CERTIFICATE

I declare that the dissertation entitled “Preconditioning of chickpea seedlings for terminal heat stress; understanding associated mechanism and HSP’s expression” has been prepared by me under the guidance of Dr. Sanjeev K. Thakur, Assistant Professor, Centre for Biosciences, School of Basic and Applied Sciences, Central University of Punjab. No part of this thesis has formed the basis for the award of any degree or fellowship previously.

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CERTIFICATE

I certify that Renu Yadav has prepared her dissertation entitled “Preconditioning of chickpea seedlings for terminal heat stress; understanding associated mechanism and HSP’s expression” for the award of M.Phil degree of the Central University of Punjab, under my guidance. She has carried out this work at the Centre for Biosciences, School of Basic and Applied Sciences, Central University of Punjab.

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ABSTRACT

Preconditioning of chickpea seedlings for terminal heat stress; understanding associated mechanism and HSP's expression

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Keywords: Preconditioning, Mild water stress, lethal temperature stress, Electrolyte leakage, Lipid peroxidation, Antioxidative enzymes, Expression analysis

Chickpea (*Cicer arietinium* L.) is the second most important pulse crop grown worldwide. Changes in the cropping system, competition from other cash crops (wheat) and global warming are pushing chickpea to relatively warmer growing environment. In northern part of country chickpea come across with terminal high temperature stress during reproductive stage which lead to reduced grain yield. Therefore to prevent the plant from incoming heat stress, 11 day chickpea seedling were preconditioned with mild drought stress, then put on recovery for six days and then recovered seedlings were exposed to lethal stress (where temperature was increased step wise from 30˚C to 36˚C). This study revealed that % EL, Lipid peroxidation increased with the increase in temperature while percent TTC reduction and total protein content decreased with the increase in temperature. Antioxidative enzymes provide the major defence against the ROS generated during the abiotic stress, and it was found that activity of SOD, CAT and APX enzyme increased proportionately with the rising temperature. HSP's act as molecular chaperons and are over expressed at both mRNA and protein level in preconditioned seedlings exposed to high temperature stress as compared to non-preconditioned ones. To conclude the whole study, results obtained clearly reveal that preconditioning with drought stress has the ability to improve tolerance above ambient temperature (27˚C $\pm$ 7˚C), thereafter preconditioning did not have any influence in terms of the improvement in membrane damage and level of antioxidants. Higher expression of sHSP's is corroborated with the low expression of antioxidants.

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RENU YADAV

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LIST OF ABBREVIATIONS

S. No.	Abbreviation	Full Form
1.	% ELI	% Electrolyte leakage index
2.	^{0}C	Degree Celsius
3.	ANOVA	Analysis of Variance
4.	APX	Ascorbate peroxidase
5.	APS	Ammonium persulfate
6.	BLAST	Basic local alignment search tool
7.	CAT	Catalase
8.	cDNA	Complementary deoxyribonucleic acid
9.	dNTP	Deoxyribonucleotide triphosphates
10.	ETC	Electron transport chain
11.	EC.NO.	Enzyme commission number
12.	FAOSTAT	Food and agriculture organisation statistical database (united Nation)
13.	FW	Fresh weight
14.	G	Gram
15.	hrs	Hours
16.	ha	Hectare
17.	Hsf	Heat shock factor
18.	HSP	Heat shock protein
19.	HSG	Heat shock granules
20.	ICRISAT	International Crop Research institute for semi arid tropics
21.	MDA	Malonaldehyde
22.	Mg	Miligram
23.	min	Minutes
24.	mL	Mililiter
25.	mM	Millimolar
26.	mt	Million tonnes
27.	NBT	Nitroblue tetrazolium
28.	NCBI	National centre for biotechnology information
29.	PAGE	Polyacrylamide gel electrophoresis
30.	PCR	Polymerase Chain Reaction
31.	PVP	Polyvinylpyrrolidone
32.	PPFD	Photosynthetic photon flux density
33.	RNA	Ribonucleic acid
34.	ROS	Reactive oxygen species
35.	RT-PCR	Reverse transcriptase polymerase chain reaction
36.	RuBisCO	Ribulose bisphosphate carboxylase/oxygenase
37	SDS	Sodium dodecyl sulphate
38.	SOD	Super oxide Dismutase
39.	sHSP	Small heat shock protein
40.	TBA	Thio-barbituric Acid

41.	TBARS	Thiobarbituric acid reactive substances
42.	TCA	Trichloroacetic acid
43.	TTC	2,3,5 Triphenyl Tetrazolium Chloride
44.	TEMED	Tetramethylethylenediamine
45.	U	Unit
46.	V	Volt
47.	µl	Microliter

CHAPTER 1

INTRODUCTION

Plants are sessile organism so they are constantly being exposed to various abiotic factors which include cold stress, drought stress, high light intensity and change in temperature (Mittler, 2006). Abiotic stress is responsible for the average yield loss of 50% in most of the crop plants (Rodziewicz *et al.*, 2014). Due to climate change and global warming, high temperature plays a crucial role on plant growth, and would affect yield (Summerfield *et al.*, 1984; Krishnamurthy *et al.*, 2010). Severity of loss by high temperature depends on the stage of the plant at which stress occur and the intensity of stress. In cereals and legumes high temperatures affect vegetative growth as well as reproductive development (Rodziewicz *et al.*, 2014). It is also speculated that the increases in temperature would have more adverse effects on winter season crops (e.g. chickpea) than the rainy-season crops (Kumar and Rao, 2013).

Legumes and cereals are the major source of nutrition for the population around the world. In India, Chickpea (*Cicer arietinum* L.) is the second most important pulse crop. India contributes about 40% of global production of chickpea from 67% of the global area. (Krishnamurthy *et al.*, 2010). Chickpea fulfils a remarkable nutritional requirement of population of developing nation as it is a rich source of carbohydrate (40-59%), proteins (13.5-13.7%), vitamins, minerals, polyunsaturated fats and dietary fibres (Gangola *et al.*, 2013). Although India being the largest chickpea producing country, a deficiency occur in domestic production and the demand is fulfilled through import from other countries.

Chickpea is a Rabi crop, optimum temperature for growth of chickpea seedling is 23-30˚C (Singh, 1997). Abiotic stresses prevent its productivity, amongst them temperature is the most important which limit chickpea yield (Summerfield *et al.*, 1984; Basu *et al.*, 2009)**.** Because of inclusion of chickpea in new cropping systems it is exposed to high temperature (Devasirvatham *et al.*, 2012). During reproductive stage, terminal drought and heat stresses (>35˚C) are major limitation to chickpea production, which severely affects membrane stability, fertilization, seed germination, photosynthesis, respiration, fruit maturation, quality of seeds and yield (Basu *et al.*, 2009).

Heat stress sensing take place at the plasma membrane of cells, which cause oxidative damage and cause lipid peroxidation (Ruelland and Zachowski,

2010). Oxidative stress is produced as a secondary stress during the heat stress response, and produce abundant reactive oxygen species (ROS) in plants. ROS are highly reactive which damage proteins, lipids, carbohydrates and DNA. ROS also affect the expression of a number of genes and control many processes like growth, cell cycle, programmed cell death (PCD) and abiotic stress responses (Gill and Tuteja, 2010). Plants possess very efficient enzymes (superoxide dismutase, SOD; catalase, CAT; ascorbate peroxidase, APX; glutathione reductase, GR; guaiacol peroxidase, GPX and glutathione-S- transferase, GST) which work in unison to protect plant cells from oxidative damage. Among various antioxidant enzymes, ascorbate peroxidase (APX) and catalase (CAT) primarily act as the scavengers of ROS under heat stress in plants (Wahid *et al.*, 2007; Timperio *et al.*, 2008). Antioxidative enzyme activities increase with the increase in temperature and thus play an important role in defence against heat stress (Balla *et al.*, 2009; Hasanuzzaman *et al.*, 2013).

In legumes heat stress factor (Hsf), Heat shock protein (HSP) and transcription factors play a central role in acquired thermotolerance. While Hsf serves as the terminal component of signal transduction and mediates the expression of HSP (Wang *et al.*, 2004; Kotak *et al.*, 2007). HSP are conserved proteins, which play a vital role for survival of plants under both normal and extreme conditions by preventing the aggregation of non-native proteins, refolding of denatured ones and removal of harmful polypeptides during stress conditions (Kotak *et al.*, 2007; Timperio *et al.*, 2008). Another important role of HSP is their role in gene regulation and signalling; their synthesis increases above a threshold temperature and varies within different plant species (Burdon, 1993). sHSP's are the most abundant class of HSP found in plants and prevent the aggregation of cellular proteins along with HSP70 and are essential for acquiring stress tolerance (Vierling, 1997).

The important element in preventing the damage from lethal abiotic stress in plants , is their ability to retain an imprint /memory of the previous exposure to mild stressful condition (also known as preconditioning), through the activation of mechanism involving antioxidative enzymes, regulation of gene expression by HSP (Goswami *et al.*, 2010). It may happen due to activation of same kind of defence mechanism in both the heat stress and drought stress.

Chickpea gets over with existing cold stress especially in the late February, but in the month of March there is abrupt increase in the temperature, which further adds extra damage to the maturing crop. Our hypothesis is that Non-lethal stress at seedling stage in chickpea may help in developing the thermotolerance to incoming high-temperature stress at later stages of plants. The present work would help in better understanding of the mechanism underlying how sublethal stress would prevent incoming lethal stress.

CHAPTER 2

REVIEW OF LITERATURE

Chickpea (*C. arietinum* L.) is the widely cultivated species of the genus Cicer and belongs to the subfamily Faboideae of the Fabaceae family. Its genome size is 740Mbp, and it is a self-pollinated diploid crop (2n = 16) (Arumuganathan and Earle, 1991). Among the grain crops, chickpea was first to be cultivated, which date back to the eighth millennium BC (Zohary and Hopf, 1987)**.** It is a major grain legume used for food since ancient times. In India, Gujarat, Andhra Pradesh, Gujarat, Uttar Pradesh, Madhya Pradesh, Gujarat, Maharashtra, Rajasthan, and Karnataka, are the major chickpea producing states contributing 95% production (ICRISAT).

2.1. Area and Production

Chickpea was originated in Turkey but later on its cultivation got shifted to other parts of the world, which includes 45 countries: South Asia, West Asia, North Africa, East Africa, North Africa, North America, South America, South Europe and Australia. India accounts for the largest production of chickpea in the world followed by Turkey. In 2012, total area under cultivation of Desi chickpeas and Kabuli was 9,701,078 ha and 2,007,200 ha from 7,136,000 and 1,273,300 Mt respectively (FAOSTAT 2010-11). From past few years there has been a considerable shift in the cultivation of chickpea from north India to central and southern India due to competition from wheat-paddy cropping system and changed environmental condition.

2.2. Major climatic factors affecting chickpea production

In Indian subcontinent, during reproductive stages, chickpea experiences cool (5–8˚C) and frosty nights (0 to 18˚C) in the early vegetative stage and warm (20–27˚C) to hot (>38˚C) air temperature during the day (Summerfield *et al.*, 1984; Berger and Turner, 2007). Productivity of chickpea is constrained by several abiotic stresses (Singh;1997; Gaur *et al.*, 2008). Among various factors, temperature is one of the most important abiotic factor responsible for crop growth which may limit chickpea yield (Basu *et al.*, 2009). In general, the winter season food legumes (lentil, peas, faba bean and chickpea) are more prone to heat stress than warm season legumes (cowpea, soybean, groundnut, pigeon pea, and mung bean). Krishnamurthy *et al* (2010) described distribution of chickpea globally on the basis of climate analysis and showed that the present area of chickpea crop

has declined due to change in climatic condition and increase in temperature and may lead its extension to cooler areas. Genetic base of chickpea is low as compared to other crops is another reason high-temperature has a detrimental effect on growth and reproductive physiology (Abbo *et al.*, 2003). In north India, yield of chickpea decreased by 301 kg ha^{-1} in Haryana and 53 kg ha^{-1} in Uttar Pradesh per 1˚C increase in seasonal temperature (Devasirvatham *et al.*, 2010).

2.3. Heat stress/injury

2.3.1. Cell membrane permeability

Terminal heat stress is a major factor affecting chickpea production that occur during the reproductive stage and have a detrimental effect on growth and reproductive physiology which lead to reduced yield.

Heat stresses disrupts the membrane and make the lipid bilayer more fluid by either denaturation of proteins, which increase the permeability of membranes and cause increased loss of electrolytes (Maestri *et al.*, 2002). During high-temperature stress, total lipid content in membranes decreased to nearly one-half and the ratio of unsaturated to saturated fatty acids decrease to one-third of the levels at normal temperatures (Qu *et al.*, 2013). Membrane fluidity caused by increased temperature can activate the expression of HSP genes (Horvath *et al.*, 2012). Nakamoto and Vigh (2007) summarise that small heat shock proteins play an important role in membrane quality control and are associated with membrane upon stress (Maestri *et al.*, 2002). Hence, membrane fluidity is considered as a sensing device of heat (Ruelland and Zachowski, 2010).

2.3.2. Effect of heat stress on physiology

Total chlorophyll content and rate of photosynthesis are important physiological parameters in plants. Heat stress in chickpea directly affects photosynthesis including photosystem II in chickpea. The rate of photosynthesis decreases with an increase in temperature due to a reduction in source and sinks activities, which lead to a severe reduction in economic yield and harvest index (Devasirvatham *et al.*, 2012). High-temperature change the activities of carbon metabolizing enzymes, mainly the RuBisCO (Ribulose-1,5-bisphosphate carboxylase/oxygenase) thereby change the regeneration rate of RUBP (Ribulose

1,5-biphosphate) by the interruption of electron transport and inactivation of the oxygen releasing enzymes of Photosystem II. Heat shock also reduces the amount of photosynthetic pigment, soluble proteins, RuBisCO binding proteins (RBP), large subunit (LS) and small subunits (SS) of RuBisCO (Wahid *et al.*, 2007).

2.3.3. Effect of heat stress on reproductive phase and yield

During the reproductive phase in legumes, heat stress is associated with inadequate pollination, abscission of flower buds, flowers and pods with considerable yield loss (Summerfield *et al.*, 1984). The intensity of high-temperature stress of 35˚C during reproductive development would lower the yield. Duration of anthesis and seed set which are important for the development of organs are critical stages for exposure to heat stress (Devasirvatham *et al.*, 2010).

2.4. Oxidative stress and antioxidants

Abundant reactive oxygen species (ROS) are produced in plants in response to the abiotic stress which are highly reactive and cause damage to DNA, proteins, lipids and carbohydrates which ultimately results in oxidative stress. ROS comprises both free radicals ($OH^{\cdot}$ Hydroxyl radical; $HO_2^{\cdot}$ Perhydroxy; $RO^{\cdot}$, alkoxy radicals and $O_2^{\cdot -}$**,** superoxide radicals) and non-radical forms ($^1O_2$, singlet oxygen and H_2O_2 hydrogen peroxide). ROS are generated at plasma membrane level or extracellularly in apoplast in plant. In chloroplasts, photosystem I and II are the major sites for the production of $^1O_2$ and O_2. In mitochondria components of electron transport chain (ETC) ; complex I, ubiquinone and complex III of electron transport chain (ETC) are the primary sites for the generation of O_2 (Gill and Tuteja, 2010). Thus, ROS are considered as cellular indicators of stress and act as secondary messengers involved in the stress-response signal transduction pathway. Under normal growth conditions, the production of ROS in cells is low (240 μM $s^{-1}O_2^-$ and a steady-state level of 0.5 μM H_2O_2 in chloroplasts) whereas during stresses cell enhance the production of ROS (240–720 μM $s^{-1}O_2^-$ and a steady-state level of 5–15 μM H_2O_2) (Mittler, 2002). ROS accumulation during stress is counterbalanced by antioxidative enzyme systems that include (CAT; Catalase, APX; Ascorbate peroxidase, SOD; Superoxide dismutase, GR; Glutathione reductase, GPX; Glutathione peroxidase, GPX; GPOX; Guaiacol

peroxidase, and GST; Glutathione-S- transferase). Antioxidative enzyme scavenge ROS and control the series of uncontrolled oxidation (Gill and Tuteja, 2010).

