxygen and peroxides (Pourcel et al.,2007). Ascorbic acid also maintains prosthetic metal groups of certain enzymes in its reduced state. Foliar application of Ascorbic acid inhibits increase in the leaf proline concentration (Bastam, 2013) and can reduce the electrolytic leakages in plants and can diminish the injurious effect to chickpea and might be helpful in improving the crop productivity. The acid may also contribute in maintenance of photosynthesis, gene expression, synthesis of anthocyanins, hormones, flavonoids etc. (Gest et al. 2013). Acid can be easily absorbed by the exogenous application (Hameed et al. 2010) and circulates within the plant tissues. Therefore the foliar spray can improve the salt tolerance in chickpea.

Table 1. Effects of Foliar spray of Ascorbic acid in some plants.

Sr.No.	Plants	Effects	References
1	Maize	Increased growth, yield and maize quality	Chattha et al.,2015.
2	Wheat	Improved yield productivity and chlorophyll content during water stress	Hafez et al.,2016.
3	Peach	Sustain the antioxidant activity, improve quality of fruit and yield	Sajid et al.,2017

Hypothesis

To investigate the effect of foliar spray of ascorbic acid on growth of chickpea in relation to salt tolerance.

Objectives

To study the effects of foliar spray of Ascorbic acid in chickpea cultivar PBG5 under salinity stress on

- a. Growth and biomass
- b. Oxidative status
- c. Antioxidative defense system

Chapter 2

Review of literature

Chickpea (*Cicer arietinum* L.) belongs to the family fabaceae (Leguminosae) grown in arid and semi-arid areas around the world (Varshaney et al.,2009). It is diploid, self-pollinated annual crop having 740 Mb genome size (Garg et al.,2011). Chickpea plays an important role in nitrogen fixation and maintenance of soil fertility.

2.1. Salinity injury

Salinity is the global problem affecting 20% irrigated lands and reduces crop yields significantly (Qadir et al.,2014). Physiological response of salinity to plants are complex and manifold. Basically, the salinity response in plant is categorised into two phases: first shoot ion independent response and second ion dependent. In first phase of response, salinity can be important in causing stomatal closure and prevention of leaf expansion (Negrao et al.,2017). The second phase is involved in building up of ions causing toxicity in older leaves and their senescence (Munns and Tester, 2008).

Chickpea is a salt sensitive species and its growth is significantly decreased at the low concentration of salt even 20mM and plant dies when critical salinity level is exceeded. Salinity causes reduction in the plants to utilize the water (Munns 2002). In Chickpea, salt stress leads to leaf necrosis, reduced vegetative growth and impede flower formation (Vadez et al.,2007). Deleterious effects of salinity on plants can be associated with the low osmotic potential, imbalance in nutrition, ion toxicity and specific ion effect. Repair strategies include osmotic adjustment, antioxidant production, stress proteins inductions such as LEA proteins, chaperonin etc. and shoot or root modifications (Manchandra and Garg, 2008).

2.2. Salt tolerance mechanism

Three main salinity tolerance mechanism as proposed by Munns and Tester 2008 are ion exclusion- excluding ions from shoots, tolerance- compartmentalising toxic ions into tissues, cells

and subcellular organelles and shoot independent tolerance-maintaining water uptake independent of sodium concentration. The other mechanisms for tolerance include optimization of water status, transpiration efficiency (Barbieri et al.,2012), antioxidant production (Ashraf, 2009) etc. several anatomical features are considered to be essential in plant adaptation to a highly saline environment like leaf succulence i.e. thickening of leaf tissue and resulting increase in leaf sap (Shabala and Mackay, 2011). Salt bladders and glands are arguably the most remarkable anatomical feature related to salinity. The main function of salt glands and bladders is the elimination or sequestration of excess salt from metabolically active tissues (Jou et al, 2007).

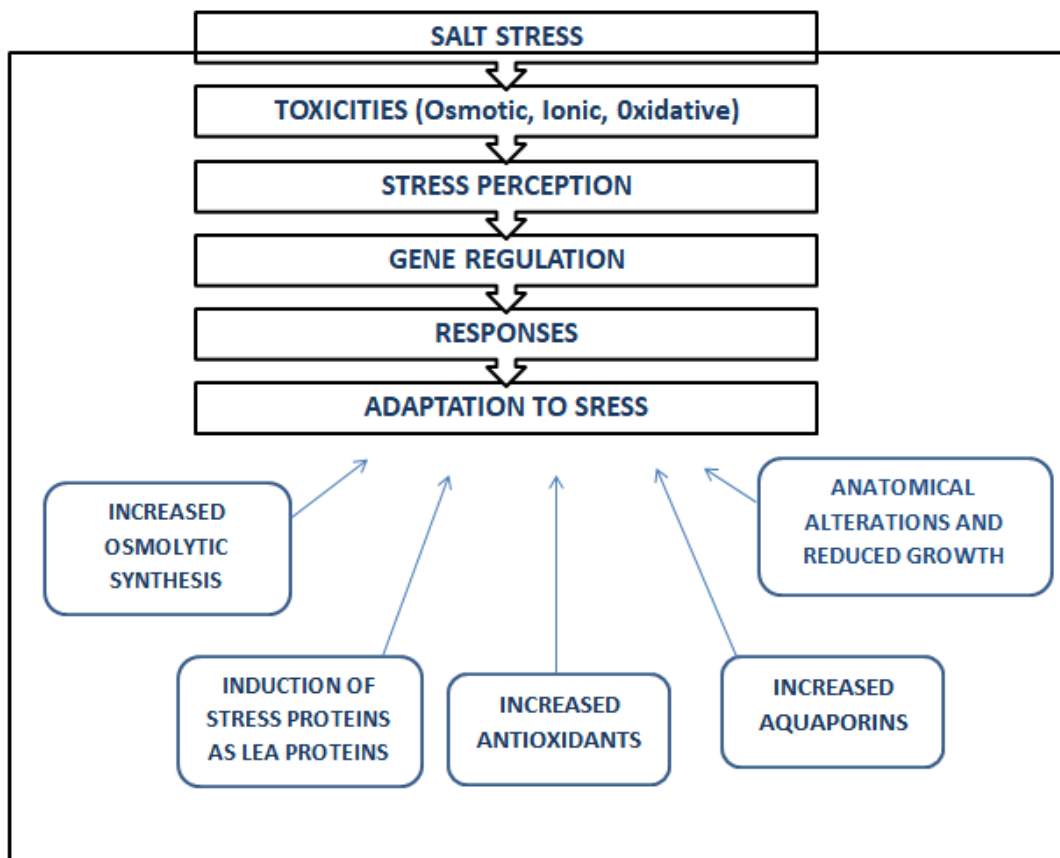


Fig 1: Strategies involved in prevention of salt stress damages in plants. (Manchandra and Garg, 2008).

