MORPHO-PHYSIOLOGICAL AND BIOCHEMICAL TRAITS FOR COLD TOLERANCE OF CHICKPEA (*Cicer arietinum* L.) GENOTYPES

BY

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CERTIFICATE – I

This is to certify that this thesis entitled "**Morpho-physiological and biochemical traits for cold tolerance of chickpea (*Cicer arietinum* L.) genotypes**" submitted for the degree of **Master of Science** in the subject of **Plant Physiology**, to the **Chaudhary Charan Singh Haryana Agricultural University**, **Hisar**, is a bonafide research work carried out by **Mr**. **Mahesh Kumar** under my supervision and that no part of this dissertation has been submitted for any other degree. The assistance and help received during the course of investigation have been fully acknowledged.

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CERTIFICATE – II

This is to certify that this thesis entitled "**Morpho-physiological and biochemical traits for cold tolerance of chickpea (*Cicer arietinum* L.) genotypes"** submitted by **Mr**. **Mahesh Kumar** to the **Chaudhary Charan Singh Haryana Agricultural University, Hisar** in partial fulfilment of the requirements for the degree of **Master of Science** in the subject of **Plant Physiology**, has been approved by the Student's Advisory Committee after an oral examination on the same.

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It is certified that the thesis submitted by Mr. **Mahesh Kumar**, Admn. No. **2014BS34M(R)**, an M.Sc student of **Department of Botany and Plant Physiology** has been checked and found as per specifications of the format circulated by Dean, PGS vide Memo. No. PGS/A-1/09/6926-90 dated 26/8/2009.

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LIST OF ABBREVIATIONS

APS	-	Ammonium per sulphate
ATP	-	Adenosine tri phosphate
CAT	-	Catalase
CD	-	Critical difference
CSI	-	Chlorophyll stability index
DAE	-	Days after exposure
DAS	-	Days after sowing
DMSO	-	Dimethyl sulphoxide
DW	-	Dry weight
EDTA	-	Ethylene diamine tetra acetic acid
FW	-	Fresh weight
HI	-	Harvest index
MDA	-	Malondialdehyde
MPa	-	Mega Pascal
MW	-	Molecular weight
NBT	-	Nitro blue tetrazolium
PS1	-	Photosystem I
PSII	-	Photosystem II
POX	-	Peroxidase
PVP	-	Polyvinyl pyrolidone
RBD	-	Randomized block design
ROS	-	Reactive oxygen species
RSI	-	Relative stress injury
RWC	-	Relative water content
SOD	-	Superoxide dismutase
TBA	-	Thiobarbituric acid
TCA	-	Trichloroacetic acid
TSC	-	Total soluble carbohydrates
TTC	-	2, 3, 5–triphenyl tetrazolium chloride
ψ_w	-	Water potential
ψ_s	-	Osmotic potential

CHAPTER – I

INTRODUCTION

Chickpea (*Cicer arietinum* L.), the world's third most important food legume, is currently grown in about 52 countries around the world and grown in many geographical regions including, South Asia, West Asia, North and East Africa, Southern Europe, North America, South America and Australia (Taylor and Ford, 2007; Varshney *et al*., 2009). In India, Madhya Pradesh, Rajasthan, Maharashtra, Uttar Pradesh, Andhra Pradesh, Karnataka, Chhattisgarh, Bihar and Jharkhand are major chickpea producing states contributing more than 95% to the total chickpea production. Madhya Pradesh is the single largest producer in the country accounting for over 40% of total production while Rajasthan, Maharashtra, Uttar Pradesh and Andhra Pradesh contribute about 14%, 10%, 9% and 7%, respectively. The share of Andhra Pradesh and Karnataka has consistently been rising during the past one decade. Further, states like Jharkhand and Chhattisgarh are expanding their area and production of chickpea crop (AICRP, 2014-15).

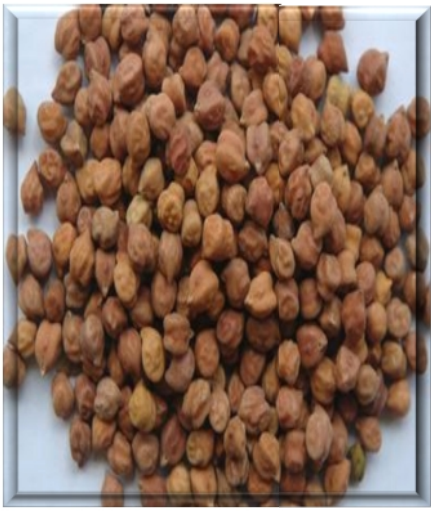
Chickpea belongs to the Fabaceae family and it is a self pollinated diploid (2n = 16) with a relatively small genome size of 740 Mbp (Garg *et al*., 2011). The chickpea seed is a good source of carbohydrates and proteins, which together constitute 80% of the total dry seed weight. The crude protein content of chickpea varies from 17% to 24% containing the essential amino acids like tryptophan, methionine and cysteine (Williams and Singh, 1987). Thus chickpea serves as a main source of dietary protein for more than 80% of the Indian population which is vegetarian in nature. Chickpea acquires importance as it provides food for humans as well as for livestock. Furthermore, chickpea pod covers and seed coats can also be used as fodder. It is consumed as a fresh immature green seed, whole seed, dhal and flour. In the Barind region of Bangladesh, where extremely low rains result in lesser choice of crops for farmers, the top twig from each chickpea plant is consumed as a green vegetable. It is also consumed as a delicacy in India during the chickpea-growing season. Chickpea nitrogen fixation plays an important role in maintenance of the soil fertility, particularly in the arid and low rainfall area (Roy *et al*., 2010).

Within cultivated chickpea, two distinct groups of cultivars are found- large seeded, cream-coloured chickpea called *kabuli* type and small seeded, dark coloured chickpea called *desi* type (Plate 1). It is known with different regional names like Dzelbana or Hamas (Arabic), Keker (Dutch), Bengal Gram (English), Café Franzais (Franch), Kichererbse (German), Garbanzo (Spanish), Cece, Ceci (Roman), Ovetichie harokh (Russian), Shimbra, (Ethiopia) Lablabi, (Turkey) and locally 'chana'.

It is one of the leading cool season food legume in the world with 14.8 million hectare area under cultivation and 14.3 million tonnes produced annually (FAO, 2014). In India, among various legumes grown, chickpea ranked 1st covering 10.7 million hectare area and produced 9.8 million tonnes with average seed yield of 919 kg ha^{-1} during 2014-15 and in Haryana 53000 tonnes produced in 47000 hectare area during 2012-13 (Agriculture profile of Haryana, 2015). The production of the cool season grain legume chickpea is constrained by low temperatures across much of its geographical range. The northern parts of the Indian subcontinent are the most affected regions due to chilling as the temperature is below 15 ^{0}C at flowering (Bakht *et al*., 2006; Berger 2007). Cold related stress is among the least understood of the adversities that affects the plants and can be defined in terms of either chilling between 0 ^{0}C and 10 ^{0}C or freezing below 0 ^{0}C without snow cover (Wery *et al.,* 1993). Low temperature (0-10 ^{0}C) prevails in the month of December, January and early February is a major factor leading to unstable yield in many current production areas. Being a cool season food legume, chickpea faces low temperature to the tune of 0-5 ^{0}C for about 15-20 days in the northern states as crop is highly sensitive to mean temperature below 15 °C at flowering leading to flower dropper pod abortion that results in yield loss.