2.4.1. Superoxide Dismutase (SOD) (EC.NO: 1.15.1.1): SOD is found in almost all cellular compartments including the water–water cycle in chloroplasts. It provide the primary line of defense in plant stress tolerance and against the toxic effects of increased levels of ROS. SOD catalyse the dismutation of $O_2^{\cdot -}$ into oxygen and hydrogen peroxide. It removes $O_2^{\cdot -}$ and hence decreases the risk of OH formation via the metal catalysed Haber-Weiss-type reaction. SODs are classified by their metal cofactors it contains into three types: copper/zinc (Cu/Zn-SOD), manganese (Mn-SOD) and the iron (Fe-SOD), which is localized in different cellular compartments. Isoforms of SOD are located in the different compartment of the cell. Fe-SODs are located in the chloroplast, Mn-SODs in the mitochondria and peroxisome, and Cu/Zn-SODs in the chloroplast (Maestri *et al.*, 2002)**.**

$$O_2^{\cdot -} + O_2^{\cdot -} + 2H^+ \longrightarrow 2H_2O_2 + O_2$$

2.4.2. Catalase (CAT) (EC.NO: 1.11.1.6): CAT is present in peroxisome and is tetrameric heme containing enzymes which dismutate H_2O_2 into H_2O and O_2 and is indispensable for ROS detoxification during stressed conditions. H_2O_2 generated in peroxisomes is removed by oxidases enzyme involved in β-oxidation of fatty acids, photorespiration and purine catabolism. CAT has the highest turnover rates for all enzymes

$$H_2O_2 \longrightarrow H_2O + {}^{1}/_{2}O_2$$

2.4.3. Ascorbate Peroxidase (APX) (EC.NO: 1.11.1.11): APX family consists of at least five different isoforms. Different isozymes of APX are found in the cytoplasm of the plant cell and some of its organelles. It detoxifies peroxide such as H_2O_2 using ascorbate as a substrate. It is an important component of glutathione-ascorbate cycle. APX has a higher affinity for H_2O_2 (mM range) than CAT and POD (peroxidase) and play an essential role in the ROS generated during stress.

$$H_2O_2 + \text{Acsorbate} \longrightarrow 2H_2O + \text{Dehydroascorbate}$$

The different affinities of APX (μM range) and CAT (mM range) belongs to two different classes of H_2O_2 suggest that they belong to two different classes of H_2O_2 scavenging enzymes. APX causes the fine modulation of ROS for signaling, whereas CAT removes excess ROS during stress (Gill and Tuteja, 2010). Heat stress causes the oxidative stress which induces the expression of antioxidative enzymes and HSP; thus there exist an interactive mechanism between heat and oxidative stress (Kotak *et al.*, 2007; Al-Whaibi, 2011).

2.5. Heat Shock Proteins (HSP's): Master Players for Heat Stress Tolerance

Heat stress and other abiotic stresses induce the heat shock gene expression which triggers some mechanisms of defense that was not expressed under normal conditions. Genotypically it result in an increase in the synthesis of protein groups called ''heat-shock proteins'' (HSP) or ''Stress-induced proteins'' (Al-Whaibi, 2011). High temperature induces constitutive expression of HSP which protect intracellular proteins from denaturation maintain their stability and help to return to equilibrium during recovery. Thus, HSP acts as molecular chaperones (Timperio *et al.*, 2008; Hasanuzzaman *et al.*, 2013).

HSP were first discovered in the salivary glands of fruit flies (*Drosophila melanogaster*) in response to heat shock (Ashburner and Bonner, 1979). *Invitro* presence of HSP in higher plants was discovered using cell culture technique in tobacco and soyabean. Ten new proteins were found, when soyabean was subjected to 4 h treatment, but disappeared after 3 h treatment at 28 °C (Barnett *et al.*, 1979; Al-Whaibi, 2011). HSP's are expressed at certain developmental stage of plants like during embryogenesis, microsporogenesis, seed germination and fruit maturation (Lubaretz and zur Nieden, 2002).

2.5.1. Genes involved during heat stress

During pod filling and seed developing stage of the crop, terminal drought occurs which is a major limitation to chickpea production. Using functional genomics, the various stress-responsive mechanisms in plants were found. In eight diverse chickpea cultivars about ten abiotic stress responsive genes were partially amplified using gene specific primer pairs, like AKIN (SNF-1 related protein kinase), AMADH (Aminoaldegyde dehydrogenase), DHN (Dehydrin gene),

DREB (Dehydration response element binding protein), CAD (Cinnamyl alcohol dehydrogenase), EREB (Ethylene-responsive element binding protein), LEA (Late embryogenesis abundant), SAM (S-adenosylmethionine synthetase), and STPK (Serine/threonine protein kinase) (Roorkiwal and Sharma, 2011; Jain *et al.*, 2013). The evolutionary response of HSP's expression with cellular resistance to high temperature, formulate that HSP protect cells from the damaging effects of high temperature, and their accumulation lead to increased thermotolerance (Sorensen *et al.*, 2003).

2.5.2. Classes of HSP

All HSP's are characterized by the presence of a carboxylic terminal domain called heat shock domain (Helm *et al.*, 1993). In plants, HSP are classified in to five classes according to their approximate molecular weight, amino acid sequence homology and function. (1) Hsp100 family (2) Hsp90 family (3) Hsp70 family (4) Hsp60 family and (5) small HSP family (Al Whaibi, 2011).

2.5.3. Mechanism of HSP

Heat stress induces the expression of HSP by using conserved heat shock elements (HSEs) located in the promoter region which initiates transcription in response to heat. The cis-acting elements i.e; HSE consist of the palindromic nucleotide sequence (5'AGAANNTTCT3') which act as binding site for heat shock factors (HSF's) (Baniwal *et al.*, 2004). The transcription of HSP genes is under the control of regulatory proteins called heat stress transcription factors, located in the cytoplasm in an inactive state, which are considered as transcriptional activators for heat. HSF's are constitutively expressed in most of the plant species. In the absence of heat stress, HSP exist as monomer and is bound with HSP70 in the cytoplasm. During heat stress (increase in temperature), HSP70 dissociates from monomeric HSF and then HSF enters into the nucleus and associate into trimers and bind to specific sequence elements in DNA called HSEs. HSF's recruit other transcriptional components, resulting in gene expression within minutes with increased temperature (Baniwal *et al.*, 2007; Wahid *et al.*, 2007). In the absence of stressing factors, HSF's are present in the cytoplasm as single and free as there is no binding activity with DNA, but when stress starts factors aggregate in triplet and accumulate in the nucleus (Wang *et al.*, 2004). Unlike animals and yeasts, which

have four or fewer HSF's, plants have multiple copies of these genes (Baniwal *et al.*, 2004). HSF's that are heat inducible depend on the time and intensity of the stress (Kotak *et al.*, 2007).

2.5. Small heat shock protein

sHSP proteins are produced in response to high-temperature stress and have a molecular mass of 15 to 42 KDa. Plants exhibit the greatest diversity of sHSP. They accumulate over 1% of total leaf proteins (Waters, 1995).

2.5.1. Classes of sHSP

sHSP are arranged in six classes based on DNA sequence similarity, immunological cross-reactivity, and intracellular localization. Three classes (classes CI, CII, and CIII) of sHSP are localized in the cytosol or in the nucleus and the other three in the plastids, the endoplasmic reticulum, and the mitochondria (Scharf *et al.*, 2001). All sHSP share a conserved 90-amino acid carboxyl-terminal domain called the alpha-crystallin domain (ACD) or heat shock domain, which is important for chaperon activity (Wang *et al.*, 2004). Formation of large oligomeric structures and conformational changes are associated with the chaperone activity of sHSP. Therefore, the amino-terminal region is not only necessary for oligomerization but also for chaperone activity (Sun *et al.*, 2002).

2.5.2 sHSP production in plants

During normal growth conditions, most sHSP are detected in the vegetative tissues but are rapidly produced in response to heat. The extent of sHSP accumulation depends on the temperature and the duration of the stress period. They are quite stable with half-lives of 30–50 hrs, which support that sHSP are rquired for the recovery as well. They are unique in that they form heat shock granules (HSGs), which are approximately 40 nm in diameter, during long-term heat stresses. HSGs store temporarily sHSP oligomers with denatured protein complexes that disintegrate during the recovery period (Helm *et al.*, 1993; Howarth and Ougham, 1993).

2.5.3. Functions of sHSP

sHSP are unique in that they cause the degradation of the proteins that have unsuitable folding, and their activity is independent of ATP (Miernyk, 1999). sHSP cannot refold non-native proteins, but they can bind to partially folded or denatured substrates proteins, and prevent irreversible unfolding or wrong protein aggregation. Sun *et al* (2002) suggested that during stress condition mitochondrial sHSP protect cellular proteins by scavenging ROS and maintain membrane integrity (Nakamoto and Vigh, 2007).

2.6. Preconditioning of plants against abiotic stress

Preconditioning is a phenomenon in which plants pre-exposed to some abiotic stress will survive when they are later exposed to some other abiotic stress that would be lethal to them (Hong *et al.*, 2003). Very few studies have been conducted on preconditioning with abiotic stress. This concept has been applied to some turfgrasses and cedar seedlings. Preconditioning of a crop is popular way to avoid abiotic stress as there is activation of similar kind of mechanism by two different stresses (Ladjal *et al.*, 2000). Dehydration and high- temperature stress induce activation of signalling pathway, which are interconnected and hence can influence each other defence mechanism.

2.7. Objective

The present study was carried out with the following objectives:

To study the effect of various levels of heat stress (30˚C, 32˚C, 34˚C, and 36˚C) in chickpea at seedling stage, mechanism associated with heat tolerance and HSP expression.

CHAPTER 3

MATERIAL AND METHODS

Plant material: Chickpea (*C. arietinum* L.); released variety PBG1 and PBG5 were used to study the preconditioning response on membrane damage, antioxidative enzyme activity and sHSP gene expression at different temperature. The germplasm consisting of two varieties PBG1 and PBG5 were procured from Punjab agriculture university, Ludhiana.

3.1. Experimental design

To achieve the objective Completely Randomized Block Design was followed.

3.1.1 Experimental setup (Raising seedling and preconditioning treatment)

Initially, healthy seeds were picked and sown in thermocol pot, 9 cm x 7cm containing 200 gm of sand. Moisture content of sand was determined. After germination (72 hours) 16/8 hrs (light/dark) photoperiod was provided. The optimum condition (temperature 27˚C) was maintained in a climate-controlled chamber, illumination was provided by cool-white fluorescent lamp with photosynthetically active radiation (PPFD) of 200µmol 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 to have enough biomass (Hoagland and Arnon, 1950). On 11th day, shoot samples were harvested and preceded for electrolyte leakage, lipid peroxidation, TTC reduction, total proteins, antioxidative enzymes activity and RNA expression analysis. On the same day, the seedlings were exposed to mild water stress condition by withholding irrigation for a period of three consecutive days. On 14th day samples from control and treatment were harvested and previously mentioned parameters were repeated. The remaining samples were put in to recovery by irrigating it for another five days. After recovery again on 20th day, samples from the shoot of control and treatments were harvested and same assays were repeated to check the damage to the system. On 21st day control (non-preconditioned) and treated + recovered (preconditioned) seedlings were exposed to differential lethal heat stress of 30˚C, 32˚C, 34˚C, and 36˚C for 12 hours in growth chamber with a photoperiod under 1200 lux light intensity . Third or fifth leaf from the top of the plants was sampled from the control plant kept at 27˚C. After 12 hours of lethal exposure of heat stress the experiment was terminated and status of different parameters was checked by harvesting

samples. Experiment for all parameters for both varieties and tissues were performed in triplicates.

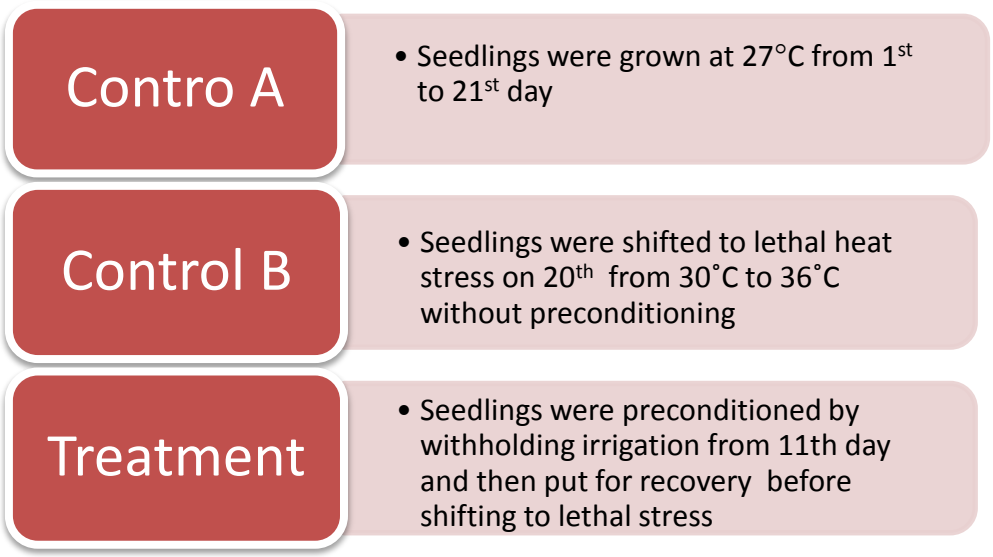

Fig 3.1.Experimental design

3.1.2. Percent seed germination of PBG1 and PBG5 Variety: 100% germination was recorded to check the seed viability of two genotypes. The shoot samples harvested were processed for fresh matter analysis and expression analysis using various parameters.

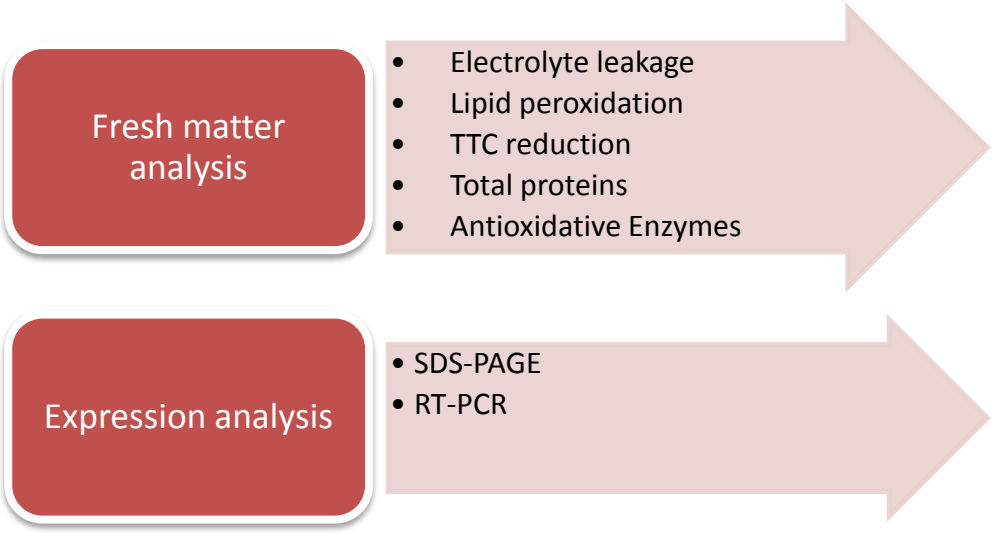

Fig 3.2.Fresh matter and Expression analysis of chickpea seedlings

3.2. Fresh matter analysis:

3.2.1. Membrane Damage

3.2.1.1. Electrolyte Leakage index (Lutts *et al.*, 1996)

The fifth leaf from the top of the seedling was sampled for membrane damage test for electrolyte leakage. 50 mg of shoot tissues were harvested from seedlings and washed three times to remove surface adhered electrolytes and immersed in 10 mL distilled water and kept at 25˚C for 24 hours, and conductivity (L1) was recorded. After this, the samples was completely damaged by boiling it for 20 minutes or autoclaving it at 120°C for 20 minutes. Final conductivity (L2) was recorded with conductivity meter and electrolyte leakage was calculated as EL (%) = (L1/L2)*100.