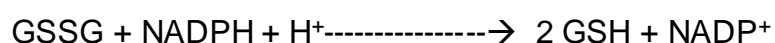
2.3. Antioxidant Systems

Salinity induces oxidative damage generating reactive oxygen species (ROS) as superoxide anion, hydrogen peroxide and hydroxyl radicals (Munns and Teaser, 2008). To fix the damages, plants exploit usage of antioxidant system for protecting nucleic acids, RNA and lipids etc. (Fahad et al., 2014). The antioxidant system involves SOD, POX, APX and GR and non-enzymatic antioxidants as ascorbate, glutathione etc. (Hossain et al., 2013). Accumulation of hydrogen peroxides by abscisic acid generate NO activating the MAPK and hence up regulate the antioxidant system to scavenge the ROS (Lu et al., 2009).

2.4. Glutathione Induced Antioxidant protection against salinity stress

Glutathione is one of the powerful antioxidants i.e. responsible for balance between oxidation and antioxidation. It protects cells from the free radicals and POXs (Pompella et al., 2003). It is the main component of Glutathione ascorbate cycle that scavenge the hydrogen peroxides. Glutathione is synthesised from glutamate and cysteine by Glutamate Cysteine ligase that is conserved and found exclusively in plastids of plants and by Glutathione synthetase (Hicks et al., 2007).

The enzyme Glutathione reductase converts oxidized Glutathione disulphide (GSSG) to Glutathione (GSH). GSH is key enzyme in the regulation of ROS levels in plants (Anjum et al., 2010).



Ascorbate Glutathione cycle.

In ascorbate glutathione cycle, hydrogen peroxide is reduced by enzyme ascorbate peroxidases. Electron donor in the reaction is ascorbate. The oxidised ascorbate is then regenerated by monodehydroascorbate reductase (MDHAR). Monodehydroascorbate is disproportionated if not reduced to ascorbate and Dehydroascorbate (DHA) by MDHAR. DHA is also reduced to ascorbate by DHAR at GSH expense, yielding GSSG. GSSG is

reduced by GR using NADPH as donor. The cycle operates in plastids, mitochondria, cytosol and peroxisomes of plants (Meyer et al.,2009).

2.5. Ascorbic acid (AsA)

AsA is an important antioxidant in plant synthesised in cytosol of higher plants. The compound have important role in physiological processes in plants. The main function of it is to reduce free radicals and to minimize the oxidative damage. AsA act as cofactor for the oxygenase and hydroxylases enzyme. It can also directly scavenge and regenerate tocopherol and thus providing the membrane protection (Mohamed et al. 2010). Under stress condition plant shows different capacity for AsA metabolism. This can be due to variation in synthesis and regeneration of AsA. Agarwal and Saheen (2007) have reported increase in AsA concentration in Momordicaleaves in NaCl stress compared to control.

Exogenous application of Ascorbic acid helps *Lycopersiconesculentum* seedlings to recover from salt stress (Shalata and Neumann 2001). Addition of exogenous AsA decrease lipid peroxidation. Ascorbic acid peroxidase (APX) activities increased with increase in salt concentration in salt tolerant genotypes of rice but activities in salt sensitive BRR1 genotype decrease (Hasanuzzaman et al. 2013).

2.6.Tocopherol

Tocopherols are also the potentive antioxidant and scavenger of ROS and lipid radicals (Hollander Czytko et al.,2005). These substances are found all over the plant and play both as an antioxidant and non-antioxidant. Tocopherol as Vitamin E has highest antioxidative activity because of three methyl groups. The chloroplasts comprises of substantial amount of the tocopherols to protect against photooxidative damage (Ashraf et al. 2009). Tocopherols and carotenoids both protect the thylakoid membrane by preventing chain propagation step during the lipid oxidation

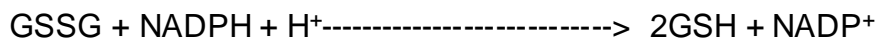
(Ashraf et al. 2009). One tocopherol molecule can scavenge upto 120 Oxygen molecule by resonance energy transfer.

2.7. Enzymatic antioxidants

Enzymatic antioxidants include Superoxide Dismutase (SOD), Catalase (CAT), APX and GR. All these enzyme reduce the superoxide and hydrogen peroxide in plants.

2.7.1. Glutathione reductase (EC 1.6.4.2)

Glutathione reductase converts the oxidized Glutathione GSSG into its reduced form GSH using NADPH cofactor. It is key enzyme in ROS regulation in different subcellular compartments. Glutathione reductase seemed to accelerate hydrogen peroxide scavenging system particularly under stress conditions. Mandhania et al. (2006) studied the salt stress in Wheat varieties and concluded increase in GR with increase in salt stress.



2.7.2. Catalase (EC 1.11.1.6)

Catalases helps in decomposing the hydrogen peroxide to water (Chelikani et al., 2004). The enzyme is present in peroxisomes of all aerobic cells and serve to protect from toxic effect of the hydrogen peroxide (Boon et al., 2007). Catalases are encoded by the nuclear genes. The enzymes perform two functions, Peroxidatic activity and Catalytic activity. Former activity cause the reduction of iron peroxide and later activity helps in conversion of peroxide to water.

2.7.3. Superoxide dismutase (EC 1.15.1.1)

SOD is the first line of defence against ROS. It catalyse the dismutation of oxygen radical to molecular oxygen and hydrogen peroxide. SODs are multimeric metalloprotein and have different isoforms based on the metal species. Most common isoforms are Cu/Zn SOD, Mn SOD, Fe-SOD and Ni-SOD. Fe SOD are located in

chloroplast, Mn SODs in mitochondria and peroxisomes, Cu-Zn SOD in chloroplast and cytosol (Alscher et al., 2002).