Cold stress is important factor responsible for the greatest agricultural loss by affecting the various growth, physiological and biochemical activities in plants and thus limiting the yield and productivity (Basu *et al*., 2009; Chinnusamy *et al.,* 2007; Heidarvand et al., 2011). Growth process i.e. crop growth rate (CGR) and relative growth rate (RGR) directly influence the economic yield and have been idendified as the major determinants of yield (Ozalkan *et al.,* 2010). Temperature below 10 ^{0}C is known to alter a variety of physiological processes ranging from plant water status, photosynthesis to reactive oxygen species (ROS) and solute accumulation and has adverse effect on chickpea yield and results in losses from 15-20 % (Ali and Kumar, 2005; Bakht *et al.,* 2006; Chaturvedi *et al.,* 2009). A number of biochemical changes occur during cold-acclimation like alteration in membrane lipid composition, the appearance of new isozymes, increased level of sugars and soluble proteins contents and increased levels of proline and other organic acid etc. (Levitt, 1980). Wery *et al.* (1994) observed the altered membrane functions during chilling stress by lipid peroxidation and leakage of electrolytes from chickpea cells. Survival at low temperature depends to a large degree on the ability of plants to maintain the integrity of membranes as they constitute the main damage site. Some of these changes, such as alteration in lipid composition, appear to have a role in bringing about the increased cold tolerance of acclimated plants (Steponkus and Lynch, 1989). The most advantageous temperature range for normal flowering, fertilization and seed set is 10 to 14 ^{0}C as minimum temperature and 25 to 31 ^{0}C as maximum temperature. Low temperature (<10 ^{0}C) is detrimental for reproductive growth of chickpea and induces abortion of flowers, pods, impaired seed growth and reduced

***Desi* chickpea plant**



***Desi* chickpea seed**

***Kabuli* chickpea plant**

***Kabuli* chickpea seed**

Plate 1: Types of chickpea cultivated in Haryana

yield (Srinivasan *et al.*, 1999; Nayyar *et al.* 2005, 2007).The underlying mechanism of chickpea metabolism at low temperature has not been fully understood.

The determination of physiological and biochemical responses to cold stress is vital. Although resistance to cold temperature involves several complex tolerance and avoidance mechanisms, the membrane is thought to be a site of primary physiological injury by cold and measurement of solute leakage from tissue can be used to estimate damage to membranes. Chlorophyll fluorescence indicates that photochemical efficiency and photochemical quenching coefficient decreased under low temperature and electron transfer from PS II is also cold sensitive.

The adverse effects of cold stress can be mitigated by developing crop plants with improved cold tolerance using various morpho-physiological and biochemical approaches. Information on tolerance to low temperature stress in chickpea genotypes is still limited. Keeping this in view, the present study was undertaken to compare different genotypes of chickpea for their morpho-physiological and biochemical parameters under chilling stress i.e. below 5 ^{o}C with the following objectives:

1. To study the morpho-physiological and biochemical characters for low temperature tolerance in chickpea
2. To correlate the morpho-physiological and biochemical characters with yield for low temperature tolerance in chickpea

CHAPTER – II

REVIEW OF LITERATURE

In chickpea, temperature is a major environmental factor regulating the timing of flowering thus influencing grain yield (Summerfield *et al*., 1990; Berger *et al.,* 2004). Both low and high temperatures can limit the growth and grain yield of chickpea at all phenological stages. The adaptation and productivity of chickpea is often limited by low and high temperatures. Cold stress generally occurs in the late vegetative and reproductive stages across the geographical areas of chickpea production. Cold and freezing temperatures (−1.5°C to 15°C) are considered a major problem during the seedling stage of winter-sown chickpea in Mediterranean areas and autumn-sown crops in temperate regions (Singh, 1993). The parts of north India are most affected by chilling temperatures at flowering and high day and night temperatures (>30/16 °C) may cause damage during the reproductivestage on winter-sown chickpea. (Berger *et al.,* 2011).

Plants have developed strategies to tolerate the range of temperatures common to their habitant, injury may occur in a new environment, or when temperature extremes exceed the norm.Cold stress injury occurs at all stages of active plant growth and early seedling growth. However, in chickpea sensitivity of chilling temperature increases as plant progresses from germination to flowering stage (Singh *et al.,* 1995). A number of physiological and biochemical changes occur in plants to extent and ability to withstand these changes, determine their sensitivity or resistance to chilling at that temperature which provides an important fundamental requirement in the screening and development of the cold tolerant lines for characterization of these stresses (Srinivasan *et al.,* 1998).

The literature on research work carried out in India and abroad on **"Morpho-physiological and biochemical traits for cold tolerance of chickpea (*Cicer arietinum*L.) genotypes"** is being discussed here under the following headings:

2.1 Phenological studies (Days to 50% flowering, 50% podding and physiological maturity)

Phenology (time to flowering, podding and maturity) plays critical role in adaptation of chickpea cultivars to different environments (Berger *et al*., 2004, 2006). Flowering time or days to flowering (number of days from sowing to appearance of first flower) can be recorded with high precision and provides fairly good indication of succeeding phenological traits (time of podding and maturity). Early phenology is a key trait for adaptation of chickpea to short-season environments as it helps crop to escape from end-of-season stresses (drought, temperature extremities). Lower temperatures, shorter photoperiods and optimal soil moisture, individually or in combinations, help in extending growth period, while higher

temperatures, longer photoperiods and moisture stress conditions are known to shorten all developmental phases thereby reducing the crop duration (Summerfield *et al.,* 1990). During reproductive phase, low temperature, (<15 °C) is detrimental to normal flowering and pod development, which causes prolonged reproductive phase, floral abortion, poor pollen germination, impaired ovule development, failure in podset and reduction in seed filling that drastically affects the crop productivity (Nayyar and Gupta, 2006). According to Wery *et al.* (l993) the critical temperature during the reproductive phases, which includes flowering, filling and enlargement of seeds of chickpea, plays an important role in the productivity. Gaur *et al.* (2008) observed that crop maturity depends on genotype, soil moisture, time of sowing, latitude and altitude.

The semi-arid tropics (SAT) observed that early maturity is a key trait for adaptation of chickpea and helps to escape from moisture and heat stresses at reproductive stage. The cultivars ICCV 37 and JG 11 (ICCV 93954) with early maturity and high resistance to fusarium wilt led to enhancement of chickpea production in some SAT regions. Kumar and Abbo (2001), Leviit (1972) and Gaff and Slinkard (1980) observed that plants escape drought with the ability to complete its life cycle with rapid phenological development (early flowering and early maturity).Chickpea genotypes with high growth vigor are early maturing. Selection for high growth vigor enhances chance for escaping terminal drought stress (Sabaghpour and Kumar 2002). Early maturity is an important trait to avoid drought stress due to onset of severe water deficits.Yield potential and early flowering are two major components of drought escape in lentil and chickpea (Saxena *et al*., 1993; Silim and Saxena, 1993).