3.2.1.2. Lipid Peroxidation (Heath and Packer, 1968)

Perturbations in membrane composition lead to oxidative stress further which illustrates the membrane damage. MDA (malondialdehyde) content formed is a product of lipid Peroxidation. Shoot samples were harvested and homogenized in 0.1% TCA (SRL, India) using chilled pestle and mortar. Homogenized material was centrifuged at 15000 g for 5 minutes at 4°C. To 1mL of supernatant, 4 mL of 0.5 % TBA (Thiobarbituric acid – prepared in 20% TCA) was added. The glass tubes containing a mixture were incubated for 30 minutes at 95°C. Reaction was stopped by placing the tubes immediately in ice bath after incubation. A light pink color appeared which was read at 532 nm and a non-specific absorbance was recorded at 600 nm, which was subtracted from a specific value of absorbance at 532 nm. The final MDA content formed was calculated by using extinction coefficient 155$mMol^{-1}cm^{-1}$and expressed as $nMol^{-1}cm^{-1}$MDA g^{-1}fresh weight.

3.2.2. Cellular respiratory capacity using TTC reduction method (2,3,5-triphenyl tetrazolium chloride) (Steponkus and Lanphear, 1967)

TTC reduction was performed on shoot tissues from seedlings from all stages (Control, Non-lethal treatment of drought for 3 days, Recovery and Lethal treatment). 50 mg of tissue was taken and processed by taking six replicates for each treatment. Three replicates of each were taken in glass tubes and immersed

in 1 mL of water and boiled for 2 minutes at 95°C (Heated samples). Heated and normal samples then incubated in 0.6 % TTC (in 0.1M phosphate buffer (pH 7.6) for 24 hours followed by decantation of excess of dye after 24 hours. The samples were washed with distilled water 2-3 times to remove excess dye and immersed in 3mL of 98 % ethanol and incubated at 80˚C for 20 minutes to extract formazan formed. The extracted formazan was read at 530 nm. TTC reduction was demonstrated as percent reduction which was calculated as TTC reduction (%) = (T1-T2)/ (C1-C2)*100. In which, T1 - Treated samples, T2 - Treated heated samples, C1 – Normal control and C2 – Heated control.

3.2.3. Total proteins (Bradford, 1976)

50mg shoot samples was homogenized in 2 mL ice-cold 100 mM phosphate buffer with pH 7.8, containing, 0.5 M EDTA with pH 8.0 (Ethylenediaminetetraacetic acid), 20 mM β-mercaptoethanol, triton x-100 and 80% glycerol. Samples were centrifuged (Remi compufuge) at 15,000 rpm; 4˚C for 30 min. Protein estimation was done using Bradford method.

3.2.4. Antioxidative Enzyme extraction

All steps were carried out at chilled conditions of 0-4˚C. 50 mg shoot sample were homogenized in chilled pestle and mortar with 2 mL of an ice-cold medium containing 50mM potassium phosphate buffer (pH 7.8), 0.1mM (EDTA), 1% (w/v) polyvinyl-pyrrolidone (PVP). In the case of APX, leaves were homogenized in the presence of 200µl of 5 mM ascorbic acid. The homogenate mixture was centrifuged at 14,000 g for 20 min and the supernatants were used for protein concentration determination and antioxidative enzyme.

3.2.4.1. Superoxide dismutase (SOD) (Dhindsa and Matowe, 1981)

Reagents:

I. 50mM sodium phosphate buffer (pH 7.0)
II. 13mM methionine
III. 25mM NBT
IV. 2 µM riboflavin

Procedure: The 25 mL of 50mM Phosphate buffer contained 35 mg methionine, 4 mg NBT and 7.5 µl Triton X-100. The reaction was started by adding the 10 µl riboflavin and placing the tubes 30 cm from six 15 W fluorescent tubes. The reaction was stopped by switching off the lights and covering the tubes with black paper. Tubes without enzymes developed maximum colour. A non-irradiated reaction mixture served as blank and absorbance was read at 560 nm. One unit of SOD (U) was defined as the amount of enzymes that produced a 50% inhibition in comparison with tubes lacking enzyme. SOD activity was expressed as units per min per gram.

$$\text{SOD (U/gm)} = \frac{\text{Reference O.D -- Treatment O.D}}{\text{Reference O.D}} \times 100$$

3.2.4.2. Catalase (CAT) activity (Badiani *et al.*, 1990)

Reagents:

I. 50mM sodium phosphate buffer (pH 7.0)
II. .1 mM H_2O_2

Procedure: To the cuvette added 860µl sodium phosphate buffer, 100µl of 1mM H_2O_2 and spectrophotometer was set to zero. Reaction was started by adding 40ul of enzyme and absorbance was recorded at 240 nm for 1 min. One unit (U) of Catalase was defined as the amount of enzyme which decomposes 1 µmol of H_2O_2 (extinction cofficient, 39.4$mM^{-1}cm^{-1}$) per min at 25°C at 240 nm.

$$\text{CAT (U/gm)} = \frac{\text{Absorbance}_{0\ min} - \text{Absorbance}_{1\ min}}{\text{Extinction coefficient}}$$

3.2.4.3. Ascorbate peroxidase (APX) activity (Gossett *et al.*, 1994).

Reagents:

I. 50 mM Phosphate buffer (pH 7.8)
II. .2mM Ascorbic acid
III. 20µM H_2O_2
IV. .1mM EDTA

Procedure: To the cuvette added 930 µl of sodium phosphate buffer, 10µl of enzyme extract, 20 µl of EDTA and 20 µl of APX was added and spectrophotometer was set to zero. Addition of 20 µl of H_2O_2 initiated the reaction and absorbance was recorded at 290 nm for 1 min. Extinction coefficient of monodehydroascorbic acid (MDHA) has the value of 2.8 $mM^{-1}cm^{-1}$. 1 unit of APX is defined of MDHA formed min^{-1} g^{-1} of protein.

$$\text{APX (U/gm)} = \frac{\text{Absorbance}_{0\ min} - \text{Absorbance}_{1\ min}}{\text{Extinction coefficient}}$$

3.3. Expression analysis

3.3.1. RNA isolation by Trizol method

Total RNA was extracted using Trizol reagent (SRL), according to Trizol isolation method (Appendix A). The quality of samples was checked on 1.5 % agarose gel containing ethidium bromide and RNA concentration was determined by assessing the absorption at 260/280 nm using thermoscientific Nano drop spectrophotometer (Thermo scientific Nano Drop 2000).

3.3.1.1 Two step RT-PCR analyses

RT-PCR analyses were performed with GeNei^{TM} two step RT-PCR kit. 100 ng of total RNA and 1µl of oligo $(dT)_{18}$ primers were taken and final volume made upto 10µl with nuclease free water. To remove any secondary structure, it was then maintained at 65˚c for 10 min in a dry bath and then kept at room temperature for 2 min. After this 10 µl of the reaction mixture of cDNA synthesis containing 1µl of RNAsin, 1µl of 100mM DTT, 4µl of 5X Assay buffer, 2µl of 30mM dNTP mix, 1µl of M-MULV RT and 1µl of nuclease free water was added. RT-PCR mix was than incubated at 37˚c for 60 min in dry bath, and then incubated at 94˚c for 2 min, quickly placed on ice. This step denatures the RNA-cDNA hybrids (Appendix B). For semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR), beta-actin gene was used as a positive internal control. After the PCR reaction, quality of cDNA was analysed on (1.5%) agarose gel and cDNA was stored at -20˚C.

Primers for RT-PCR reactions were designed from the deduced sequence corresponding to the chickpea (*C.arietineum* L.) gene using Prime 3 primer designing software. Eppendorf gradient thermal cycle PCR in 20 µl reaction was run for each primers at five different temperatures from 55˚C, 57˚C, 58˚C, 59˚C and 60˚C (Appendix C). T_m and number of cycles for each primer were standardized. The T_m of HSP 18.5 was 55˚C for 32 cycles and for HSP 22.7 is 57˚C for 32 cycles.

PCR amplification for all the cDNA obtained was carried out for 25 µl reaction. It contained 2µl cDNA, .5µl 30 mM dNTP, 5µl each of 10µM of forward and reverse primer, .5µl of 0.3U of Taq DNA polymerase (SRL) and 2.5µl of 10X assay buffer. The amplification outline was: initial denaturation for 2 min at 94°C proceeded by 30 cycles of denaturation at 94°C for 45 seconds, annealing was specific for each target gene (Appendix E) and extension for 1 minute at 72˚C. Final extension was allowed for 1 min at 72°C and storage at 4°C (Appendix D). Amplified PCR products were resolved on 1.2% agarose gel.

3.3.1.2. Electrophoresis

About 5µl of the PCR Product was mixed with 1µl of 5X loading dye and loaded onto 1.5% agarose gel along with molecular marker (100bp). Electrophoresis was done at 75 V for 30 min. The buffer used was 1x TAE of pH 8.0. The bands in the gel were visualized using a gel documentation system (Bio-Rad Gel Doc™ XR system). Band percentage was determined using Image analysis version 1.1 software for densitometric analysis.

3.3.2. SDS-PAGE

Leaf soluble proteins were separated by SDS-PAGE with a discontinuous buffer system , having composition of 4% stacking gel, and 12% separating gel, as described by (Laemmli, 1970) using a Mini Protean II Dual Slab Cell (Tarson system) (Appendix E and F). Equal amount of proteins from plants after being exposed to different treatments were loaded. Protein samples were mixed with 5 X SDS gel-loading buffers and denatured by heating at 95°C for 5 min before loaded into the gel. Dalton Mark Standard Mix (14–95 kDa, BioLit™) was used as a ladder to determine the molecular mass of the protein. Electrophoretic

separation was performed at 120 V for 20 min with a constant current of 25 mA, followed by 85 V for 1 hr, till the dye enters the separating gel. Gel was incubated in 20% TCA for 20 min. and then stained with 0.25% Coomassie brilliant blue R-250 staining solution and then kept for destaining overnight. (3:1:6; methanol: glacial acetic acid: water)

3.4. Statistical analysis: Analysis of variance (Two way ANOVA) was performed using sigma plot 12. Software and was used to assess the significance of treatment. Statistical level of significance are represented by * at $P<0.05$, ** at $P<0.01$, *** at $P<0.001$ by ANOVA.

CHAPTER 4

RESULTS

In the first phase of experimentation, two varieties of chickpea were grown under optimum temperature conditions (27˚C and light of 2000 PPFD light and 40% humidity) for 11 days and then exposed to mild drought stress (by withholding the water) for 3 days till plant started showing temporary wilting. Immediately the seedlings were watered for recovery, for almost double the time they were exposed to mild stress. After recovery the seedlings were exposed to lethal temperature stress (30˚C, 32˚C, 34˚C and 36˚C). Then for various parameters related to the extent of damage caused due to elevated stress and parameters responsible for providing the tolerance.

The results obtained during the present course of experiment are shown below parametrically. The main purpose of study was to measure the extent of membrane damage before exposing to the stress, after exposing to the mild drought stress (Preconditioning) after recovery and finally after exposing the preconditioned seedling to increasing high temperature (with every 2 degree rise in temperature). The main objective was whether preconditioning (mild drought stress of three days) can improve the tolerance to heat stress.

4.1. Membrane damage: Electrolyte leakage and lipid peroxidation were used as indicators of direct heat injury of membrane damage and membrane lipid peroxidation in terms of TBARS.

4.1.1. Percent electrolyte leakage index (%ELI)

The data collected in the form of percent electrolyte leakage was analysed by two way ANOVA with all pairwise comparison using Tukey test. Both genotype, PBG1 and PBG5 were significantly different ($P<0.050$) from each other in terms of sensitivity to the heat stress, as the percent electrolyte leakage was more in PBG1 as compared to PBG5, indicating the latter one more tolerant (Fig.4.1.1. A).

In PBG1 variety, individual genotype x treatments data was analysed using one way ANOVA, which revealed the significant differences among the treatment ($P<0.001$) i.e. before exposure, after exposure, and at lethal stress. Data analysis revealed PBG1 sensitive to the increasing temperature treatments, initially at 30°C there was significant damage ($P<0.05$) in terms of %EL in non-preconditioned seedlings whereas no significant change was observed in preconditioned

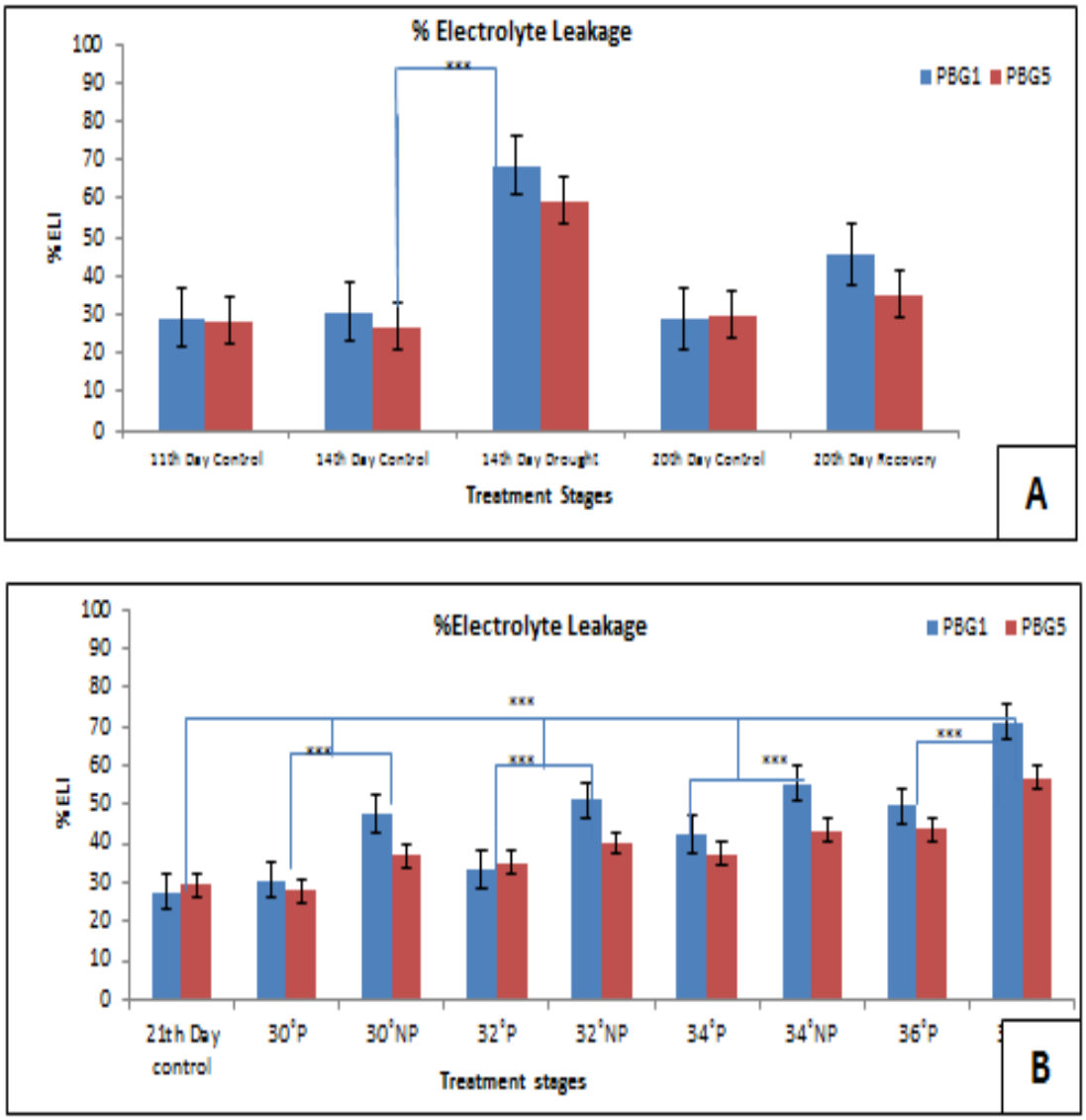

Fig 4.1.1: Electrolyte leakage in terms of %EL index in chickpea seedlings (A) Comparison of %EL of PBG1 and PBG5 till 20th day. (B) Comparison of %EL of PBG1 and PBG5 at different temperature (30˚C, 32˚C, 34˚C, and 36˚C). Statistical analysis was done with ANOVA multiple comparison using (Tukey test) at P<0.05).

seedlings over control. While further two degrees rise in temperature (32°C) had a severe impact on membrane damage in non- preconditioned seedlings whereas no significant change was seen in the preconditioned. Another rise in two degrees (34°C) did the same damage in the non-preconditioned seedlings, but the preconditioned remain unaffected with elevated temperature. At 36°C both preconditioned and non-preconditioned had significant damage over control but non-preconditioned seedlings showed significantly higher level of damage (70%)

as compared to preconditioned one (50%). In PBG5 different temperature treatments 30°C, 32°C and 34°C did not cause any significant change in the electrolyte leakage index. The %EL was at par with the control seedlings. But further rise in temperature (36°C) caused significant damage in the membrane over control. But there wasn't any difference between preconditioned and non-preconditioned seedlings. Here preconditioning did not confer tolerance in terms of preventing the membrane damage (Fig. 4.4.1. B).