Table 2: Important Antioxidants in plants. Adopted from Mittler (2002).

Antioxidant	Localisation	Respective ROS	References
<p>1. Enzymatic</p> <ul style="list-style-type: none"> ● Superoxide dismutase (EC 1.15.1.1) ● Catalase (EC 1.11.1.6) ● Ascorbate peroxidase (EC 1.11.1.11) ● Glutathione peroxidases (EC 1.11.1.12) 	<p>Chl, Mit, Per, Cyt</p> <p>Per, Gly, Chl, Mit, Per, Cyt</p> <p>Cyt</p>	<p>Superoxides</p> <p>H₂O₂ H₂O₂</p> <p>H₂O₂, lipid peroxyradicals (ROO), organic hydrogen peroxide (ROOH)</p>	<p>Mittler (2002)</p> <p>Mittler (2002)</p> <p>Mittler (2002)</p> <p>Hoque et al. (2008)</p>
<p>Non enzymatic</p> <ul style="list-style-type: none"> ● Glutathione (GSH) ● Ascorbic acid 	<p>Chl, Mit, Per, Cyt, Apo</p> <p>Chl, Mit, Per, Cyt, Apo Membranes</p>	<p>H₂O₂, hydroxyl radical (UOH), singlet oxygen (¹O₂), dehydroascorbate reductase (DHAR)</p>	<p>Mittler (2002)</p> <p>Mittler (2002)</p> <p>Holländer-Czytko et al.</p>

● α -Tocopherol		$H_2O_2, O_2,$ $U-, UOH,$ $^1O_2 \quad ^1O_2,$ $UOH, \text{ lipid}$ peroxy radicals (ROO), Organic peroxide (ROOH)	(2005)
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2.8. Improvement of salt tolerance by engineering antioxidant genes

Engineering plants overexpressing antioxidant gene had great opportunity in enhancing tolerance to salinity. Overexpression of MnSOD in Arabidopsis have increased the salt tolerance capacity two fold compared with old one (Wang et al. 2004). The overexpression of MnSOD enhanced the activities of other antioxidative enzymes as Catalase etc. The level of MDA were also lower indicating the enhanced ability of antioxidants to detoxify the ROS. Similarly in tobacco, (Yadav et al. 2005) developed the transgenics by glyoxalase enzyme overexpression. This cause resistance against accumulation of methylglyoxal (MG) in plants under salt stress. The transgenic had an enhanced activity of glutathione S transferases. Manipulating the antioxidant genes seems to be good approach for counteracting the salt stress. Number of genes responsible for salinity tolerance can be transferred to plants through genetic engineering. List of genes are provided in Table number 3.

Table 3: Genes coding to impart salinity tolerance when overexpressed.

Osmolytes	codA, cox, betA, mtlD, ostB, TPS1
Helicases	PDH45, MCM6, MH1
Antioxidative enzymes	Gly1, Gly2
Molecular Chaperones	RcHSP17.8
Signalling molecules	ZmMKK4, Ots1, Rab7, ZmSAPK8
Transcription factors	SNAC1, AtDREB1A, TaMYB2A, HARDY, ABP9, SIAREB1
Lea proteins	HVA1

Chapter 3

Material and Methods

3.1 Plant material

Chickpea (*C. arietinum* L.); released variety PBG5 was used to study the effect of salt stress response on membrane damage, water status and antioxidant enzyme activity. The germplasm consisting of variety PBG5 was procured from Punjab agriculture university, Ludhiana.

3.2 Experimental setup

Initially, healthy seeds were picked and sown in thermocol pot, 9 cm x 7cm containing 200 gm of sterilised sand. After germination (72 hours) 16/8 hrs (light/dark) photoperiod was provided. Optimum condition (temperature 25°C) were maintained in a climate-controlled chamber, illumination was provided by cool-white fluorescent lamp with photosynthetically active radiation (PPFD) of 200 μ mol m⁻² s⁻¹. Plants were watered daily to maintain 70% humidity and sub-irrigated every other day with a half-strength Hoagland's solution (Hoagland and Arnon, 1950) to have enough biomass. On 10th day, the plants were divided into three groups:

- (1) **Control plants** which were irrigated with distilled water
- (2) **Salinity stress** which were irrigated with 50 mM NaCl concentrations in separate pots
- (3) **Salinity stress + Ascorbic acid treatment** which were given 50mM NaCl + foliar spray of 10mM Ascorbic acid treatment. These treatments were given for 10 days.

On 20th day leaf samples were harvested and analysed for electrolyte leakage, relative leaf water content and lipid peroxidation, water status and antioxidant enzyme analysis. Experiment for all the parameters were performed in triplicates.