2.2 Growth studies

Growth analysis is still the most simple and precise method to evaluate the contribution of different ecological processes in plant development (Namvar *et al.*, 2011). Growth is generally a function of environmental factors (such as temperature and solar radiation) and mineral nutrition, along with genotype and production practices (Alam and Haider, 2006).

Saha *et al.* (2010) observed that when temperature falls below 15 °C growth is usually retarded and yield decreases. Growth analysis is one way to verify the crops ecological adaptation to new environments, the competition between species, crops management effects and the identification of the productive capacity of different genotypes. The dynamics of dry matter distribution to various plant organs, their yielding and productivity may be characterized by using various indices of growth analysis (Zajac *et al.*, 2005; Kibe *et al*., 2006). It provides a considerable insight into the functioning of a plant as depends on genotype or environment. The purpose of growth analysis is the determination of the increase in dry matter referred to a suitable basis for photosynthetically active tissue, leaf

area and amount of leaf protein (Ali *et al*., 2004; Gupta and Gupta, 2005; Alam and Haider, 2006; Yasari and Patwardhan, 2006).

Plant height is an important index of plant growth and its measurement used to monitor the comparison between different genotypes (Raina, 2011). He conducted a field experiment with ten chilling tolerant and two chilling sensitive chickpea genotypes and observed that plant height was significantly lesser in chilling tolerant genotypes.

Crop growth rate (CGR) is regarded as the most meaningful growth function since it represents the net results of photosynthesis, respiration and canopy area interaction (Alam and Haider, 2006). Gupta and Gupta (2005) noted that CGR is also a representative of the most common agronomic measurements such as yield of dry matter per unit land area. Due to incomplete plant cover and limited absorption of solar radiations, crop growth rate is slow at early growth stages, but with the development in plants their growth is increased rapidly because of expansion of leaf area. CGR is dealing with production potentiality of plants and yield among different cultivars is determined by this (Gulzar *et al*., 2006).

In the initial stages of the plant growth the ratio between alive and dead tissues is high and almost entire cells of productive organs are activity engaged in vegetative matter production and, consequently, the relative growth rate RGR of plants is high, while with plant aging, the metabolic activity of tissues decreases and hence the tissues cannot contribute to the growth that results in RGR decreasing (Zajac *et al.,* 2005; Alam and Haider, 2006; Kibe *et al.,* 2006). Ahmadi *et al.* (2014) observed in mustard when high RGR, this will quickly increase in size and occupy large space in both below and above ground parts and it facilitate rapid completion of plant life cycle.

2.3 Physiological studies

Low temperature stress is frequently associated with reduced water availability. Water deficits reduce the number of leaves per plant and individual leaf size, leaf longevity by decreasing the soils water potential (ψ_w). Osmotic potential (ψ_s) is another physiological parameter used for recording the extent of stress level in plants. The leaf cells contain various organic and inorganic solutes, which determine leaf ψ_s. Relative water content is the ability of plant to maintain high water in the leaves under abiotic stress conditions.

Many plants accumulate a number of compatible osmolytes such as proline and various sugars under abiotic stress conditions and these osmolytes function as osmoprotectants (Ishitani *et al.,* 1996; Igarashi *et al.,* 1997; Wanner and Juntilla 1999; Taji *et al.,* 2002). Adaptation to all environmental stress is associated with metabolic adjustments that lead to accumulation of several organic solutes like sugars and proline (Greenway and Munns 1980; Yancey *et al.,* 1982). Accumulation of sugars in different parts of plants is

enhanced in response to the variety of environmental stresses (Garham *et al.,* 1981, Wang *et al.,* 1996, Prado *et al.,* 2000).

Osmotic adjustment is a specific response to maintain water relations (turgor) under osmotic stress. A range of so-called osmotically active or compatible substances is involved, including soluble sugars, proline. Osmotic adaptation was found to be lower even absent in faba beans (Amede *et al.,* 1999; Sau and Minguez, 2000; Katerji *et al.,* 2002; Amede and Schubert, 2003) and high in both chickpea (Leport *et al.,* 1998; Amede and Schubert, 2003) and lentil (Leport *et al.,* 1998; Leport *et al.,* 2003). It is recognized that resistent plants under stress conditions develope various physiological and biochemical responses of adaptive nature. These include changes of pigment content, osmotic adjustment and photosynthetic activity (Dhanda *et al.,* 2004; Serraj *et al.,* 2004; Benjamin and Nielsen 2006; Kalefetoğlu and Ekmekçi 2009; Praba *et al.,* 2009) and this mechanisms play a key role in preventing membrane disintegration and provide tolerance against abiotic stresses (Hanson and Hitz 1982; Bohnert and Jensen 1996; Mahajan and Tuteja 2005).

Osmotic adjustment (OA) has been suggested as an important trait in postponing dehydration in water-limited environments as it maintains cell turgor and physiological processes as water deficits develop (Turner and Jones, 1980; Morgan, 1984). Variation in OA among chickpea genotypes in response to developing water deficits has been observed in several studies (Singh *et al.,* 1990; Morgan *et al.,* 1991; Lecoeur *et al.,* 1992; Leport *et al*., 1999; Moinuddin and Khannu-Chopra, 2004).

Saghfi and Eivazi (2014) studied the mechanism of cold stress tolerance in two chickpea cultivars of Flip 93-174 (resistant) and Flip92-169 (susceptible) and observed that proline increased in leaves exposed to cold stress in both cultivars. This increase was more prominent for the resistant cultivar as compared to the susceptible cultivar and same holds for leaf solublecarbohydrates, Glucose, Ramnose, mannose and Fructan for the resistant cultivar.

Proline is a compatible osmolyte occurs widely in higher plants and normally accumulates in large quantities in response to environmental stresses (Sairam and Tyagi 2004; Verbruggen and Hermans 2008). Accumulation of proline has been observed in many studies on plants exposed to abiotic stress (Kaplan *et al.,* 2004; Zuther *et al.,* 2007; Kempa *et al.,* 2008; Kaushal *et al*.,2011). Proline acts as an osmoprotectant and over production of proline results in increase tolerance to osmotic stress (Yoshiba *et al.,* 1997). In addition to proline's role as an osmoprotectants it can also act as a scavenger of free radicals (Saradhi *et al.,* 1995) and replenishes NADP+ supply thereby maintaining the redox potential in the cell (Delauney and Verma 1993). Maller *et al.* (2002) reported that increase in proline accumulation leads to increase tolerance to higher levels of cold stress

Carbohydrates increase inter-cellular concentration and prevent water loss due to cold stress (Mcvicar *et al.,* 2005). Increase in freezing tolerance during cold compatibility period is

due to storage of soluble sugar in plants (Miguelezfrade *et al.,* 2005). Effect of soluble sugars is their acting as a nutritional substance which makes plants survives in low temperatures.