It can be concluded from the above finding that PBG1 is more sensitive to elevated high-temperature stress at seedling stage. But preconditioning reduced the membrane damage from 30°C to 34°C. At the same time, 36°C treatment was proven to me more lethal where preconditioning could not prevent the membrane damage. On the other hand, PBG5 was found to be tolerant to high-temperature stress. Change in temperature from 30°C to 34°C did not caused any damage to the membrane. But further rise in temperature (36°C) was found to be lethal where preconditioning could not prevent the membrane damage.

4.1.2. Lipid peroxidation

MDA level is used as an index of lipid peroxidation under stress conditions, and measure the extent of membrane damage. There is more accumulation of saturated fatty acid in the membrane which lead to the formation of ROS under high-temperature stress (Ruelland and Zachowski, 2010). Two way ANOVA of the present data, revealed significant interaction between the various treatment but no significant interaction was seen between the two genotype and genotype x treatments except at 36˚C, where both the genotypes showed significant difference reflecting PBG1 more prone to peroxidation than PBG5 (Fig 4.1.2 A). After preconditioning treatment, when seedling were exposed from ambient 27˚C to the lethal high temperature stress of 30˚C and subsequent two degree rise in further temperature treatments up to 36˚C, preconditioning prevented the increase in the peroxidation of lipids (MDA content), whereas in non-preconditioned seedlings the MDA content was significantly ($P<0.001$) higher (31.2) and increased with proportionate extent of high temperature treatment.

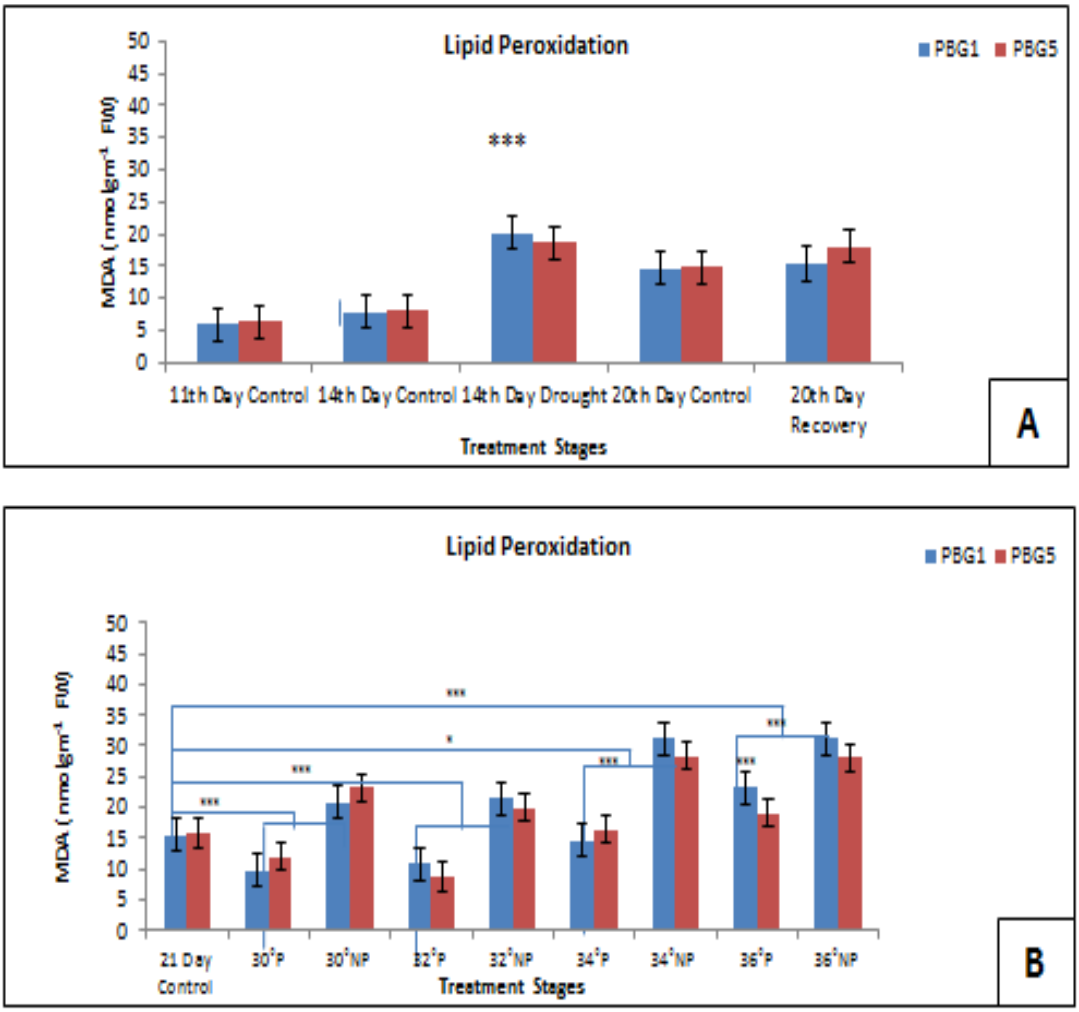

Fig. 4.1.2: TBARS in terms of MDA content (nmol^{-1}MDAg^{-1}FW) formed in chickpea seedlings. (A) Comparison of MDA content formed in PBG1 and PBG5 till 20th day. (B) Comparison of MDA content formed in PBG1 and PBG5 at different temperature. Statistical analysis was done with ANOVA multiple comparison using (Tukey test) at ($P<0.05$).

Individual analysis of PBG1 revealed that preconditioning decreased the lipid peroxidation (22.58) significantly over non-preconditioned seedlings at 30°C, 32°C, 34°C, and 36°C treatment. It is clearly indicating that preconditioning provided defence against the high-temperature stress by reducing the peroxidation of membrane lipids in this genotype. Similar trend was observed in PBG5, preconditioning treatment prevented increase in MDA content/ lipid peroxidation in all the temperature conditions (30°C-36°C) and the content was at par with control. Whereas, in non-preconditioned seedlings, MDA content was significantly higher, and the increase was proportionate with rise in the temperature (Fig.4.1.2.B).

It can be concluded that both the genotypes are equally sensitive to TBARS, but preconditioning treatment provides reduction in the formation of TBARS up to approximate 10 degree rise in the temperature from its ambient.

4.2. Percent TTC reduction

TTC test represents the respiratory activity of the mitochondria and evaluate the mitochondrial electron transport chain and. It is based on the principle of tetrazolium salt reduction to formazan by dehydrogenase activity (Towill and Mazur, 1975). Heat stress affects the photosynthetic and mitochondrial activity (Lin *et al.*, 1985).The present data was analysed using two way ANOVA and it was found that there is significant interaction between genotypes ($P<0.05$), among various treatments ($P<0.001$) and genotype x treatment ($P<0.001$).

The preconditioning treatment in general elevated percent TTC reduction as compared to the control, after recovery a significant increase in the percent TTC reduction was recorded in both the genotypes ($P<0.001$) where PBG5 outperformed PBG1 (Fig.4.2 A). When recovered seedlings of PBG1 were exposed to lethal stress of 30˚C, preconditioned seedlings showed at par results with control but in non-preconditioned seedlings the TTC reduction capacity decreased significantly. In proceeding high temperatures i.e. 32°C,34°C and 36°C a significant ($P<0.001$) down fall of TTC reduction capability was observed. Interestingly, preconditioning improved the TTC reduction capacity over non-preconditioned in all the temperature conditions. Similar trend was observed in PBG5 the only difference observed was its TTC reduction capacity over earlier genotype PBG1, above 32 degrees this genotype performed better than the PBG1.

In brief, it can be concluded that the gradual decrease in the percent TTC reduction in mild stressed seedlings and non-preconditioned exposed to lethal stress can be attributed to high temperature induced dehydrogenase dysfunctioning of mitochondrial electron transport chain. But here also preconditioning helped the seedling to outperform in all the temperature conditions over non-preconditioned.

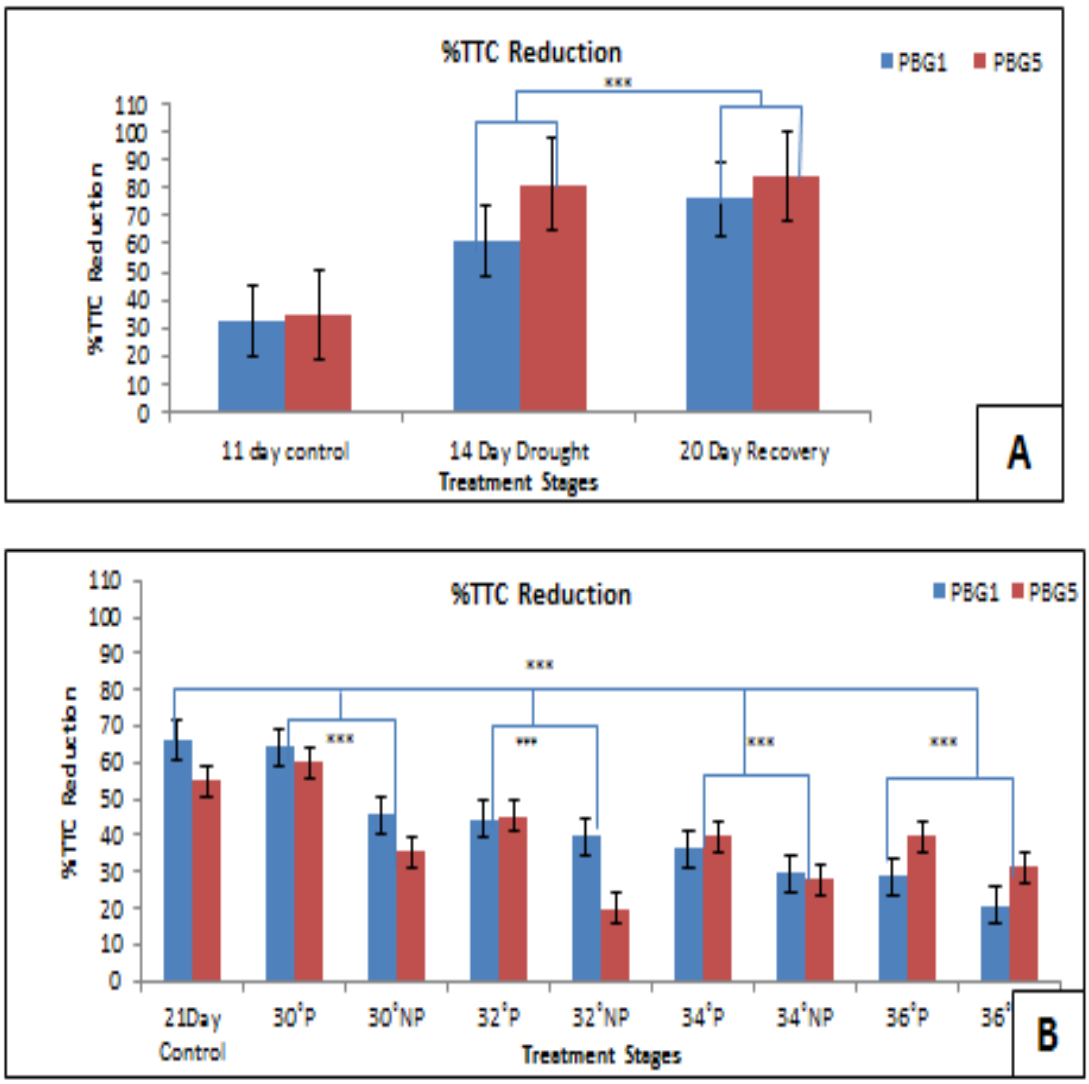

Fig. 4.2: Cellular respiratory capability of in terms of %TTC reduction in chickpea seedlings. (A) Comparison of %TTC reduction in PBG1 and PBG5 till 20th day. (B) Comparison of %TTC reduction in PBG1 and PBG5 at different temperature. Statistical analysis was done with ANOVA multiple comparison using (Tukey test) at ($P<0.05$).