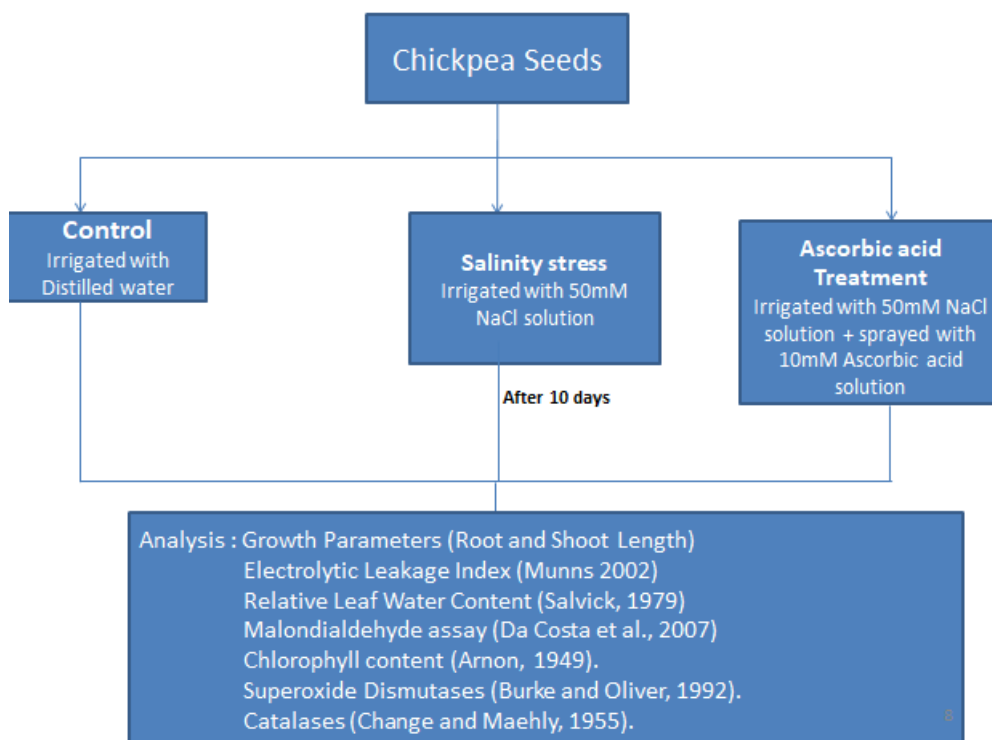


Figure 2 : Workflow diagram for experimental analysis

3.3. Electrolytic leakage index

50 mg of fresh leaves were rinsed 3 times with demineralised water and subsequently floated on 10ml of demineralised water. Then electrolyte leakage in the solution was measured after 22h of floating at room temperature using a conductivity meter (Metler Toledo, India). Total conductivity was obtained after keeping it in boiling water flask for 2 h. Results were graduated in total conductivity percentage.

L1= Conductivity before boiling

L2= Conductivity after boiling

Electrolytic leakage Index= $(L1/L2) * 100$

3.4. Malondialdehyde (MDA) Content

Method used in Davey et al., 2005 with slight modification was followed to find MDA content. 50 mg of plant tissue is crushed in 2ml of extraction buffer. Then it was heated at 95 degree for 30 mins. Reaction was stopped in ice bath. After that it was centrifuged at

15000g for 5 min and the reading of supernatant was taken at 532nm and 600nm (Da costa et al.,2007)

3.5. Leaf Relative Water Content

To obtain the fresh weight leaves were weighed immediately. The leaves were then soaked in water for 2hr and blotted with tissue paper to remove moisture on the leaves. The leaves were weighed, referred as turgid weight. The turgid leaves were oven-dried. The dried leaves were weighed and to calculate the relative water content (RLWC) following formula was proposed (Salvick, 1979)-

$$RWC (\%) = [(FW-DW) / (TW-DW)] \times 100$$

Where, FW is the sample fresh weight, TW is the sample turgid weight and DW is the sample dry weight.

3.6. Chlorophyll content

The chlorophyll was extracted with 80% acetone to ensure complete extraction and the extract filtered using whatman filter paper. The absorbance of the supernatant was read at 645, 663 nm against 80% acetone solvent (Arnon, 1949). The amount of chlorophyll present in the extract was calculated according to the following equation mentioned below.

$$\text{Chl a} = 12.7 (\text{OD at } 663\text{nm}) - 2.69 (\text{OD at } 645)\text{V}/1000*W$$

$$\text{Chl b} = 22.9 (\text{OD at } 645 \text{ nm}) - 4.68 (\text{OD at } 663)\text{V}/1000*W$$

3.7. Antioxidative enzymes:

For enzyme extract preparation, 50 mg FM was grounded to powder in liquid nitrogen and homogenized in 2ml of extraction buffer (50mM phosphate buffer pH 7.8, 0.1mM EDTA, 1mM phenylmethylsulfonyl fluoride (PMSF) and 2% PVP). Homogenized samples was centrifuged at 14,000xg for 30 minutes at 4⁰C and supernatant was used for the determination of protein content and enzyme activities. Enzyme extracts will be kept at 4⁰C for all the assays and was stored at -20⁰C for long period (Ceylan et al.,2013). We had assayed four antioxidative enzymes, superoxide dismutase (SOD; 1.15.1.1),

catalase (CAT; 1.11.1.6), as previously described Chakrabarty et al.,(2009).

3.8. Superoxide dismutase (SOD)

For SOD assay, the reaction mixture (Solution A) was prepared by mixing 25 mL of 50 mM potassium phosphate buffer pH 7.8, 35 mg of L-methionine (9.9 mM), 7.5 µl of Triton X-100 (0.025%) and 4 mg NBT (57 µmol/l). Aliquots (1 ml) of this Solution A were poured into small glass tubes, followed by 20 µl of enzyme extract and 10 µl of Riboflavin (4.4 mg/100 ml). After mixing the cocktail, the reaction mixture was illuminated for 10 min under 20W florescent tubes at 25°C. Sample in the control tube was replaced by 20 µl of buffer. The reading were taken at A560. Fifty percent of inhibition was taken as equivalent to 1unit of SOD activity (Chakrabarty et al.,2009).

3.9. Catalase (CAT)

Catalase activity was performed as follows. 1ml reaction mixture contained 50 mM potassium phosphate (pH 7.0), 0.1 mM H₂O₂ and 40µl enzyme extract, and the initial linear rate of decrease in absorbance at 240 nm was used to calculate the activity. The activity of catalase was calculated by using extinction co-efficient 43.6 M⁻¹ cm⁻¹ and expressed as µM min⁻¹ mg⁻¹ FW (Chakrabarty et al.,2009).

3.10. Statistical Analysis

Experimental data of physiological and biochemical sections of this research were subjected to a One Way ANOVA (analysis of variance) using Sigma Plot 11.0 software and all pairwise comparison of the mean responses to the different treatment groups were performed by Tukey Test at p<0.001.

Chapter 4

Results

4.1 Growth of Root length and shoot length.

The root and shoot length of control, salt treated and salt+AsA treated plant were measured. Salt cause increment in the root and decrement in the shoot of the plant as compared to control. Exogenous spray of AsA caused had no significant.

Table 4: Root and Shoot length parameter of salt treated and salt treated with foliar spray of ascorbic acid.

Parameter	Control	NaCl treated	NaCl + Ascorbic Acid treated
Root Length (cm)	6.0±0.1	10±0.1	8.0±0.1
Shoot Length (cm)	22±0.2	20±0.1	20±0.2



Fig 3: Root and shoot length of A) Control B) NaCl treated C) NaCl + AsA treated

4.2 Leaf Injury

Leaves of control, NaCl and NaCl+AsA treated plants were analysed and found that salt cause chlorosis in leaves. Spray of AsA was significant in suppressing the chlorotic effect of salt on leaves.