In addition to preserving osmotic pressure inside the cells, these sugars, through binding to two-layer lipid membrane, protect cellular membrane from damages arising from water loss, freezing and phosphorylation of lipid membranes (Yuanyuan *et al.,* 2009). In other words, any increase in accumulation of soluble sugars in the cell, promotes membrane stability against cold. Membrane stability is a prerequisite for making a cell resistant to freezing.

Prado *et al.* (2000) reported that an adaptation to abiotic stress has been attributed to the stress induced increase in carbohydrates level. The most abundant, accumulated sugar in response to low temperature is sucrose (Kaurin *et al.,* 1981).

Leaf measurement of chlorophyll fluorescence can give quantitative assessment of inhibition or damage to electron transfer (Li *et al*., 2009). The technique is rapid, sensitive, non-destructive, relatively cheap and able to detect the injury even before visible symptoms appear (Maxwell and Johnson 2000). Chilling cause inhibition of chlorophyll synthesis which may have an inhibitory effect on the progress of photosynthesis. Unfavorable environmental conditions also effect movement of the products of photosynthesis at the loading stage, during phloem transport and also during its unloading (Wardlaw 1990). Pinhero and Fletcher (1994) observed decline in total chlorophyll content in corn plants after exposure to the chilling temperature.

Turan and Ekmekci (2011) reported that plants subjected to chilling temperatures after cold-acclimation were more tolerant with respect to chlorophyll fluorescence parameters, and chilling tolerant cultivar had better photosystem II (PSII) photochemical activity. In the chilling treatments, total chlorophyll (a+b) content reduced, especially at 2ºC.

Several physiological traits have shown promise for evaluation against cold stress response in other species (e.g. conductivity measurements of injured tissues, fatty acid composition of membrane lipids) but few reports on their use in grain legumes are available (Stoddard *et al.,* 2006).

2.3.1 Membrane interiority

The cellular membranes play an important function in maintaining integrity of cell, by involving in signal transduction and ion homeostasis under environmental stress (Kaur and Gupta, 2005; Tuteja and Sopory, 2008).

Kaur *et al.* (2011) reported that the stress injury measured as oxidative stress, electrolyte leakage, loss of chlorophyll and decrease in leaf water content was mitigated significantly in proline- chilling stressed treated plants.

When chickpea seedlings were subjected to temperatures ranging from 1 to 7ºC, that chilling injury was observed as electrolyte leakage (EL) and 2,3,5-triphenyltetrazolium (TTC) reduction activity. Calcium appeared to alleviate the chilling-induced primary effects like

membrane damage through more effective diminution of oxidative damage while ABA probably acted through modulation of solute levels. The recovery process was facilitated more with calcium as compared to ABA treatment (Nayyar *et al.,* 2005).

Talebi *et al.* (2013) studied on 35 chickpea genotypes and observed that abiotic stress cause membrane injury. Deleterious responses to abiotic stress can include reduction of growth, decrease in chlorophyll, increase in hydrogen peroxide, which causes lipid peroxidation and consequently membrane injury (Mukherjee and Choudhuri, 1983).

2.4 Biochemical studies

The generation of reactive oxygen species (ROS) is one of the earliest biochemical responses of eukaryotic cells to biotic and abiotic stresses. The production of ROS in plants, known as the oxidative burst, is an early event of plant defense response to water-stress and acts as a secondary massager to trigger subsequent defense reaction in plants. ROS, which include oxygen ions, free radicals and peroxides, form as a natural by product of the normal metabolism of oxygen and have important role in cell signaling. However, during abiotic stress, ROS levels increase dramatically resulting in oxidative damage to proteins, DNA and lipids (Apel and Hirt, 2004). Being highly reactive, ROS can seriously damage plants by increasing lipid peroxidation, protein degradation, DNA fragmentation and ultimately cell death.

Lipid peroxidation is a natural metabolic process under normal aerobic conditions and it is one of the most investigated consequences of ROS action on membrane structure and function (Blokhina *et al*., 2003). Lipid peroxidation is a commonly utilized stress indicator of membrane damage. Farooq *et al*. (2009) observed that abiotic stress induces oxidative stress in plants by generation of reactive oxygen species (ROS).The ROS such as O_2^-, H_2O_2 and $^{\bullet}OH$ radicals, can directly attack membrane lipids and increase lipid peroxidation (Mittler, 2002) and overproduction of ROS increases the content of malondialdehyde (MDA) .The content of MDA has been considered an indicator of oxidative damage (Moller *et al.,* 2007). MDA is considered as a suitable marker for membrane lipid peroxidation. A decrease in membrane stability reflects the extent of lipid peroxidation caused by ROS. Furthermore, lipid peroxidation is an indicator of the prevalence of free radical reaction in tissues. Moreover, oxygen uptake loading on the tissues as both processes generate reactive oxygen species, particularly H_2O_2 that produced at very high rates by the glycollate oxidase reaction in the peroxisomes in photorespiration.

There is a defensive system in plants, that is to say, plants have an internal protective enzyme-catalyzed clean up system, which is fine and elaborate enough to avoid injuries of active oxygen, thus guaranteeing normal cellular function (Horváth *et al*., 2007). The balance between ROS production and activities of antioxidative enzyme determines whether oxidative signaling and/or damage will occur (Moller *et al*., 2007). To minimize the affections of oxidative stress, plants have evolved a complex enzymatic and non-enzymatic antioxidant

system, such as low-molecular mass antioxidants (glutathione, ascorbate, carotenoids) and ROS-scavenging enzymes [superoxide dismutase (SOD), peroxidase (POX), catalase (CAT), ascorbate peroxidase (APX)] (Apel and Hirt, 2004). Non-enzymatic antioxidants cooperate to maintain the integrity of the photosynthetic membranes under oxidative stress. The enzymatic components may directly scavenge ROS or may act by producing a non-enzymatic antioxidant.

Yang *et al.* (2009) exhibited that in abiotic stress, increased specific activities of CAT, SOD and POX destruction of $O_2^-$and H_2O_2 in plant cells requires the concerted action of antioxidants. O_2^-can be dismutated into H_2O_2 by SOD in the chloroplast, mitochondrion, cytoplasm and peroxisome. POX plays a key role in scavenging H_2O_2 which was produces through dismutation of O_2^-catalyzed by SOD.

2.5 Pollen viability

Low temperature during early flowering leading to excessive floral abortion is a major cause of low pod and seed set in chickpea (Saxena, 1980; Srinivasan *et al.*, 1998, 1999). Such loss is considered an adaptive mechanism that stimulates vegetative growth and provides additional nodes for production of flowers and pods (Saxena, 1984), this is only true in environments that are not limited by soil moisture towards the end of growing season. Chohan and Raina, 2011 observed that pollen viability percentage at 5 ºC and 10 ºC temperatures were recorded highest in chickpea chilling tolerant genotypes as compared to sensitive genotypes.

Sharma and Singh (2013) reported that low temperature less than 5°C affected the pollen fertility. However, if minimum temperature falls below 3°C and that too for 3 to 4 days consecutively, pollen viability was greatly reduced.

Srinivasan *et al.* (1999) studied on four chickpea genotypes and observed distinct genetic variation in pollen viability. The greater pod-setting ability of tolerant lines (ICCVs 88502 and 88503) than the sensitive cultivars (Annigeri, Pant G 114, etc.) was associated with a higher pollen viability at low temperature in the former.