4.3. Total proteins

High-temperature stress causes changes such as protein denaturation or altered protein synthesis but at very high temperature plant synthesize heat shock proteins (Levitt, 1980). Total proteins present in plants were quantitatively measured using Bradford method. It was observed that significant interactions were found between the various treatment ($P<0.001$), genotype x treatment, and the genotypes ($P<0.001$). This clearly indicates that seedling has got differential response to different high-temperature treatments and after preconditioning and

non-preconditioning. Total protein content was significantly high in PBG1 followed by PBG5 (Fig.4.3 A)

After the preconditioning treatment, the protein content decreased to a significant level (P<0.001). PBG1 had more prominent loss of the proteins than PBG5. After recovery similar trend was observed. In general when the recovered seedlings were exposed to different temperatures the protein content decreased gradually with every two degrees rise in temperature. But exceptional rise in protein content was observed at last high-temperature treatment (36°C). In PBG1, total protein content increased with increase in temperature, (P<0.05) from 30°C 34°C both on preconditioned and non-preconditioned seedlings. But at 36°C the total protein content was exceptionally high when compared to lower the

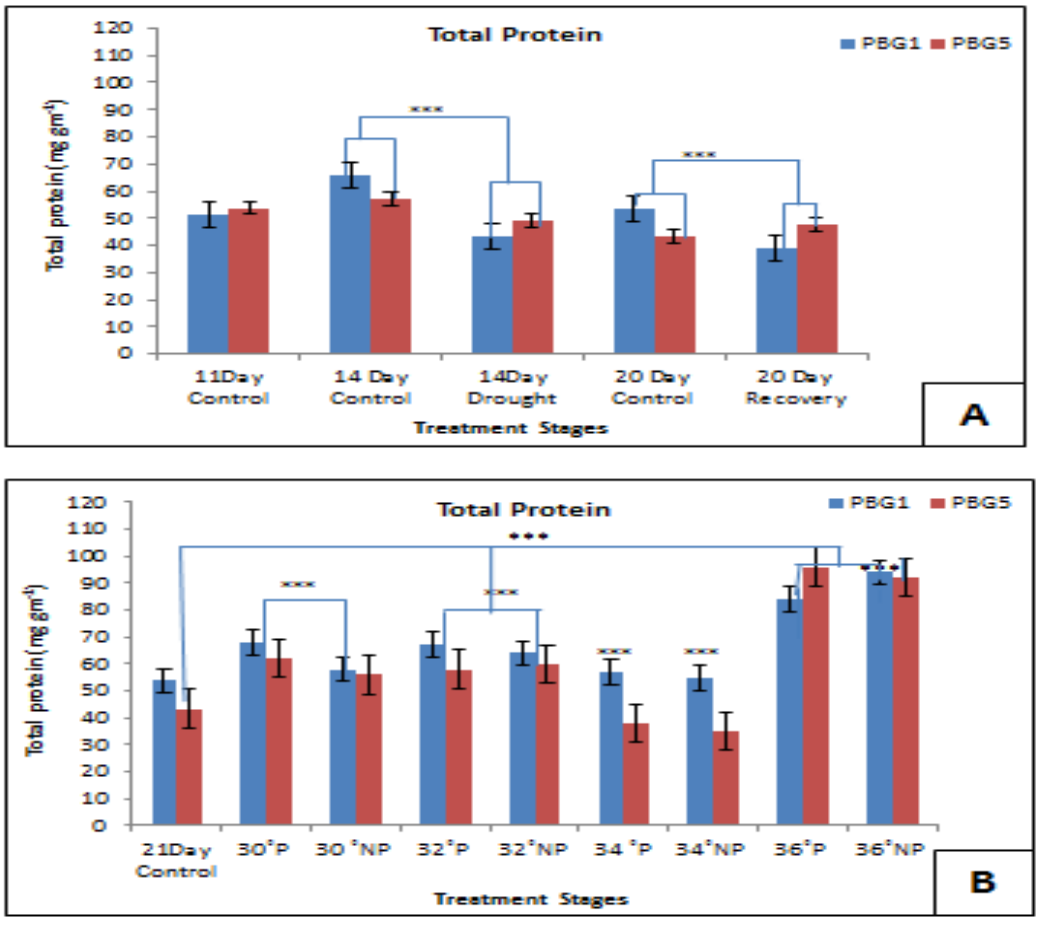

Fig.4.3: Soluble proteins in terms of total protein content in chickpea seedlings. (A) Comparison of total protein content in PBG1 and PBG5 till 20th day. (B) Comparison of total protein content in PBG1 and PBG5 at different temperatures. Statistical analysis was done with ANOVA multiple comparison using (Tukey test) at (P<0.05).

temperature as well as control (P<0.001) (Fig. 4.3.B). Interestingly in preconditioned the protein content was significantly higher than the non-preconditioned. In PBG5, drought reduced the protein content significantly which remained low even after recovery. After exposing to the lethal stress this genotype behaved absolutely identical to PBG1 but as explained earlier the content was lower than the PBG1 (P<0.001). Preconditioning improved (increased) the protein content than the non- preconditioned seedlings.

4.4. Heat temperature and endogenous Antioxidative Enzyme Activity

4.4.1. Superoxide dismutase Activity ($Ugm^{-1}FW$)

The peroxidation of membrane lipid is a result of free radical production which reflects stress-induced oxidative damage at the cellular level. Endogenous antioxidative enzymes play an important role in scavenging the ROS (Reactive oxygen species) generated during the abiotic stress conditions (Jain *et al.*, 2001). SOD provide the primary defence against oxidative stress generated by ROS during the stress. One unit of SOD is defined as the amount of enzyme which causes a 50% inhibition of the SOD-inhibitable NBT reduction. Two way ANOVA revealed significant interactions between treatments and genotypes. It was observed that there was a significant difference between the treatment, genotype, treatment X genotype (P<0.001).

Between the genotypes PBG5 had more pronounced activity than PBG1. In both the genotypes, the enzyme activity increased after exposing the plant to mild stress (P<0.05) (Fig.4.4.1.A). Even after recovery the enzyme activity increase over control. Within genotypes both the genotypes responded in the same manner at 30˚C, but at 32˚C PBG5 increased the SOD activity. A reverse scenario was observed at 36˚C where PBG1 had more activity than PBG5. In PBG1 non-preconditioning had higher SOD activity in all the temperature conditions whereas preconditioning reduced the activity of SOD from (30˚C-36˚C).

Preconditioned seedlings had almost similar/ at par SOD activity at 30˚, 32˚, 34˚C and 36˚C. Similarly in PBG5 preconditioning has more pronounced effect causing decrease in the activity of SOD from (30˚C- 34˚C), while a significant decrease in the activity of SOD was found to occur at 36˚C (P<0.001) (Fig 4.4.1

B). Whereas the activity was much significantly higher in non-preconditioned seedlings (Fig 4.4.1). In short, it can be concluded that preconditioning id negatively regulating the SOD activity

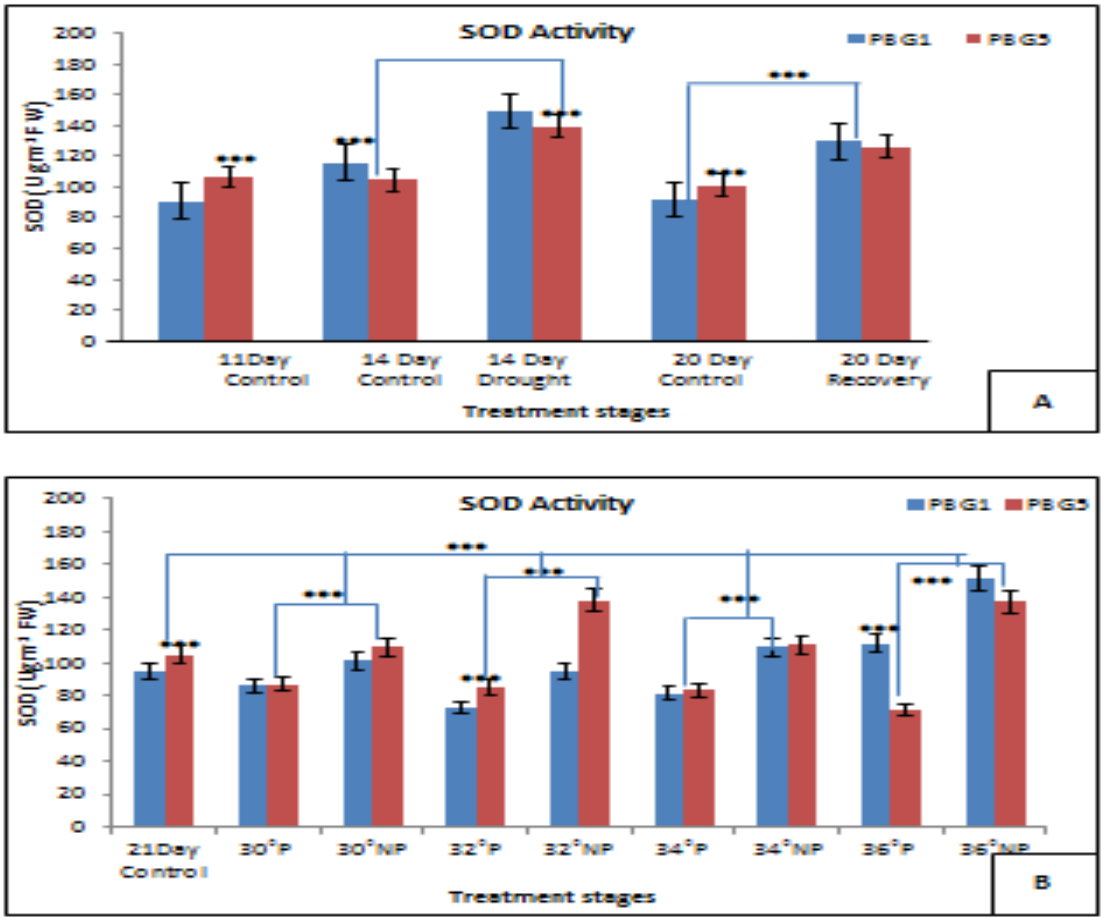

Fig. 4.4.1: Antioxidative enzyme activity in terms of SOD activity (Ugm^{-1} FW) in chickpea seedlings. (A) Comparison of SOD activity in PBG1 and PBG5 till 20th day recovery. (B). Comparison of SOD activity in PBG1 and PBG5 at different temperature. Statistical analysis was done with ANOVA multiple comparison using Tukey test with significance level ($P<0.05$).

4.4.2. Catalase Activity ($Ugm^{-1}FW$)

Catalase is another endogenous antioxidant enzyme which primarily quenches H_2O_2. Significant difference were observed between the genotype, treatments ($P<0.001$) and genotypes x treatment ($P<0.05$). In general CAT, activity increased with preconditioning treatment which decreased when kept for recovery (Fig.4.4.2 A).

After exposure, when analysed for CAT activity, it was observed that with increase in temperature the activity increased significantly in all treatments (P<0.001). PBG5 had much higher activity as compared to PBG1. Non-preconditioned seedlings had much higher activity than preconditioned ones. Up to 32˚C no change in CAT activity was seen but with further increase in temperature (34˚C and36˚C) CAT activity increased to the highest extent. Over all, Preconditioning reduced the CAT activity in both the genotypes but in PBG1 preconditioning lowered the CAT activity at par with control in all the temperature treatments. Concurrently, it was significantly high in subsequent non-preconditioned seedlings. It can be concluded that up to 36 degrees preconditioning did not allowed the lethal temperature to enhance the production of H_2O_2 (Fig 4.4.2.B).

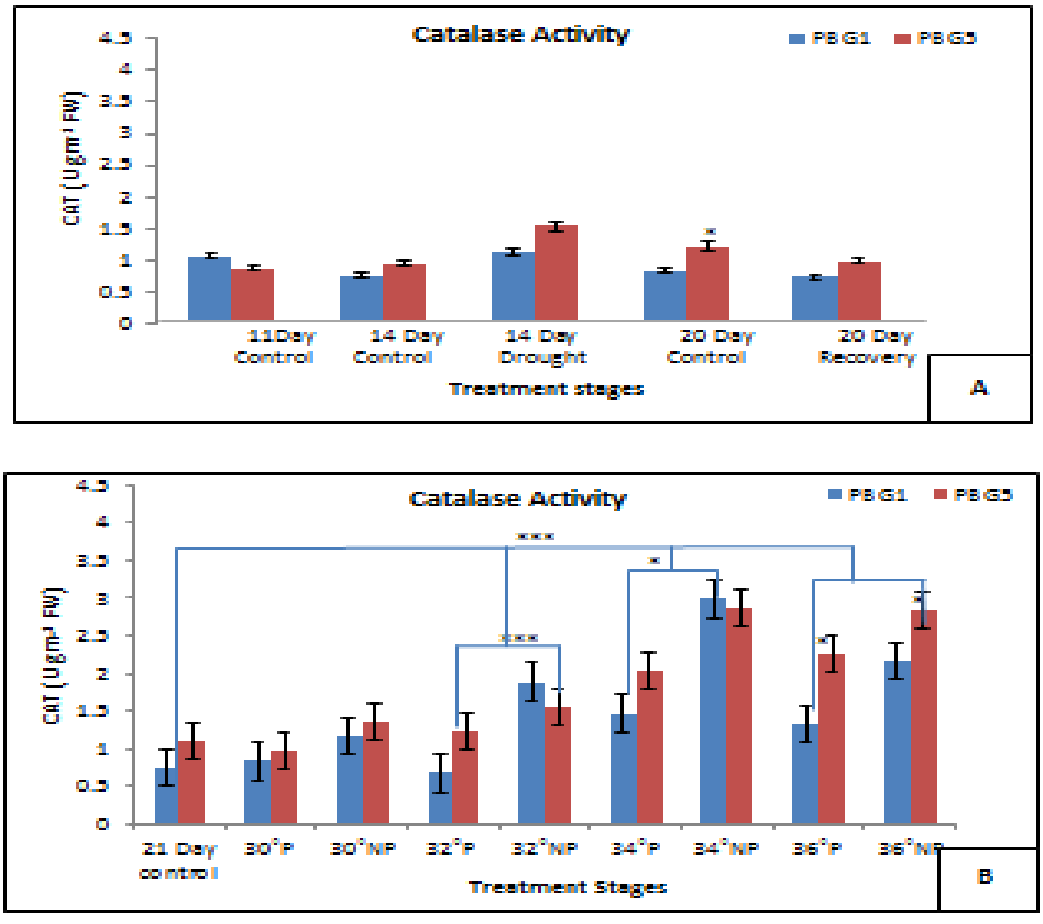

Fig: 4.4.2: Antioxidative enzyme activity in terms of CAT activity ($Ugm^{-1}FW$) in chickpea seedlings. (A) Comparison of SOD activity in PBG1 and PBG5 till 20th day recovery. (B) Comparison of SOD activity in PBG1 and PBG5 at different temperature. Statistical analysis was done with ANOVA multiple comparison using Tukey test with significance level (P<0.05).

4.4.3. Ascorbate Peroxidase Activity

APX is a potential endogenous scavenger of reactive oxygen species which mainly quenches H_2O_2. Data obtained was subjected to two way ANOVA, the analysis revealed significant interactions among genotypes, treatments and genotypes x treatments (P<0.001). Mild drought stress (preconditioning) enhanced APX activity in both the genotypes, further at recovery the activity was high as compared to control (P<0.001) (Fig.4.4.3 A). Similarly when preconditioned and non-preconditioned seedlings were exposed to lethal range of temperature both the genotypes showed enhanced APX activity especially in non-preconditioned where as it was significantly less in the preconditioned ones.

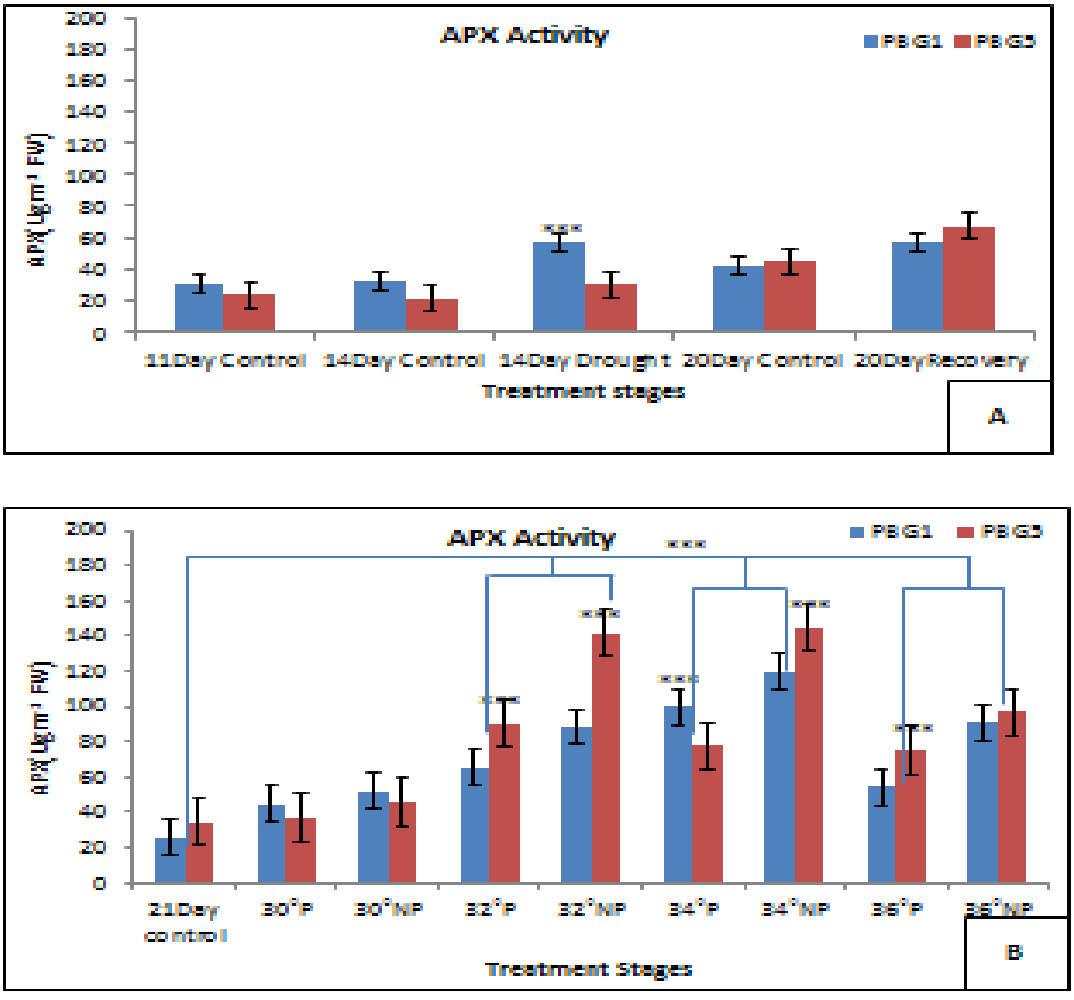

Fig.4.4.3: Antioxidative enzyme activity in terms of APX activity ($Ugm^{-1}FW$) in chickpea seedlings. (A) Comparison of SOD activity in PBG1 and PBG5 till 20th day recovery. (B) Comparison of SOD activity in PBG1 and PBG5 at different temperature. Statistical analysis was done with ANOVA multiple comparison using Tukey test with significance level (P<0.05).