Fig 4: Leaf injury in A) 50mM NaCl with treatment of 10mM foliar spray of AsA B) Control irrigated with distilled water and C) 50mM NaCl treated.

4.3 Membrane damage

Cell membrane is primary site of damage during abiotic stress. Membrane damage was measured by percentage electrolyte leakage index (%ELI) and lipid peroxidation.

4.3.1. Percentage electrolyte leakage index (ELI)

Data collected for determining electrolyte leakage in the form of %ELI was analyzed by One Way ANOVA using Tukey's test, showing significant interaction between treatments (p-value <0.001).

Between treatments (i.e., before exposure to salt stress, after exposure to salt stress and after application of ascorbic acid treatments) significant difference was observed. In PBG5 seedlings, damage to tissue on 10th day was 62.73%. There was a slight increase in damage due to ELI in control plants (63.73%) on 20th day. But there was a significant increased damage seen in salt treated plants (77.82) than the plants which were treated with ascorbic acid along with salt (71.10).

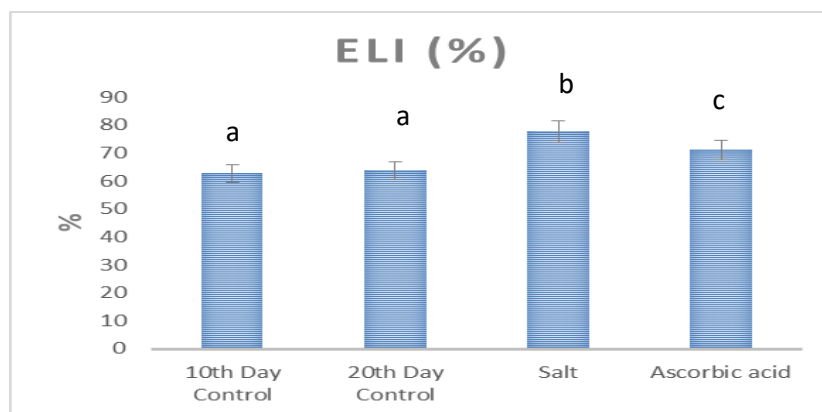


Fig 5: Graph showing %ELI content in chickpea variety PBG5. Comparison of plants at different treatments. a, b, c are significantly different at $P < 0.001$ based on Tukey's test.

4.3.2 Lipid peroxidation

Another factor for determining membrane damage is measurement of MDA content formed due to lipid peroxidation of polyunsaturated fatty acids and it is considered as marker for salt stress.

The main focus of the study was to see whether ascorbic acid spray can improve the salt tolerance or not. Data collected for lipid peroxidation was analyzed by One Way ANOVA with all pairwise multiple comparison using Tukey Test which show significant interaction between treatments (p value < 0.001).

MDA content on 10th day in plants was $65.11 \mu\text{M g}^{-1}$ FW. On 20th day, the MDA content in control decreased significantly to $50.40 \mu\text{M g}^{-1}$ FW. There was significant increase in MDA content in both salt stressed ($81.54 \mu\text{M g}^{-1}$ FW) and ascorbic acid sprayed plants ($73.80 \mu\text{M g}^{-1}$ FW). But significantly less damage in terms of lipid peroxidation was seen in ascorbic acid sprayed plants.

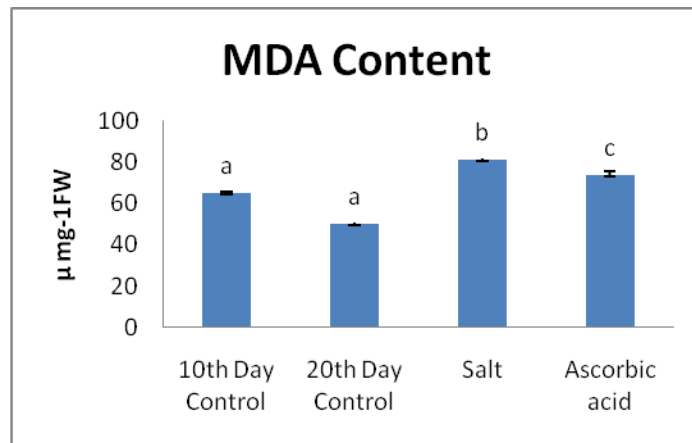


Fig 6: Graph showing MDA content in chickpea variety PBG5. Comparison of plants at different treatments.a, b, c are significantly different at $P < 0.001$ base on Tukey's test.

4.4 Relative Leaf Water Content

%RLWC was analyzed by one way ANOVA using Tukey's test. The analysis showed that there is statistically significant interaction between all the treatments. The RLWC on 10th day in plants was 79.28. On 20th day, %RLWC in control was 78.36. There was significant reduction in salt stressed (63.71 %) as well as ascorbic acid sprayed plants (67.77 %) in terms of % RLWC. % RLWC was better in ascorbic acid sprayed as compared to ascorbic acid sprayed plants.

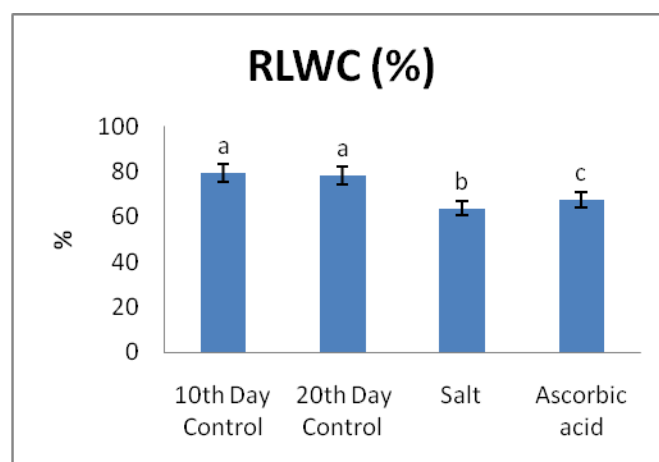


Fig 7: Graph showing RLWC content in chickpea variety PBG5. Comparison of plants at different treatments.a, b, c are significantly different at $P < 0.001$ base on Tukey's test.