The chilling stress (<10 ºC) at reproductive phase of chickpea results in abortion of flowers and pods leading to poor yield (Kumar *et al.*, 2011). Kaur *et al.* (2011) observed that the proline-treated chilling-stressed plants showed significant improvement in retention of flowers and pods leading to better seed yield compared to the untreated ones. The proline-applied plants also had greater pollen viability.

Clarke *et al.* (2004) recognized the flower colour as marker for chilling tolerance and demonstrated that pollen from purple chilling tolerant parent was 4-5 times more competitive than pollen from white chilling sensitive parent at low temperature. Pollen viability though did not show appreciable reduction but was considerably lower in aborted flower. This should be due to some inhibitory effect on pollen maturation stage (Srinivasan *et al.*, 1999).

2.6 Yield and yield attributes

Cold stress is one of the most significant abiotic stresses of agricultural plants, affecting both plant development and yield (Lang *et al.,* 2005). It is a major factor in determining the natural distribution of plants (Repo et al., 2008), and phenology and yield potential of agricultural crops (Hayashi, 2001). Cold temperatures are the cause of enormous agricultural losses, especially in sub-tropical and temperate grain crops. The reproductive period is a vital phase in the life cycle of all annual plants, and metabolism during this phase ultimately determines crop yield. Plants exposed to cold temperature during reproduction show reduced metabolic rates leading to low yields (Thakur *et al.,* 2010).

Low temperature-induced yield reduction is a common phenomenon in many crops (Nahar *et al.,* 2009; Kalbarczyk, 2009; Riaz-ud-dinsubhani *et al.,* 2010; Kumar *et al.,* 2011). Thakur *et al.,* (2010) concluded that low temperature often causes flower abortion, pollen and ovule infertility, breakdown of fertilization, poor seed filling, decreases in seed setting which ultimately reduce the grain yield.

The retarding effect of low temperature on the emergence, growth and flowering of legumes has been reported for chickpea (Croser *et al.,* 2003; Regan *et al.,* 2006) and in common bean (Melo *et al.,* 1997). Gunawardena *et al*. (2003) demonstrated that when rice plants encountered low temperature during the reproductive stage reduces the yield.

A declining trend in number of pod set and distinct genetic variation in pod set was observed in response to decreasing temperatures in chickpea cultivars (Srinivasan *et al.,* 1998). Iquebal *et al*. (2012) studied 44 chickpea genotypes and observed genetic variability and association of different traits towards yield. Mallu *et al.* (2014) evaluated sixty chickpea genotypes and observed that in abiotic stress decline in the yield and its attributes viz. number of pods per plant, test weight and seed yield. Maqbool *et al*. (2010) observed that in cool season grain legume crops, chickpea, lentil and faba bean and field pea are the most susceptible to radiant frost injury during the reproductive stages and to a drastic reduction in yield and quality.

CHAPTER – III

MATERIAL S AND METHODS

The experiment was conducted in the experimental area of Pulses Section, Department of Genetics and Plant Breeding, College of Agriculture and the laboratory work was carried out in the Department of Botany and Plant Physiology, College of Basic Sciences and Humanities, Choudhary Charan Singh Haryana Agricultural University, Hisar, 125 004, Haryana. Six chickpea genotypes namely ICCV 88506 (Tolerant, National check), HC-1, H03-56, H07-120, H08-71 and H09- 96 differing in their sensitivity were selected for the experiment. Seeds of chickpea genotypes were sown on 15th October, 2015 (early sown condition in Haryana) to expose the plants to chilling temperature at flowering stage. Seeds of selected chickpea genotypes were grown in a randomized block design (RBD) with three replications in the field of pulses section. The experimental plot was 1.2m x 4m in size (Plate 2). The chickpea genotypes were evaluated for their growth behavior, physiological, biochemical and yield traits on the basis of their adaptability to low temperature in the cropping season *Rabi* 2015-16.

3.1 Climate and weather conditions of experimental location

Hisar is located in Indo Gangetic plains of North-West India at 215.2 meters above mean sea level with latitude of 29° 10′ North and longitude of 75° 46′East. The climate of Hisar can be classified as tropical, semiarid and hot which is mainly dry with very hot summer and cold winter except during monsoon when moist air of oceanic origin penetrates into the district. There are four seasons in a year. The hot weather season starts from mid March to last week of the June followed by the south west monsoon which lasts upto September. The transition period from September to October forms the post monsoon season. The winter season starts late in November and remains upto first week of March. The average rainfall varies from 300-500 mm and the total rains as well as its distribution are subjected to great variations. About 80-90 per cent of the total rains are received from South-West monsoon during the month of July to September. The minimum temperature in this area reaches upto 0.5 ºC in December and January and the maximum temperature in the area reaches upto 48 ºC during May or June. Weekly weather parameters recorded at observatory of the CCS Haryana Agricultural University, during *Rabi* 2015-16 are depicted in table 3.1 and figure 3.1.

3.1.1 Air temperature

Weekly mean maximum temperature prevailed in *Rabi* 2015-16 (40th to 15th standard meteorological weeks) ranged from 15.3-38.6 °C, whereas, the weekly mean minimum temperature varied from 2.4-21.2 °C.

EXPERIMENTAL VIEW

(A) 50-60 days after sowing (DAS)

(B) 80-90 days after sowing (DAS)

Plate 2: Experimental view of chickpea genotypes under early sown condition

3.1.2 Relative humidity

The weekly morning relative humidity for the season varied from 70.0-98.4 per cent, whereas the weekly evening relative humidity varied between 21.1-86.0 per cent throughout the season.

3.1.3 Wind velocity

Monthly mean wind velocity for the season was 2.7 km h^{-1} which was less than normal (4.0 km h^{-1}).Fastest wind blew in 11^{th} SMW with velocity of 5.1 km h^{-1}.

3.1.4 Sunshine hours

The average sunshine hours during the season were 6.7 h day^{-1}, which was below normal (7.9). The maximum sunshine hours were recorded in 12^{th} SMW (March) with a value of 9.9 h day^{-1}, while the minimum sunshine hours were recorded in 4^{th} SMW (January) with a value of 1.8 h day^{-1}.

3.1.5 Pan evaporation

The average pan evaporation was recorded as 2.7 mm day^{-1}, which was less than the normal (4.2 mm day^{-1}). The minimum pan evaporation was 0.7 mm day^{-1} in 2^{nd} SMW (January) and highest pan evaporation was 6.7 mm day^{-1} in 40^{th} SMW (October).

3.1.6 Rainfall

The total rainfall received at Hisar during the *Rabi* 2015-16 was 280.3 mm which was nearly five times more than normal (61.4 mm). Average rainfall received during the season was 1.4 mm day^{-1}. Crop received the maximum rainfall during 9^{th} SMW, which was 16.5 mm day^{-1}.