Individually in PBG1, APX activity increased significantly (P<0.001) with increasing temperature up to 34˚C, but a sharp decrease in enzyme activity was seen at 36˚C, though it was less but at the same time higher than control (P<0.001).In PBG5, a steep rise in the enzyme activity was found at 32˚C and 34˚C (P<0.05) and declined at 36°C (Fig 4.4.3 B). In both the genotypes similar trend was seen, pre-conditioning significantly reduced the APX activity compared to non-preconditioned. It can be inferred from the above results that pre-conditioning prevents the oxidative stress.

4.5. Expression Analysis

4.5.1. small Heat Shock Protein (sHSP)

The mRNA expression of small Heat Shock Proteins 18.5 and HSP 22.7 was studied in *C.arietinium* L. seedlings subjected to preconditioning (mild drought) and later exposed to varying levels of heat stress. The results were analysed using Image analysis version 1.1 software for densitometric analysis and compared for constitutive verses induced expression of both the proteins. Beta actin was used as positive control.

sHSP 18.5

In PBG1, after preconditioning (mild drought stress) there was 2.07 folds increase in expression as compared to the control, while after recovery there is 1.12 fold increase in expression as compared to control. After preconditioning, plants were exposed to varying temperature treatments, at 30˚C there was 1.27 folds increase in expression as compared to non-preconditioned. At 32˚C during preconditioning there was 1.49 fold increase in expression as compared to control, but in non-preconditioned plants the expression was 1.87 fold higher than control. Further rise in temperature at 34˚C lowered the expression both in non-preconditioned and preconditioned plants. Interestingly at 36˚C preconditioned plants showed 2.73 fold increase in the expression of sHSP 18.5 as compared to the control and non-preconditioned (Fig 4.5.1.1).

In PBG5, almost similar pattern was seen after preconditioning, 1.61 fold increase in expression was seen as compared to the control and same was recorded after recovery (1.26 fold) as compared to control. At 30˚C preconditioning

improved the expression by 1.11 fold than the non-preconditioned and control. At 32 ˚C preconditioning enhanced the expression by 1.16 fold as compared to the control but non-preconditioned had much higher expression than other treatments. No apparent change was recorded at 34˚C, only preconditioning showed 1.28 fold increase as compared to non-preconditioned. At 36˚C both the genotype showed similar response except that at 36˚C PBG1 showed enhanced response in preconditioned than the non-preconditioned (Fig 4.5.1.2).

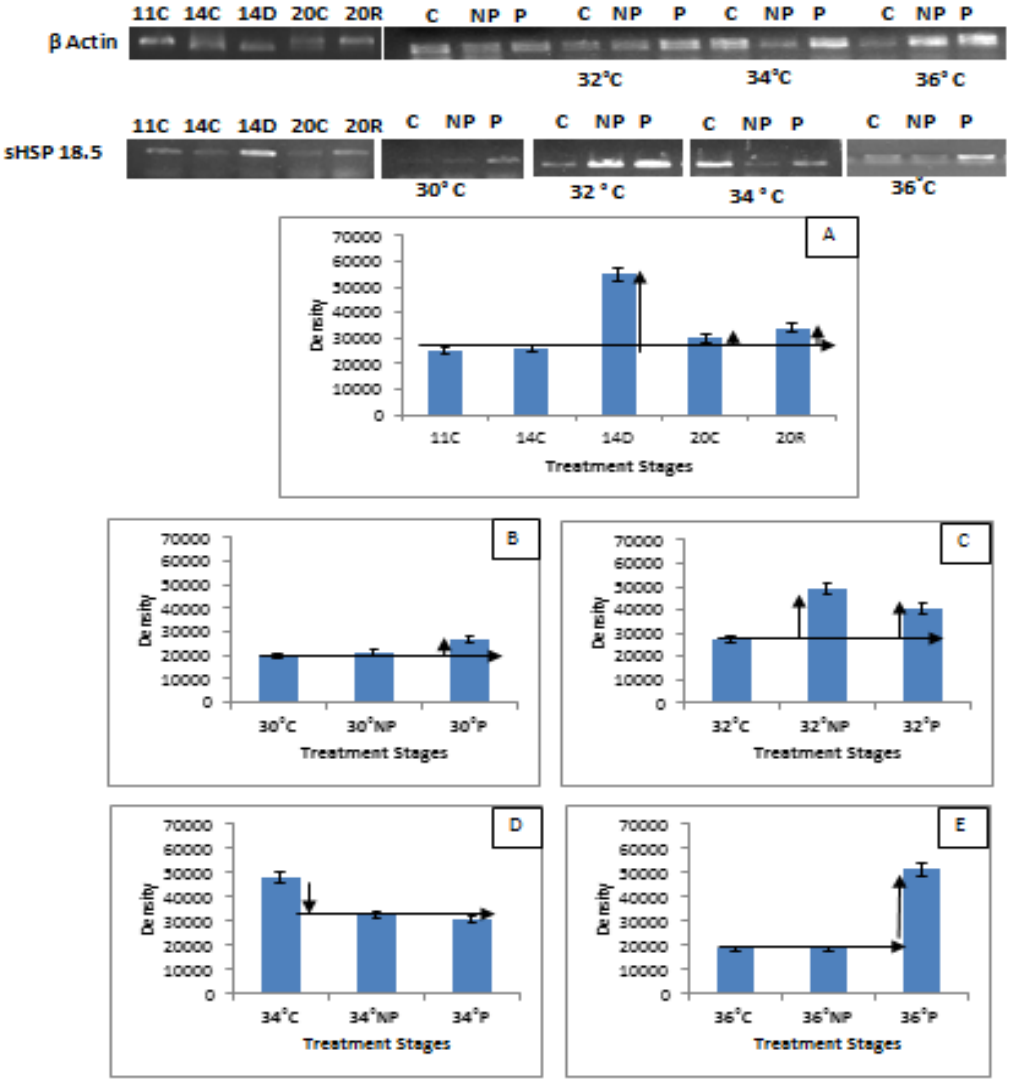

Fig. 4.5.1.1. RT-PCR amplification product of HSP 18.5 of PBG1 variety at different conditions: (A) 11C, 11th day control; 14C, 14th day control; 14D, 14th day drought; 20C, 20th day control;20R, 20th day recovery, (B) 30˚C, 30˚C Control; 30˚NP, 30˚C Non-preconditioned; 30˚ P, 30˚C preconditioned; (C) 32˚C Control; 32˚C NP, 32˚C Non-preconditioned; 32˚C P, 32˚C preconditioned; (D) 34˚C Control; 34˚C NP, 34˚C Non-preconditioned; 34˚C P, 34˚C preconditioned;(E) 36˚C Control; 36˚C NP, 36˚C Non-preconditioned; 36˚C P, 36˚C preconditioned.

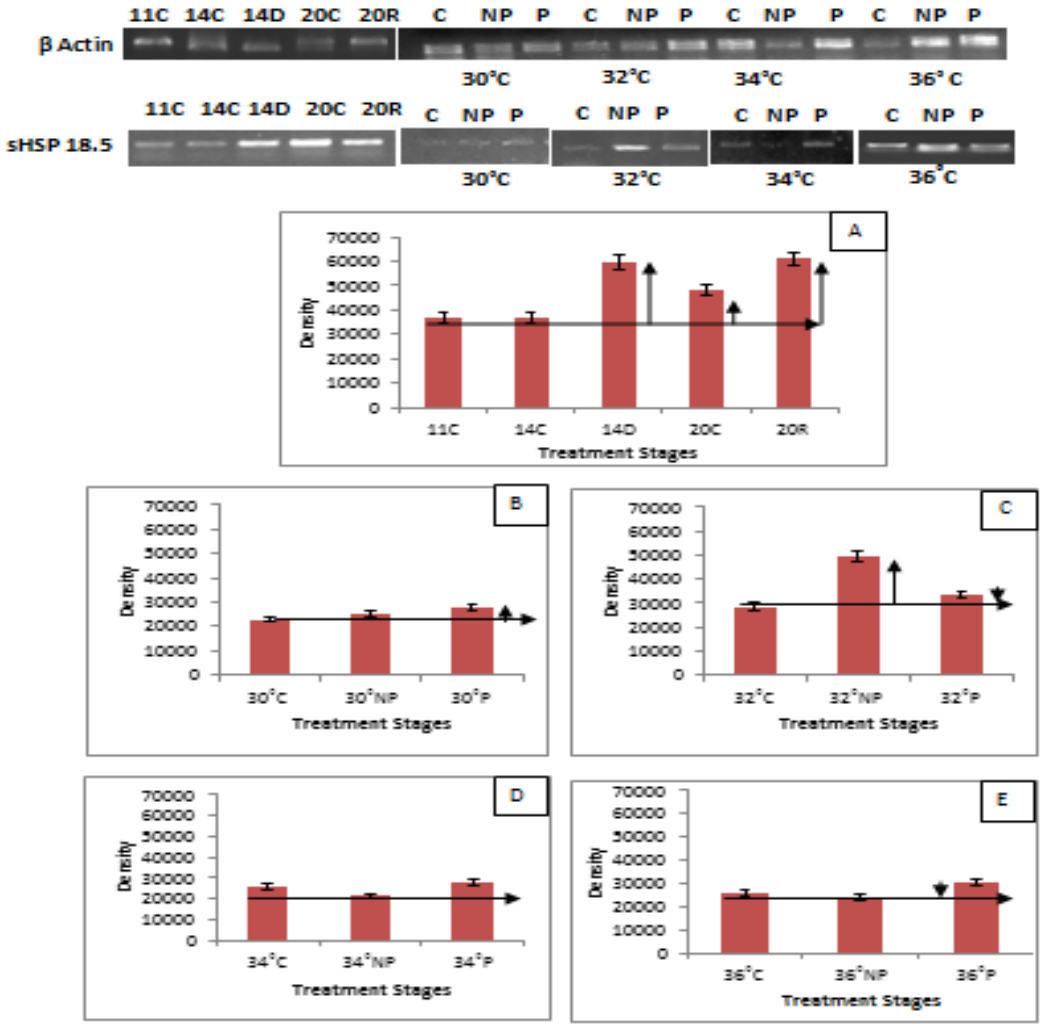

Fig.4.5.1.2. RT-PCR amplification product of HSP 18.5 of PBG5 variety at different conditions: (A) 11C, 11th day control; 14C, 14th day control; 14D, 14th day drought; 20C, 20th day control;20R, 20th day recovery, (B) 30˚C Control; 30˚C NP, 30˚C Non-preconditioned; 30˚C P, 30˚C preconditioned; (C) 32˚C Control; 32˚C NP, 32˚C Non-preconditioned; 32˚C P, 32˚C preconditioned; (D) 34˚C Control; 34˚C NP, 34˚C Non-preconditioned; 34˚C P, 34˚C preconditioned;(E) 36˚C Control; 36˚C NP, 36˚C Non-preconditioned; 36˚C P, 36˚C preconditioned.

sHSP 22.7

In PBG1, it was observed that preconditioning had negatively regulated HSP 22.7 expression over control as the control had 1.7 fold increased expression as compared to the drought stressed plants, and it didn't show any improvement during recovery. When these plants were exposed to 30˚C, preconditioned chickpea seedlings showed 2.1 fold increase over control and 4 fold increase in expression as compared to non-preconditioned samples. At 32˚C, 2.4 fold

decreased expression was observed in preconditioned plants over control and non- preconditioned plants. At 34˚C the HSP expression was positively regulated both in non-preconditioned and preconditioned plants though the expression was higher in non-preconditioned. Further rise in two degrees, at 36˚C, both preconditioned and non-preconditioned plants had 1.42 and 2.8 fold higher expression in comparison to the control. It can be concluded here that the HSP 22.7 expression varied with varying temperatures at 32˚C, preconditioned didn't show any increase in expression when compared with non-preconditioned, but showed improvement later on at 34˚C, and 36˚C when compared with the control samples (Fig.4.5.1.3).

In PBG5 after preconditioning no change in the HSP expression was seen whereas recovery showed 1.27 fold increase. Later when exposed to lethal stress, preconditioning increased the expression at 30˚C, whereas decrease in expression was observed at 32˚C. But at 34˚C much higher elevated expression (2.7 and 2.0 fold) was observed in preconditioning over non-preconditioned and control. At 36˚C again preconditioning enhanced 1.8 and 2.1 fold HSP expression compared to non-preconditioned and control. It can be inferred from the above findings that preconditioning had positively regulation of this HSP which is getting induced with high temperatures 30˚C, 34˚C and 36˚C. At 34˚C the expression was negatively regulated which needs further clarification (Fig.4.5.1.4).

4.5.2. Total protein profile

Since the gene expression pattern alone is insufficient for the description of one system due to the posttranscriptional changes and posttranslational modifications of the polypeptides under stress (Gygi et al., 1999; Timperio et al., 2008) hence supportive protein pattern analysis using SDS-PAGE was done.