4.5 Chlorophyll Content (mg/gm)

In case of chlorophyll a content, there was no significant change in 10th day (.082 mg/gm) and 20th day control (.069mg/gm) readings. But in salt stressed plants (.038mg/gm), significantly less chlorophyll content was seen. In ascorbic acid treated plants (.031mg/gm), there was no significant difference in comparison to salt treated plants. Similar trend was seen in case of chlorophyll b. This clearly indicates that ascorbic acid is not able to influence any change in the chlorophyll content.

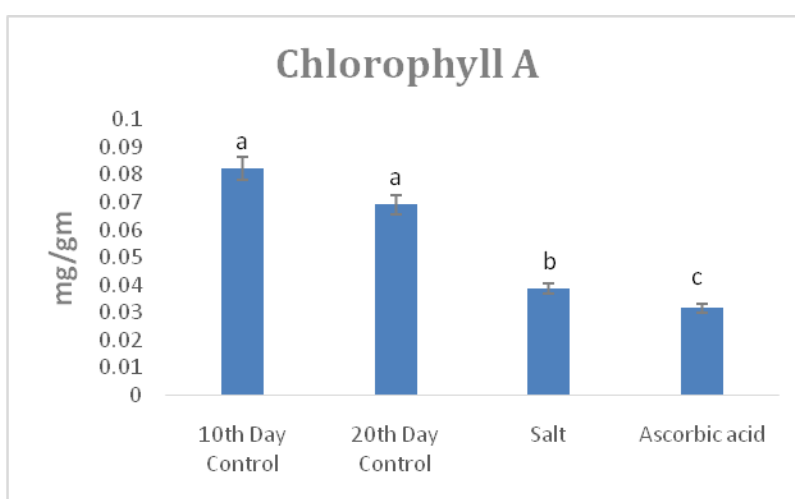


Fig 8: Graph showing Chlorophyll a content in chickpea variety PBG5. Comparison of plants at different treatments. a, b, c are significantly different at $P < 0.001$ based on Tukey's test.

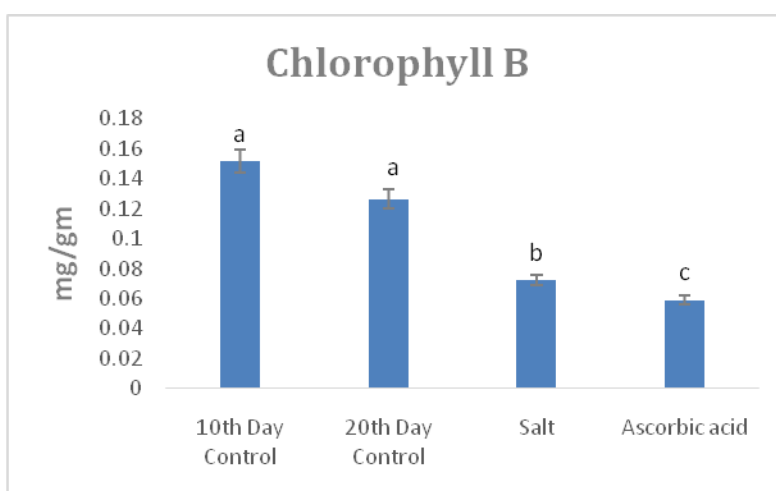


Fig 9: Graph showing Chlorophyll b content in chickpea variety PBG5. Comparison of plants at different treatments.a, b, c are significantly different at $P < 0.001$ base on Tukey's test.

4.6 Antioxidative enzymes

4.6.1 Superoxide dismutase (SOD) ($U \mu g^{-1} FW$):

SODs are a group of enzymes that are involved in the primary line of antioxidative defense by catalyzing the dismutation reaction of superoxide anion radical ($O_2^{\cdot -}$) to oxygen (O_2) and hydrogen peroxide (H_2O_2).

Data for SOD activity was measured as unit enzyme required for 50% reduction of NBT and analyzed by One Way ANOVA using Tukey test. Significant interactions were seen between the treatments (p value < 0.001).

The SOD activity increased ($7.55 U \mu g^{-1} FW$) on 20th day in control plants as compared to 10th day control ($5.56 U \mu g^{-1} FW$) but it is not significant. There was a significant increase in SOD activity in plants exposed to salt stress ($17.94 U \mu g^{-1} FW$) and plants sprayed with ascorbic acid ($12.64 U \mu g^{-1} FW$). This clearly indicates that ascorbic acid is able to influence change in the SOD activity.

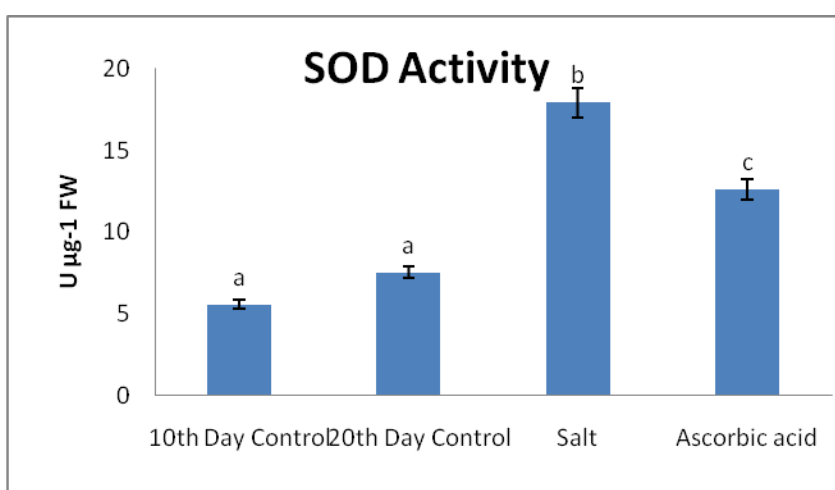


Fig 10: Graph showing SOD activity in chickpea variety PBG5. Comparison of plants at different treatments.a, b, c are significantly different at $P < 0.001$ base on Tukey's test.

4.6.2 Catalase (CAT) ($\mu\text{g min}^{-1} \text{mg}^{-1} \text{FW}$):

Catalase is the tetramericheme containing enzyme, catalyzes the dismutation of two H_2O_2 molecules to water and O_2 . Catalase activity was measured spectrophotometrically and data collected was analyzed by One Way ANOVA using Tukey Test. Significant interactions were observed between treatments (p value < 0.001).