3.2 Observations

In this experiment, the various observations with regard to morpho-physiological, biochemical and yield traits of chickpea genotypes were recorded. Five plants were randomly selected in each plot. The field observation were recorded after 4 days exposure to temperature below 5 ºC (chilling stress) and above 5 ºC (control) (Fig. 3.2)

3.2.1 Phenological Traits

3.2.1.1 Days to 50% flowering

Considering the overview of each plot total number of days taken by the plant from the date of sowing to the period of 50% flowering was recorded.

3.2.1.2 Days to 50% podding

The number of days from seeding to the formation of 50% pods in the plots was recorded.

3.2.1.3 Days to physiological maturity

The number of days taken from seeding to physiological maturity in selected plants was recorded.

Table 3.1: Standard meteorological weekly data of the experimental site (October 2015- April 2016)

SMW	Temperature (°C)		Relative humidity (%)		Average wind velocity (km h^{-1})	Bright sunshine (h)	Pan evaporation (mm day^{-1})	Rainfall (mm)
	Max.	Min.	M	E				
40	37.7	21.2	70.0	23.0	4.0	9.2	6.7	0.0
41	34.6	19.0	83.0	42.0	3.7	7.4	4.9	0.0
42	29.6	13.2	75.7	32	0.8	7.9	3.6	0.3
43	33.8	15.7	72.0	34	1.0	7.7	3.7	0.0
44	30.2	14.3	89	23	1.8	5.7	3.8	0.0
45	30.2	15.4	88	53	1.8	7.0	3.5	0.0
46	29.9	12.7	77	53	2.4	7.4	3.5	0.0
47	28.4	8.9	82	21.1	1.2	8.3	2.8	0.0
48	25.2	10.5	88	32	2.5	8.3	2.9	0.0
49	26.7	8.1	91	35	1.9	8.4	2.6	0.0
50	21.6	6.0	88	57	2.9	5.0	1.6	0.8
51	20.2	2.4	91	63	5.0	4.2	0.9	0.0
52	23.4	4.8	91	49	1.2	5.5	1.0	0.0
1	17.1	6.6	95	67	1.8	3.3	1.0	0.5
2	22.4	7.5	98.4	65	0.5	3.8	0.7	0.2
3	15.3	4.5	92	63	3.1	5.5	1.1	0.0
4	21.4	4.6	93	61	2.2	1.8	0.9	2.9
5	22.6	6.6	91	67	3.0	6.8	1.9	0.0
6	23.7	4.4	90	57	1.3	8.4	2.1	0.0
7	22.8	7.0	91	41	2.0	7.6	2.3	0.0
8	28.0	9.4	81	31	2.1	5.9	2.4	0.0
9	30.9	10.4	91	35	3.7	6.7	1.6	16.5
10	29.9	12.8	89	37	3.0	7.7	3.0	4.3
11	30.4	13.7	89	86	5.1	5.1	2.0	2.9
12	33.5	14.4	72	59	3.0	9.9	4.3	0.0
13	34.8	15.2	70	38	4.1	7.2	3.3	1.9
14	38.6	20.1	85	55	4.2	8.5	3.7	9.2
15	37.4	17.9	76	43	3.7	7.6	4.8	0.5

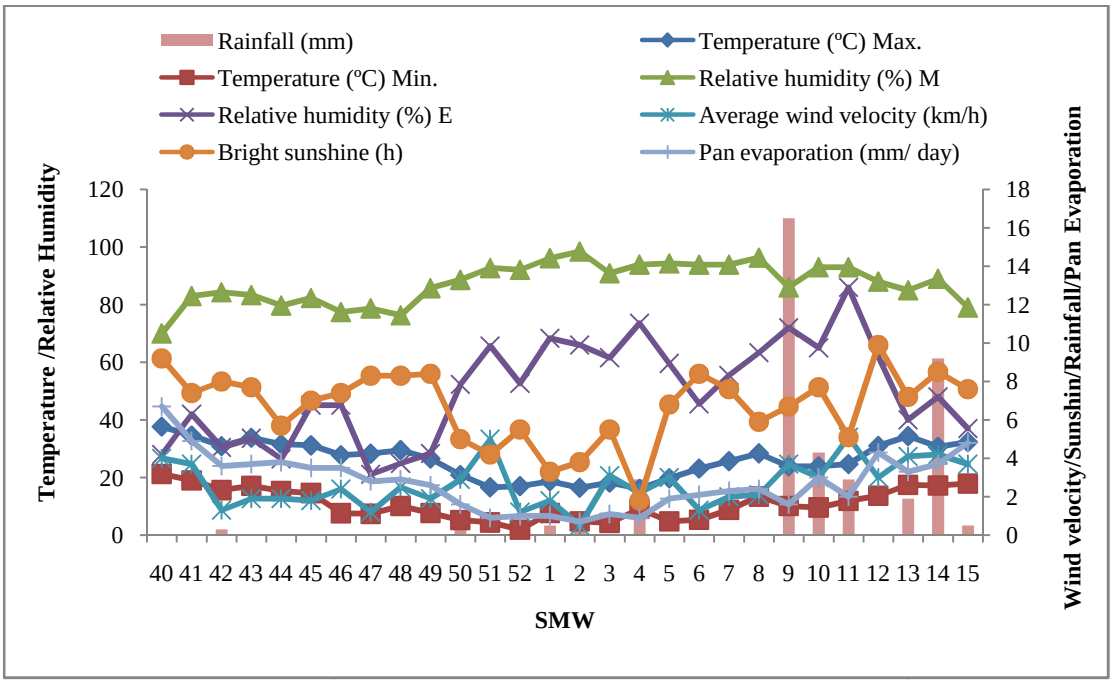


Fig. 3.1: Mean weekly meteorological data (October 2015- April 2016)

Source- Department of Agricultural Meteorology, CCS HAU, Hisar, Haryana, India.