Total protein profile of PBG1 revealed that during drought extra protein bands were seen at ~85Kda and after recovery protein bands of ~70, ~85, ~95 KDa were observed (Fig.4.5.2.A). In PBG5 the proteins bands at ~85, ~95, ~100 KDa were missing in 14 day drought exposed plants. At recovery proteins bands of ~50 KDa and ~ 55 KDa were missing. The protein profile during preconditioning clearly indicates that both the genotypes have differential sensitivity and response

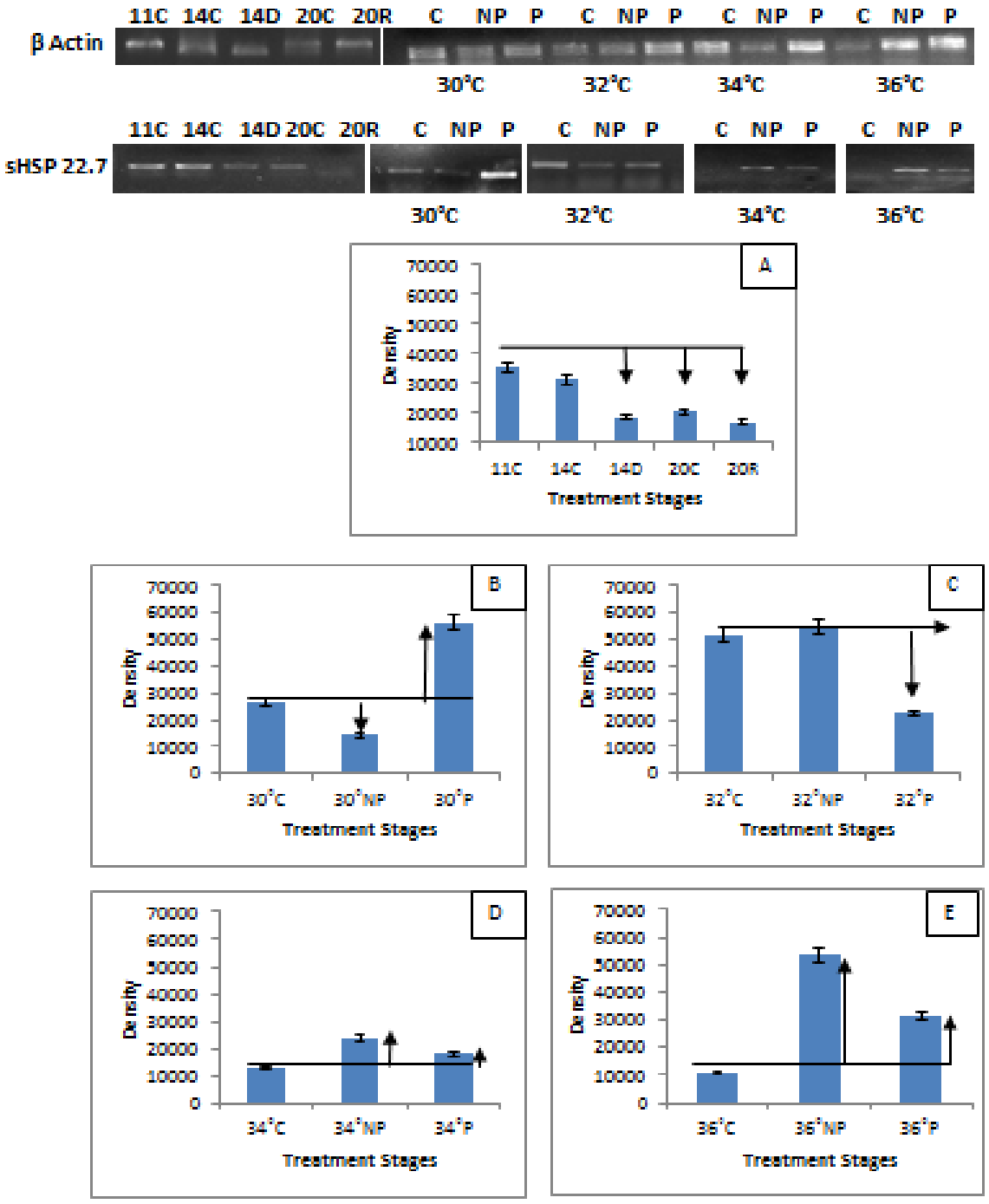

Fig. 4.5.1.3 : RT-PCR amplification product of HSP 22.7 of PBG1 variety at different conditions: (A) 11C, 11th day control; 14C, 14th day control; 14D, 14th day drought; 20C, 20th day control;20R, 20th day recovery, (B) 30˚C Control; 30˚CNP, 30˚C Non-preconditioned; 30˚C P, 30˚C preconditioned; (C) 32˚C Control; 32˚C NP, 32˚C Non-preconditioned; 32˚C P, 32˚C preconditioned; (D) 34˚C Control; 34˚C NP, 34˚C Non-preconditioned; 34˚C P, 34˚C preconditioned;(E) 36˚C Control; 36˚C NP, 36˚C Non-preconditioned; 36˚C P, 36˚C preconditioned.

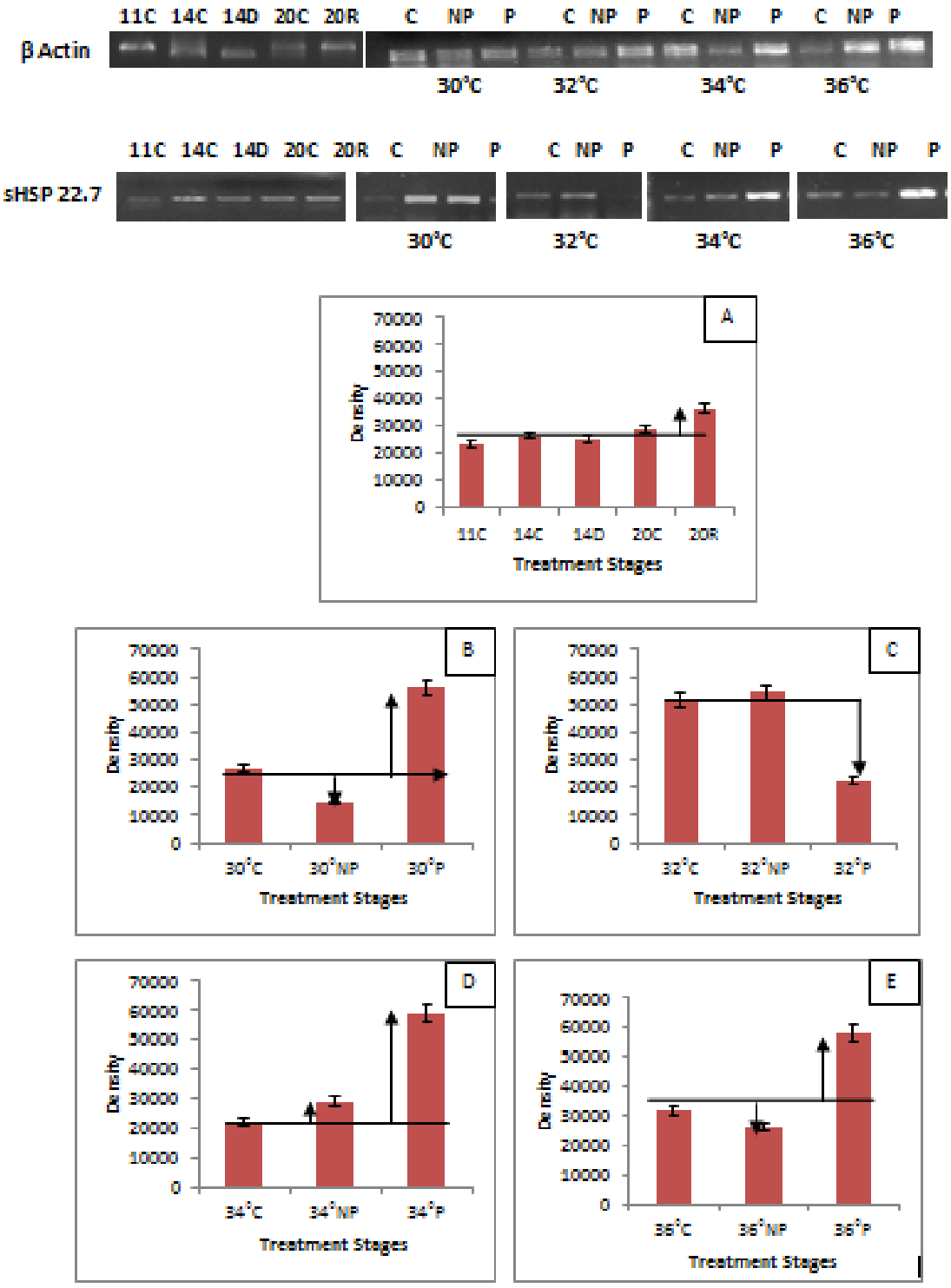

Fig. 4.5.1.4. RT-PCR amplification product of HSP 22.7 of PBG5 variety at different conditions: (A) 11C, 11th day control; 14C, 14th day control; 14D, 14th day drought; 20C, 20th day control; 20R, 20th day recovery, (B) 30˚C Control;30˚C NP, 30˚C Non-preconditioned; 30˚C P, 30˚C preconditioned; (C) 32˚C Control; 32˚C Non-preconditioned, 32˚C Non-preconditioned; 32˚C P, 32˚C preconditioned; (D) 34˚C Control; 34˚C NP, 34˚C Non-preconditioned; 34˚C P, 34˚C preconditioned;(E) 36˚C Control; 36˚C NP, 36˚C Non-preconditioned; 36˚C P, 36˚C preconditioned.

preconditioning (Fig.4.5.2.B). In both the genotype it was also observed that RuBisCo level also decreased during in the course of experimentation (arrow).

After preconditioning the plants were exposed to varying levels of high temperature stress 30°C, 32°C, 34°C and 36°C°. In PBG1, at 30˚C some new protein were seen in preconditioned plants at ~70KDa and missed at ~55KDa but altogether the protein expression was comparatively low (Fig.4.5.2.C). No clear differentiation was seen in PBG5 except the low protein profile compared to control and its competitor genotype. At 32˚C both PBG1 and PBG5 showed more protein expression in non-preconditioned plants was higher at than preconditioned and control while no proper interpretation could be made (Fig.4.5.2.D). At 34˚C and at 36˚C the protein pattern was much more differentiable in preconditioned plants. Only difference within the genotypes was the level of expression which was high in PBG1 (Fig.4.5.2.E,F). It can be concluded here that the protein expression varies with the level of temperature stress. The expression of proteins of ~70, ~85, ~95 and ~100 KDa can be corroborated with the new proteins expressed due to preconditioning.

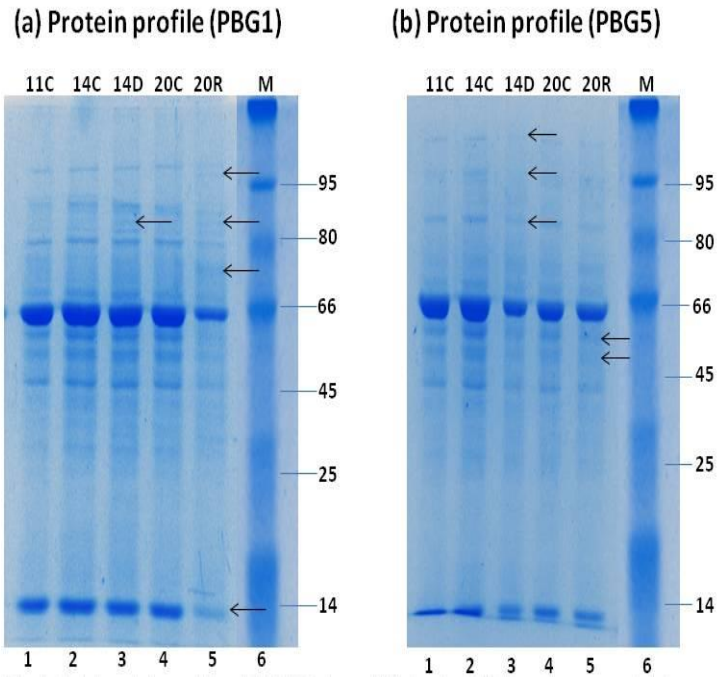

Fig. 4.5.2.1. (A). Total protein profile of PBG1 from 11th day to 20th day recovery (B) Total protein profile of PBG5 from 11th day to 20th day recovery

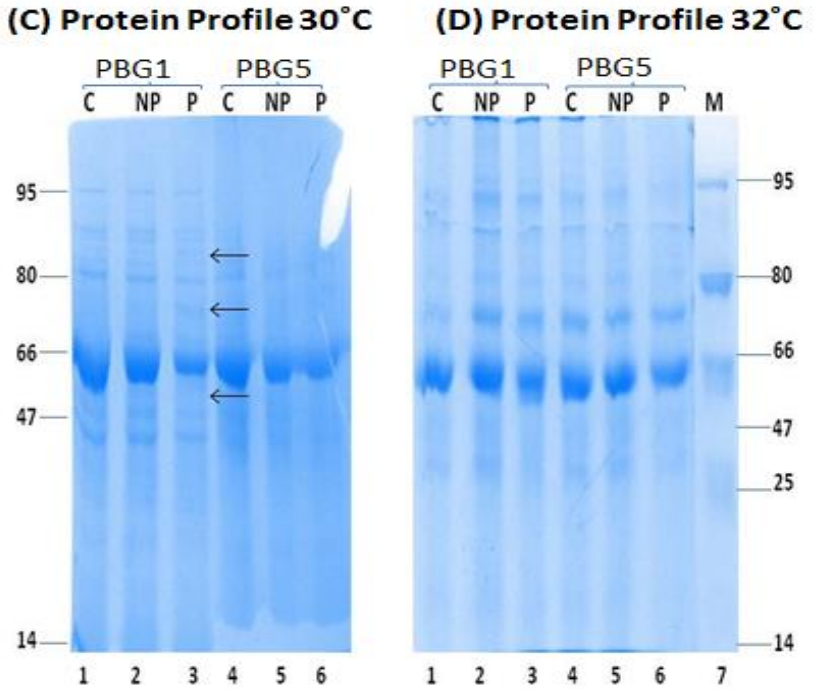

Fig. 4.5.2.2 (C) Total protein profile of PBG1 and PBG5 at 30˚C (D) Total protein profile of PBG1 and PBG5 at 32˚C.

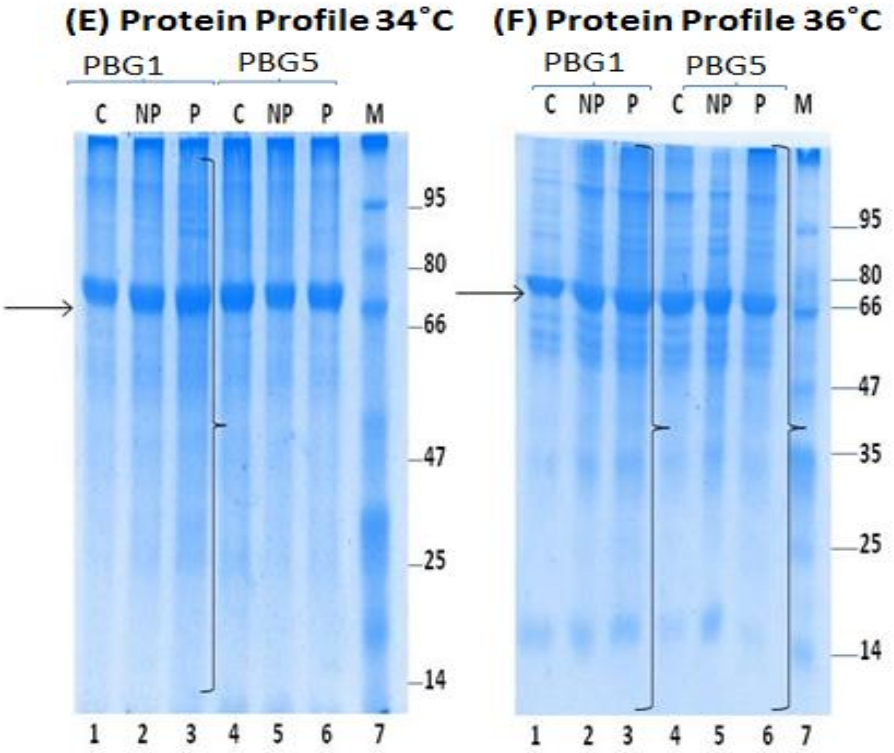

Fig.4.5.2.3. (E). Total protein profile of PBG1 and PBG5 at 34˚C (F).Total protein profile of PBG1 and PBG5 at 36˚C.

Data validation

sHSP 18.5 and sHSP 22.7 regions of PCR products were amplified using sHSP 18.5 and sHSP 22.7 forward primer (designed from primer 3 software) respectively. These were subsequently subjected to bidirectional Sanger sequencing (Applied Biosystem 3730 X 1 Genetic Analyzer, Foster city, CA, USA). Sequences were BLAST in NCBI tool for similarity search. Our sample, sHSP 18.5 showed similarity with *Cicer arietinium* L. having accession number (502180001), e- value 131 and showed 100% identity with chickpea sample, while sHSP 22.7 having accession number (502116450), e value 107 and showed 99% similarity with the chickpea sample.