There was a significant difference in catalase activity on 10th day ($0.84 \mu\text{g min}^{-1} \text{mg}^{-1} \text{FW}$) and on 20th day ($0.64 \mu\text{g min}^{-1} \text{mg}^{-1} \text{FW}$). The catalase activity increased significantly in salt stressed plants ($1.53 \mu\text{g min}^{-1} \text{mg}^{-1} \text{FW}$) and ascorbic acid treated plants ($1.32 \mu\text{g min}^{-1} \text{mg}^{-1} \text{FW}$) 10th and 20th day with respect to control plants. Significant decrease in catalase activity in ascorbic acid treated plants were observed.

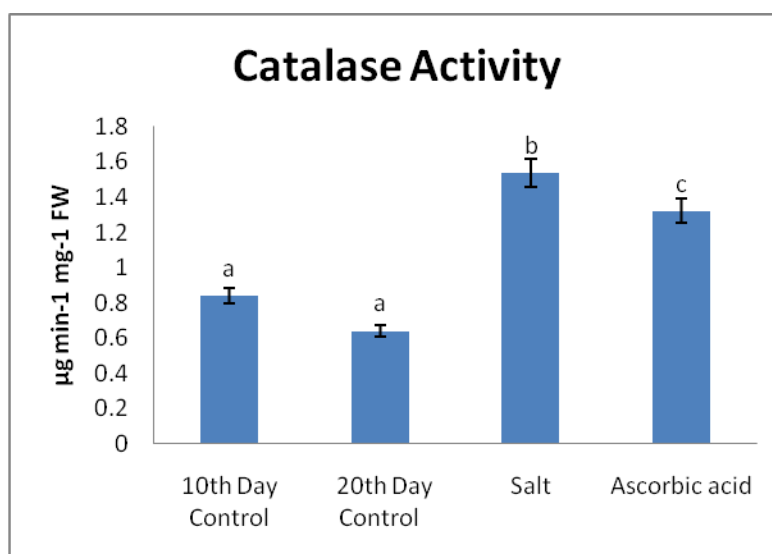


Fig 11: Graph showing Catalase activity in chickpea variety PBG5. Comparison of plants at different treatments. a, b, c are significantly different at $P < 0.001$ base on Tukey's test.

4.7 Biomass (mg)

Biomass on 10th day in plants was 1276 mg which reduced to 1171 mg on 20th day control plants. There was significant reduction in salt stressed (63.71) but no significant reduction in biomass in

ascorbic acid sprayed plants in comparison to control as well as salt stressed plant. Thus ascorbic acid spray did not show any improvement in terms of biomass.

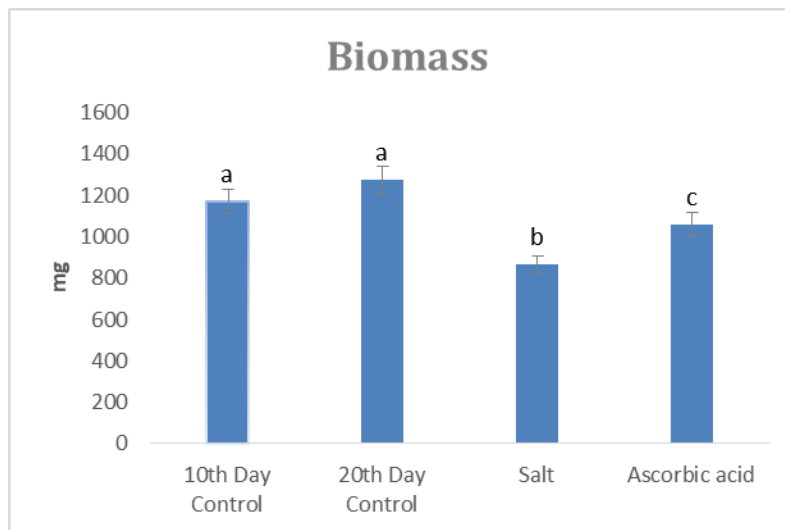


Fig 12: Graph showing biomass in chickpea variety PBG5. Comparison of plants at different treatments. a, b, c are significantly different at $P < 0.001$ based on Tukey's test.

Chapter 5

Discussion

Salinity is a major threat of irrigated agriculture in arid and semi-arid regions, which affects crop productivity. Unlike drought, salinity stress is a complicated phenomenon which includes osmotic stress, specific ion effect, nutrient deficiency etc., thereby affecting various physiological and biochemical mechanisms associated with plant growth and development. Plants have developed various combating mechanisms to cope with the deleterious effects of salinity stress. Among the several approaches to solve the problem of saline soils, foliar spray of ascorbic acid could be one approach to reduce the effect of salinity. The present study evaluates the effect of ascorbic acid spray on plants during salt stress by examining various biochemical parameters.

5. Membrane stability

Cell membranes are one of the first targets of many plant stresses (Shahandashtiet al.,2013). ELI and MDA content were analyzed to check the effect of ascorbic acid on membrane integrity during salt stress conditions.

5.1 Electrolyte leakage Index

Salinity leads to dehydration and increase in the electrolyte leakage (Munns 2002).It can be inferred from the result that ascorbic acid spray improved the membrane stability in response to salinity stress. Similar results were seen in Agami, 2014 on barley.

5.2 Lipid peroxidation

Lipid peroxidation is a well-known parameter for determination of oxidative damage to membranes (Da Costa et al.,2007). The adverse effects of the salt on membranes are results of the accumulating toxic ions and ROS. The ion balance of membranes changed by Na⁺ and Cl⁻ accumulation. These ions replace K⁺ and Ca²⁺ ions, which

have important roles in the functioning of the membrane proteins, under salt stress. On the other hand, ROS, especially the hydrogen peroxide and hydroxyl radicals, damage the membrane lipids and result in lipid peroxidation, damaging the membrane structure and integrity. Lipid molecules, in general, and unsaturated lipids, in particular, are sensitive to oxidation by ROS. Lipid peroxidation, measured as the amount of MDA, is produced when polyunsaturated fatty acids in the membrane undergo oxidation by the accumulation of free oxygen radicals.