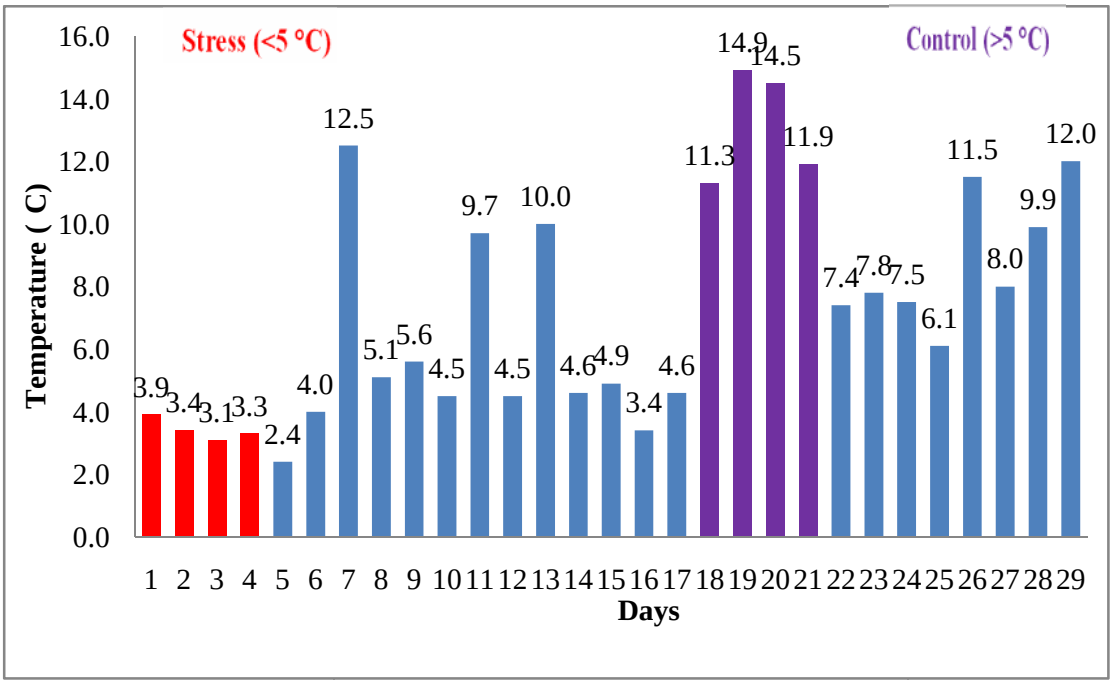


Fig. 3.2: Profile of daily minimum temperature of the field during February, 2016 when observations were recorded

3.2.2 Growth parameters

3.2.2.1 Plant height (cm)

The perpendicular distance from the ground level to the tip of the plant was measured in centimeter at different growth stages *i.e* 30, 60, 90 and120 DAS (days after sowing).

3.2.2.2 Branches plant^{-1} (no)

The number of branches emerging directly from the main stem counted at different growth stages *i.e* 30, 60, 90, and 120 DAS on five randomly selected plants and the average was recorded.

3.2.2.3 Dry weight of stem and leaves plant^{-1} (g)

The five randomly selected plants from each plot were uprooted and sun dried. The dry weight of stem and leaves was taken and average was recorded at growth stages 30, 60, 90 and 120 DAS.

3.2.2.4 Crop growth rate (g m^{-2}day^{-1})

Crop growth rate (CGR) indicates increase in dry weight (W) of plant in a unit time (T) per unit land area (P). The five randomly selected plants from each plot were used to record the dry matter production at 30, 60, 90 and 120 DAS. These samples were first sun dried and then kept in an oven at 65±5 ºC till a constant weight was achieved. The average was recorded as dry matter (g) per plant. CGR was measured at 30, 60, 90, and 120 DAS by using the following formula (Reddy and Reddy, 2009):

$$CGR = (W_2 - W_1) / P (T_2 - T_1)$$

Where, P is the land area and W_1 and W_2 are dry weights at T_1 and T_2 time, respectively.

3.2.2.5 Relative growth rate (RGR) (g g^{-1}day^{-1})

Relative growth rate (RGR**)** indicates the amount of growing material per unit dry weight of plant per unit time. It is also called efficiency index. It represents the rate of growth per unit dry matter. The five randomly selected plants from each plot were used to record the dry matter production at 30, 60, 90 and 120 DAS. These samples were first dried under the sun and then kept in an oven at 65±5 ºC till a constant weight was achieved. The average of five plants was recorded as dry matter g plant^{-1}. RGR was measured at 30, 60, 90 and 120 DAS by the following formula (Reddy and Reddy, 2009):

$$RGR = (\text{Loge}W_2 - \text{Loge}W_1) / (T_2 - T_1)$$

Where, W_1 and W_2 are dry weights at T_1 and T_2 time, respectively.

3.2.3 Physiological parameters

3.2.3.1 Water potential (Ψw)

Water potential of leaves was measured with the help of Pressure Chamber (Model 3005, Soil Moisture Equipment Corporation, Santa Barbara, CA, USA), between 10:00 AM to 11:30 AM. The third fully expanded leaf from the top was excised with the help of sharp edged knife and sealed in the pressure chamber one by one with the cut end protruding outside. Pressure was developed till the sap just appeared at the cut end of leaf and this pressure (-MPa) was recorded as water potential.

3.2.3.2 Osmotic potential (Ψs)

Osmotic potential was determined using Psychrometric Technique (Model 5100- B Vapor Pressure Osmometer, Wescor Inc. Logan, Utah, USA). The third leaf from the top was stored in air tight microcentrifuge tubes. The leaves frozen at -20^{0}C and crushed at room temperature. A filter paper disc was immediately dipped in the sap and placed in the concave depression of the sample holder, avoiding the touching of wet disc on the outer surface of the sample holder. Then pushed the sample slide gently into the chamber of instrument. The chamber was sealed by rotating the knob clockwise. After about one and half minutes a 'beep' tone was sounded. The osmotic potential reading (m Os kg^{-1}) displayed on the digital meter were recorded.

The osmometer was calibrated by using Osmolality Reference Standards of Sodium Chloride (WescorInc, USA) and the calculations were done as follows:

40 Osmol = -1 bar

-10 bar = -1 Mpa

3.2.3.3 Relative water content (RWC %)

The plants were sampled and third fully expanded leaf from the top was excised from the shoots. Sand was removed with the help of a soft camel hair brush. Then the leaves weighed immediately to take their fresh weight and kept in petri dishes filled with distilled water for 3 h. After that the leaves (fully turgid) were weighed again and then kept in oven at 85°C for 72 h or until a constant dry weight. These three weights were used to calculate RWC (%) of leaves according to the formula given by Weatherley, (1950):

RWC (%) = [(Fresh weight - Dry weight) / (Turgid weight - Dry weight)] ×100

3.2.3.4 Photochemical efficiency/quantum yield of PS-II

Photochemical efficiency was determined by using a amplitude modulated OS- 30P (Optic Science, Inc., Hudson, USA), at morning 9.00 to 11.00 AM by Chlorophyll Fluorometer. The clamps of the instruments were installed on the leaves to keep the leaves in the dark and to stop the light reaction of photosynthesis for 20 min. After this, the clamps were attached to the optic fiber of the device and the valves of clamped were opened. After starting the device, the 695 nm modulated light was radiated through the optic fiber towards the leaf. Subsequently, the Fv/Fm ratio that appeared on the instrument was recorded.

3.2.3.5 Relative stress injury (RSI %)

Membrane injury index was measured as percent proportion of ion leakage in to the external aqueous medium to the total ion concentration of the stressed tissue as measured by the EC of the external medium (Sullivan and Ross, 1979).

Procedure

The 200 mg of leaf were kept in 20 ml vials containing 10 ml de-ionized water at 27°C. After 5 h, the electrical conductivity (EC) of the surrounding solution was measured

and designated as EC_1. Then the samples were kept in boiling water bath for 50 min to achieve total killing of the tissue. After cooling, the EC of the solution was again measured and designated as EC_2. The membrane injury index was calculated as follows:

$$\textbf{RSI}\ (\%) = (EC_1 / EC_2) \times 100$$

3.2.3.6 Chlorophyll content and Chlorophyll stability index (CSI %)

Chlorophyll content was estimated according to the method of Hiscox and Israelstam (1979) using dimethyl sulfoxide (DMSO).