HSP 18.5

Sequence

TCTGCATTTATGAGCACACGGGTGGACTGGAAGGAAACACCAGAAGCACACG
TGTTTAAGGCTGATCTTCCTGGACTAAAGAAGGAGGATGTAAAAGTTGAAATT
GAAGATGATAGGGTTCTTCAGATAAGCGGAGAGAGGAGCGTTGAGAAAGAG
GACAAGAATGATGAGTGGCATCGGGTTGAACGTAGCAGTGGTAAATTCATGA
GAAGATTCAGATTACCTGAGAATGCTAAAATGGAACAAGTTAAAGCTGCC

Table 4.6.1

Gene	BLAST result	Max score	Total score	Query cover	E-value	Identity%
HSP 18.5	PREDICTED: C*icer arietinum* 18.5 kDa class I heat shock protein-like (LOC101505773), mRNA. GI: 502180001.	477	477	100%	2e-131	100%

2. HSP22.7

Sequence

TTAGTGGTGAAGGAAGAAGAAGAGGAAAAAAGGGTGATCATTGGCATAGAGT
GGAAAGGTCTTATGGAAAATTTTGGAGACAGTTTAGATTGCCTGAGAATGTTG
ATTTGGATTCTGTTAAGGCTAAGATGGAAAATGGTGTGCTTACTTTGACACTT
GATAAGTTGTCACGTGATAAGATTAAAGGTCCTAGATTGGTTACCATTGCTAA
TGATGGGGAGAA

Table: 4.6.2.

Gene	BLAST result	Max score	Total score	Query cover	E-value	Identity%
HSP 22.7	PREDICTED: *Cicer arietinum* 22.7 kDa class IV heat shock protein-like (LOC101497389), mRNA. GI: 502116450	398	398	100%	2e-107	99%

CHAPTER 5
DISCUSSION

Chickpea (*C. arietinum* L.) is winter season crop and ideal temperature for growth is 22-26˚C and exposure to higher temperature during reproductive stage alters its metabolism and cause the over production of ROS which can trigger oxidative damage (Devasirvatham et al., 2012). Exposing the plants to one stress can induce a response similar to those after exposure to other stresses and sometime protect plant against another incoming stress (Lurie and Klein, 1991). In present course of our study; we have tried to counteract the heat stress by preconditioning chickpea seedlings with the non-lethal water stress (drought stress) and also examined the enzymatic activities involved in the chickpea seedlings grown at different temperature from 27˚C to 36˚C.

In our present study, different parameters in chickpea seedlings for measuring the the extent of membrane damage, cellular respiratory capability, antioxidative enzymes, and changes in sHSP profile at gene and protein level during preconditioned and non-preconditioned at different temperature, was checked.

5.1. Membrane damage: The degree of membrane damage was evaluated by % electrolyte leakage and lipid peroxidation.

5.1.1. Improving Electrolyte leakage: It can be inferred from the result that preconditioning improved the membrane stability in response to high temperature till 34°C and damage was less than the non-preconditioned seedling. The % ELI was less in PBG5 variety as compared to PBG1, which makes the earlier tolerant to high-temperature range. At 36°C, both the genotypes had more than 60-70% of the damage in non-preconditioned plants but here also preconditioning reduced the damage to 50%. Similar results were seen by Kaur *et al.* (2013) while analysing the effect of preconditioning to incoming lethal low-temperature stress (Unpublished).

5.1.2. Lipid peroxidation: MDA is a marker for lipid peroxidation and showed a marked accumulation under environmental stresses. In the present research, we observed increased MDA content in non-preconditioned plants which reflect the membrane damage resultant of peroxidation of lipids. But after preconditioning there was no apparent effect of high temperature till 34°C, later it was increased at 36°C. Previous research have also indicated that an elevated temperature resulted

in high peroxidation of the cell membrane (Xu *et al.*, 2006), and increased thermo tolerance in grasses following acclimation to a gradual increase in temperatures, which was associated with enhanced expression of HSP and suppressed lipid peroxidation (Liu and Huang, 2000).

Low lipid peroxidation is supported by some endogenous mechanisms. It can be inferred from the present findings that the extra mitochondrial production of ROS is less, concomitantly; the associated defence system is also less active. What it needs is the thorough examination of mitochondrial defence system i.e. SOD, GPX and GR activity.

5.1.3. Percent TTC Reduction: TTC reduction decreased with increase in temperature, which is well supported by previous finding that percent TTC reduction decrease as the temperature increased (Chen *et al.*, 1982). In our study, we found that % TTC reduction was less when samples were put under drought stress, which is supported by the fact that drought stress reduces the cellular respiratory ability in grasses which leads to decreased reduction of TTC (Abraham *et al.*, 2008). It was also found that the % TTC reduction in PBG1 was high at 30˚C and 32˚C and decreased as the temperature was increased to 36˚C, in PBG 5 the % TTC reduction was less as compared to PBG1 at higher temperature. Our result indicated improvement in TTC reduction in preconditioned PBG1 and PBG 5.

5.1.3. Total protein: High temperature stress cause protein denaturation, or altered protein synthesis but at very high temperature plant synthesize heat shock proteins, which lead to tolerance to high temperature (Levitt, 1980). Previous studies have shown that increase in temperature decreases the total protein content in the plants (Gulen and Eris, 2004). Almost similar results were observed when pre and non- preconditioned plants were exposed to a range of increasing high temperatures, but at 36˚C an abrupt increase in total protein content was observed in both the genotypes PBG1 and PBG 5. It can be hypothesized that at high temperature (36˚C) chickpea plants synthesized some extra proteins which can be linked with the expression of sHSP 18.5 and 22.7. Previous studies, carried out in cork oak, explain that heat stress induces the synthesis of sHSP in cork oak plant, which induce thermotolerance to the plant (Correia *et al.*, 2014).

5.2. Antioxidative Enzymes

5.2.1. SOD enzyme

SOD provide the first line of the enzymatic defence and catalyses the dismutation of O^{2-} to H_2O_2 and O_2 (Oidaira *et al.*, 2000). In present research SOD activity was high in non-preconditioned as compared to preconditioned plants. High SOD activity is well justified due to high oxidative stress, but less activity in preconditioned can be attributed to less production of superoxides, low oxidative stress, eventually less synthesis of SOD. In previous research on tomato plants, SOD activity increased, where overproduction of O^{2-} is involved and increased when exposed to high temperatures (Rivero *et al.*, 2004). In the present research, PBG5 demonstrated slightly higher constitutive and induced levels of SOD under control and stress conditions, which indicate that this variety might have a better O^{2-} scavenging capacity. Similar to our findings, previously higher constitutive or induced activities of antioxidant defence enzymes were generally accompanied with an increase in SOD levels in the cotton plant (Sekmen *et al.*, 2014).

5.2.2. Catalase Enzyme

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 high temperature (Scandalios *et al.*, 2000; Rivero *et al.*, 2004); Again it can be recollected from previous parameter that preconditioned plants had low SOD activity. Here also, CAT activity was less in preconditioned as compared to non-preconditioned 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).

5.2.3. APX Activity

Ascorbate-glutathione cycle is a major hydrogen peroxide detoxifying system found in chloroplast and cytosol of plants, in which APX (Ascorbate Peroxidase) is a major enzyme (Asada, 1992; Pang and Wang, 2010). High APX activity in non-preconditioned plants at high temperature is well understood as more activity is directly proportional to more H_2O_2 production. Our results are also in accordance

to the study, in which the activity of APX enzyme increase with increased temperature, while its activity is inhibited at 36˚C, same has been studied in lily plants, the activities of APX and GR were maintained at high levels under heat stress, indicating that the Ascorbate-glutathione cycle plays a crucial role in mitigating the accumulation of H_2O_2 in lily plants under heat stress (Panchuk *et al.*, 2002; Yin *et al.*, 2008). We have no reason other than correlating low oxidative stress and low APX activity in preconditioned plants. Preconditioning is lowering down respiration (TTC reduction) which in turn produces less ROS, less peroxidation, less membrane damage and % EL, less antioxidant and more proteins.

These results are supported by the significantly enhanced antioxidant enzyme activities (SOD, CAT, and APX) in inhibiting the accumulation of ROS. The results are consistent with previous reports perennial grass *Leymus chinensis* and *Phalaenopsis* when subjected to heat stress (Ali *et al.*, 2005; Xu and Zhou, 2006).

5.3. Expression Analysis

RT-PCR analysis

Heat stress in plants induce massive transcription and translation of HSP's and that the most evident heat induced proteins of plants are the small heat shock proteins (sHSP) which explain their chaperon activity, which is in agreement with the up regulation of sHSP18.5 under drought stress as seen in RT-PCR gel (Aragoncillo *et al.*, 2008). In PBG1, the expression of HSP 18.5 was more than the PBG5, which could be explained that PBG1 was more sensitive and need more HSP expression to cope up with stress as compared to the tolerant variety PBG5. While the expression of HSP 22.7 was found to increase with the increase in temperature and maximum at 36˚C, which explains that their accumulation increase with the rising temperature (Kadyrzhanova *et al.*, 1998).

From SDS-PAGE it can be concluded that during drought stress and recovery induced the synthesis of few new proteins, previously similar results were seen when wheat seedling were exposed to drought stress (Grigorova *et al.*, 2011) and with the increase in temperature few more proteins were synthesized in

preconditioning as compared to non-preconditioned sample. After total protein profiling, it can be inferred that preconditioning has induced the synthesis of few new proteins with the increase in temperature.

CHAPTER 6

SUMMARY AND CONCLUSION

Chickpea (*C. arietinium* 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 experience high temperature stress at reproductive stage which cause reduced yield. The main factors associated with tolerance are membrane fluidity and integrity, cellular respiration ability, antioxidative enzymes and synthesis of Heat shock proteins. To counteract the heat stress, the present strategy employed is feasible especially in field condition, which may induce tolerance mechanism in chickpea.

It is known that pre-exposure to one stress creates the ability to respond to the incoming stress due to alterations at biochemical and molecular level. In present course of study, we used the preconditioning of seedlings with drought stress, which activate heat tolerance. It can be summarized from our results that percent electrolyte leakage was found to be less in preconditioned seedlings as compared to non-preconditioned seedlings in both the variety PBG1 and PBG 5 and with the gradual rise in temperature, extent of damage increase and complete damage was found at 36˚C, but the extent of damage was more in PBG1 as compared to PBG5. Similarly, MDA content formed was found to be high at 34˚C and 36˚C. It was also found that the MDA content was 2 fold more in non-preconditioned seedlings than the preconditioned seedlings, but again less improvement of preconditioning was seen at higher temperature. Percent TTC reduction decrease with the increase in temperature, and was found to be least at 34˚C and 36˚C, while the total protein content increased during drought stress, decreased with the increase in temperature, a sudden increase in protein content was found at 36˚C .

From above study the results obtained are in correlation with the expression analysis study, in which there was up regulation of sHSP 18.5 during drought stress found in both the varieties. It was also found that during preconditioning there was increased expression of HSP22.7 as compared to non-preconditioning with the increase in temperature. Antioxidant act as a major defense against free radicals generated during heat stress. Antioxidative enzyme activity of SOD increased linearly with the increase in temperature and was found to be maximum at 36˚C. PBG5 demonstrated comparatively much higher activity as compared to PBG1, while in case of CAT and APX activity increased with the increase in

temperature and was found to be maximum at 34˚C, and a steep decrease at 36˚C.

To conclude the whole study, results obtained clearly indicate that preconditioning with drought stress has the ability to improve tolerance to heat stress. Our result indicates that chickpea seedlings display a high tolerance to elevated temperature (27˚C-36˚C), which is attributed to the enhanced activities of antioxidative enzymes and increased synthesis of sHSP. By contrast, chickpea show symptoms of oxidative stress at 36˚C, as indicated by enhanced electrolyte leakage, MDA content, and total protein content which may be due to inhibition of antioxidative enzyme system particularly CAT and APX.

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APPENDICES

Appendix A: Protocol for RNA extraction

1. Homogenize the tissue sample (100mg) in liquid nitrogen, and then add 1mL of Trizol to the homogenate.
2. Centrifuge the homogenate at 12000g for 10 minutes and remove the supernatant that contain RNA and allow the sample to stand for 5 minutes at room.
3. Add 200µl of chloroform and shake vigorously for 15 seconds and allow it to stand for 15 minutes at room temperature.
4. Centrifuge the mixture at 12000g for 15 minutes at 4˚C, which separate into three phases: lower phase which contain Protein, an interphase which contain DNA and upper phase which contain RNA.
5. Transfer the upper phase to a fresh tube and add 500µl of Isopropanol and mix gently.
6. Allow the sample to stand at 10 minutes at room temperature.
7. Centrifuge the mix at 12000g for 10 minutes at 4˚C, RNA will form a pellet. Wash the RNA pellet by adding 1mL of 75% ethanol
8. Vortex the sample and then centrifuge at 7500g for 5 minutes at 4˚C
9. Air dry the pellet for 10 minutes to remove the traces of ethanol
10. Dissolve the pellet gently in 50 µl of elution buffer (formamide/SDS) by pipetting.

Appendix B: Reaction mixture for cDNA synthesis

COMPONENTS	VOLUME
RNAsin	1µl
100mM DTT	1µl
5X Assay Buffer for M-MULV RT	4µl
30m M dNTP mix	2µl
M-MULV Reverse Transcriptase	1µl
Nuclease free water	1µl
Total volume	10µl

Appendix C: PCR reagents and their volumes for amplifying HSP18.5 and HSP 22.7 primer.

COMPONENT	CONTROL(volume)	TEST(volume)
Nuclease free water	19.5µl	9.5µl
10X assay buffer for Taq DNA polymerase	2.5µl	2.5µl
30mM dNTP Mix	.5µl	.5µl
Forward primer (10p mol)	.5µl	5µl
Reverse primer(10pmol)	.5µl	5µl
cDNA product	1µl	2µl
Taq DNA polymerase (3u/mL)	.5µl	.5µl
Total reaction mixture	25µl	25µl

Appendix D: Amplification scheme for PCR

Stage	Step	Temperature(˚c)	Time	No.of cycles
1.	Initial denaturation	94	2 min	1
	Denaturation	94	45 sec	30
	Annealing	52	30sec	
3.	Extension	72	1 min	
4.				
5.	Final extension	72	5 min	1
6.	Hold	4	α	

Appendix E: List of primers along with their Tm and no. of cycles

Primers	T_m and no. of cycles
Hsp 18.5 (F) TCCATGGCAGCTTTAACTTG (R) CAAGTTAAAGCTGCCATGGA	55˚C, 32
Hsp 22.7 (F)GGTTGGTGATTCTCTCCCCATCA (R)TGATGGGGAGAGAATCACCAACC	57˚C, 30
Actin (F) TTCCTGGTATTGCTGACCGT (R) GATGGGCCAGACTCGTCATA	55˚C, 28

Appendix F: Component of 12% SDS-PAGE

Component	12% (mL)
H_2O	3.4
1.5M Tris-HCl, pH 8.8	2.5
20%(w/v) SDS	.05
Acrylamide/Bis-acrylamide (30%/0.8%w/v)	4
10%(w/v)APS	.05
TEMED	.0066

Appendix G: Component of 4% SDS-PAGE

Component	4%(mL)
H_2O	1.5
0.5M Tris-Hcl, pH6.8	.625
20%(w/v) SDS	.012
Acrylamide/Bis-acrylamide (30%/0.8%w/v)	.33
10%(w/v)APS	.025
TEMED	.005

Appendix H: Components of 5X Sample buffer

10% (w/v)	SDS
10mM	Betamercapto-ethanol
20%	Glycerol
0.2M	Tris-HCl, pH 6.8
0.05% (w/v)	Bromophenol Blue

Appendix I: Components of 1X Running buffer

25mM	Tris-HCl
200mM	Glycine
0.1 %(w/v)	SDS

Appendix J: Standard curve for total proteins, BSA of 100µg/mL was made and diluted to various known concentrations.

S.No.	Concentration of BSA (µg/mL)	O.D at 595 nm
1.	10	.113
2.	20	.194
3.	30	.340
4.	40	.392
5.	50	.612
6.	60	.667
7.	70	.799
8	80	.901
9.	90	.963
10.	100	1.23