There was marked improvement seen in terms of lipid peroxidation by the foliar spray of ascorbic acid. These results are in agreement with the reports of higher increases in the amount of MDA with the increase in salt stress in rice (Mandhania et al.,2006). MDA has been widely used as selection to assess salt injury as criterion in various plants (Jaleel et al.,2007; Ahmad et al.,2011). MDA concentration was found the maximum in plants exposed to stress (Giannakoula et al.,2008). Lipid peroxidation can occur in both chloroplasts and mitochondria (Elstner, 1982; Bowler et al.,1992).

5.3 Relative Leaf Water content

Leaf relative water content (RLWC) is one of the several methods to measure tissue water status. The RLWC represents a useful indicator of the state of water balance of a plant, essentially because it expresses the absolute amount of water, which the plant requires to reach artificial full saturation (Gonzalez and Gonzalez-Vilar, 2001). It can be inferred from the result that foliar application of ascorbic acid improved the water status in response to salt stress and the damage was less than the salt stressed plants. Salt stress had reduced water status due to reduction in hydraulic conductance and ascorbic acid might have enhanced the hydraulic conductance by increasing stomatal conductance.

5.4 Chlorophyll Content

The decrease in chlorophyll concentration might possibly be due to changes in the lipid protein ratio of pigment-protein complexes or increased activity of the chlorophyll degrading enzyme chlorophyllases (Parida et al, 2004). The reduction in leaf chlorophyll content under NaCl stress has been attributed to the destruction of chlorophyll pigments and the instability of the pigment protein complex (Levit, 1980). It is also attributed to the intrusion of salt ions with the de novo synthesis of proteins, the structural component of chlorophyll, rather than the breakdown of chlorophyll (Jaleel et al, 2007). In case of chlorophyll content foliar spray of ascorbic acid is not able to reduce the damage caused by salt stress.

5.5 Antioxidant enzymes

In order to scavenge ROS and to avoid oxidative damage, plants possess an antioxidative system comprising of antioxidative enzymes like, SOD, APX, CAT, GR, and non-enzymes like ascorbate, glutathione (Hernandez et al.,2000; Khan et al.,2002; Bor et al.,2003), Tolerance to NaCl-stress in higher plants correlates to the levels of antioxidant systems and substrates (Koca et al.,2007; Athar et al.,2008) .To overcome the effects of salinity-induced oxidative stress, plants stimulate their antioxidant defense mechanism.

5.5.1 Superoxide dismutase (SOD)

Superoxide dismutase (SOD) is the first enzyme in detoxification process and converts superoxide radicals to H₂O₂ at a very fast rate. It is a critical responsible for the elimination of superoxide radicals are considered to be a key anti-oxidant in aerobic cells. Accumulation of ROS in cellular oxidative stress can lead to the damage of important biomolecules such as membrane lipids, proteins and DNA. In the present study, SOD activity was high in salt stressed plants as compared to ascorbic acid treated plants. High SOD activity is well justified due to high oxidative stress, but less activity in ascorbic acid

treated plants can be attributed to less production of superoxides, low oxidative stress, eventually less synthesis of SOD.

5.5.2 Catalase

Catalase is the major scavengers of H_2O_2 , which is produced through dismutation of O_2^- in peroxisomes, chloroplasts and cytosols (Asada and Takashi, 1987). Previous studies have shown that CAT activity increase during exposure to salinity stress (Ahmad et al.,2004). Again it can be recollected from previous parameter that ascorbic acid treated plants had low SOD activity. Here also, CAT activity was less in those plants as compared to salt treated plants which complement and justify the SOD activity as less H_2O_2 production and less CAT activity. Low MDA content is an example of less lipid peroxidation and formation of less ROS (Becana et al.,2000).

5.6 Biomass

In the present investigation, the responses of chick pea plants to high levels of salinity were reflected by decreases in fresh weight of the whole plant. The stressful environment in the soil solution at a concentration of 50mM NaCl attenuated the fresh and dry matter gain in plants. The inhibitory effects of salt stress on these parameters add more support to the ubiquitous findings of earlier investigations (PérezAlfocea et al.,1993; Hamada, 1996). The reduced plant growth under salt stress conditions could be attributed to the physiological drought induced by the low water potential of the soil solution and osmotic adjustments in plants as a result of increased ionic concentration in their cells, which result in deformation of macromolecules by disrupting their shell or bound water (Schwarz, 1985).

Overall 50mM NaCl was proved to be harmful to chickpea variety PBG5 as reflected by various parameters i.e. Growth and Biomass, leaf injury, ELI, LPO, Chl a and b content. However, foliar spray of 10mM AsA was proved to be beneficial by minimizing oxidative damage in chickpea PBG5.

Chapter 6

Summary

Growth and development of the plant is influenced by various environmental factors like drought, temperature, salt, and high light. When any of these exceed the optimum tolerance, this results in stress to the plant, which in turn affects its developmental, structural, physiological and biochemical processes. In arid and semi-arid regions, salinity in soil or water is one of the most important abiotic factors that limit plant growth and productivity. Sodium chloride is one of the most abundant salts that contribute to soil salinity (Koca et al, 2007). Salinity can affect growth and yield of most crops. High salinity is known to cause both hyper-ionic and hyperosmotic effects in plants, leading to membrane disorganization, increase in activated oxygen species production and metabolic toxicity. It invariably leads to oxidative stress in the plant cell due to higher leakage of electrons towards O₂ during photosynthetic and respiratory processes leading to enhancement in reactive oxygen species (ROS) generation (Asada, 1992). ROS such as hydrogen peroxide, superoxide ions, singlet oxygen, peroxides etc are toxic molecules for plant metabolism. All cellular macromolecules including DNA are damaged due to the deleterious effects of ROS. Plant systems are equipped with enzymatic and nonenzymatic antioxidants such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR), ascorbic acid (AsA), glutathione etc. They minimize the deleterious effects of ROS. Every compartment of the cell contains one or more antioxidants that act on a particular ROS and detoxifies it (introduction or over-expression of selected genes is the promising way to generate stress tolerant plants. Chickpea is highly sensitive to salinity, like many other leguminous crops. Therefore, identifying sources of tolerance to salinity will be of great practical importance. More studies are required to trace the exact tolerance mechanism involved under salinity stress through

foliar spray of Ascorbic acid. After identification of the key mechanism, resistant varieties can be developed by suitable manipulation in order to fulfil growing food demand.

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