Procedure

Third leaf from the top of plant was detached and weighed and was kept into a test tube containing 5 ml of DMSO. The test tube was then placed into oven at 60ºC for about 2 h or more (if required) to facilitate the extraction of pigment. After 2 h and attaining the room temperature the absorption was read at 645 and 665 nm on a computer added spectrophotometer. DMSO was used as blank. Calculations for different pigments were made according to Welburn (1994).

For estimation of photosynthetic pigments, 30 mg of freshly harvested young fully expanded leaf (3^{rd}) from top was taken in test tube containing 3 ml of DMSO. These tubes were then placed in an oven at 60ºC for 2 h to facilitate the extraction of the pigments. In another set, 30 mg of above mentioned leaves were taken separately in test tubes containing 10 ml of de-ionized water, and heated at 65^0C for 30 min in a water bath. After cooling, leaves were taken out of water, blotted dry and transferred to test tubes containing 5 ml of DMSO and processed for extraction of pigments as explained above. After incubation, tubes were cooled to the room temperature and absorbance of these pigment extract was read at 645 and 665 nm as a described by Kaloyereas, (1958) on a computer aided spectrophotometer (Systronic India Spectrophotometer 117) running a multiple wavelength programme. DMSO was used as blank.

Chl ‘a’ (µg/ml) $= 12.19\ A_{665} - 3.45\ A_{645}$

Chl ‘b’ (µg/ml) $= 21.99\ A_{645} - 3.32\ A_{665}$

Total chlorophyll = Chl ‘a’ + Chl ‘b’

Quantity of all these pigments were calculated in µg/gm tissue dry weight expressed as µmole g^{-1} tissue dry weight by using following relationship.

µmoles of Chl ‘a’ = mg Chl ‘a’ x 1.119

µmoles of Chl ‘b’ = mg Chl ‘b’ x 1.102

µmoles of total chlorophyll = Chl ‘a’ (µmoles) + Chl ‘b’ (µmoles)

CSI (%) = [(Total chlorophyll of non heated samples) / (Total chlorophyll of heated samples)] X 100

3.2.4 Biochemical parameters

3.2.4.1 Lipid peroxidation [In terms of MDA content]

The level of lipid peroxidation was measured in terms of malondialdehyde (MDA) present in the leaves tissues MDA is a product of lipid peroxidation and was measured by thiobarbituric acid (TBA) reaction with minor modifications of the method of Heath and Packer (1968).

Reagents

(i) 0.1% Trichloroacetic acid (TCA)

(ii) 20 % TCA containing 0.5 % thiobarbituric acid (TBA)

Extraction

Three hundred mg of fresh leaves were homogenized separately with 5 ml of 0.1 % TCA. The homogenate was centrifuged at 8000 x g for 15 min. The supernatant was collected and then directly used for the assay.

Procedure

One ml of the supernatant was taken in a test tube and precipitated by 4 ml of 20 % TCA containing TBA. The mixture was heated in a water bath shaker at 95°C for 30 minutes and quickly cooled in an ice-bath. After centrifugation at 8000 x g for 10 minutes the absorbance of the supernatant was read at 532 nm and the value for nonspecific absorption at 600 nm was subtracted. The concentration of MDA was calculated using its extinction coefficient of 155 $mM^{-1}cm^{-1}$.

3.2.4.2 Hydrogen peroxide (H_2O_2)

H_2O_2 content of the leaves was determined by a modified Patterson *et al*. (1984) method.

Reagents

(i) 5 % trichloroacetic acid (TCA)

(ii) 100 mM potassium phosphate buffer (pH 8.4)

Colorimetric reagent: It was prepared by mixing 0.6 mM M4-(2-pyridyzalo) resorcinol and potassium titanium oxalate in 1:1 (v/v) ratio. The mixture was kept once until use.

Extraction

Three hundred mg of leaves were homogenized separately with 0.2 g of activated charcoal and 5 ml of 5 % TCA. The homogenate was filtered through Whatman No.1 filter paper and centrifuged at 8000 x g for 10 min. The supernatant was filtered through three layers of Whatman No.1 filter paper.

Procedure

Two hundred µl of the extract was brought to 4 ml with potassium phosphate buffer. The pH was adjusted to 8.4 with concentrated ammonia solution. Then 2 ml of colorimetric

reagent was added to the reaction mixture. The absorbance was then read at 508 nm. H_2O_2 was determined from the difference in absorbance between sample and blank.

The H_2O_2 content was calculated using its molar extinction coefficient of 3.6x10-4 moles and the H_2O_2 content was expressed as μmoles g^{-1} DW.

3.2.4.3 Proline content

Proline content was estimated by using the method of Bates *et al*. (1973).

Reagents

(i) 3 % aqueous sulphosalicylic acid (w/v)

(ii) Acid ninhydrin (prepared by dissolving 1.25 g ninhydrin in 30 ml glacial acetic acid and 20 ml 6.0 M o-phosphoric acid until dissolved)

(iii) Toluene

Extraction

Three hundred mg of fresh leaves were separately homogenized in 5 ml of 3 % sulphosalicylic acid and then centrifuged at 5000 rpm for 15 minutes and supernatant was collected.

Procedure

2 ml of supernatant was taken in a tests tube and 2.0 ml reagent acid ninhydrin was added. This mixture was then kept in boiling water bath for 1 h at 100 °C and thereafter reaction was terminated by keeping tubes in ice-bath. Then 4.0 ml of toluene was added. After vigorous shaking, the upper coloured organic phase was taken after attainment of room temperature and absorbance was recorded at 520 nm by using toluene as blank. Standard curve was prepared by using graded concentration of proline (20-100 μg/ml). The proline content was expressed as mg g^{-1}DW.

3.2.4.4 Total soluble carbohydrates (TSC)

Total soluble carbohydrates were determined with the method of Yemm and Willis, (1954) using anthrone reagent. Two hundred mg fresh samples of leaf were homogenized separately in 80% ethanol using acid washed sand as an abrasive. The homogenate was refluxed thrice with 80% ethanol. The supernatant from different extraction was pooled and volume made to 5ml with 80 per cent ethanol. The extract so obtained was used for estimation of TSC.

Reagents

Anthrone reagent: Anthrone reagent was prepared by dissolving 0.4g anthrone in 100 ml concentrated $H_2 SO_4$.

Procedure

An aliquot from the above extract measuring 0.2 ml was evaporated to dryness in a test tube in a boiling water bath. On cooling the residue left in the tube was dissolved in one ml of distilled water and mixed with 4.0 ml of the anthrone reagent. The mixture was heated

in a water bath for 10 minutes. After cooling, absorbance was recorded at 620 nm using Spectrophotometer-117. Standard curve was prepared using graded concentration (20-100 µg/ml) of D-glucose and the data were expressed as mg g^{-1} DW of the tissue.

3.2.4.5 Antioxidant enzymes

Extraction: 500 mg of leaves were crushed in 5 ml of 0.1 M potassium phosphate buffer (pH 7.0). The extract was then centrifuged at 10,000 at 4 ^{0}C for 20 minutes. The supernatant was then used for the estimation of the following enzymes:

3.2.4.5.1 Superoxide dismutase (EC1.15.1.1)

SOD activity was estimated by the method of Giannopolitis and Reis, (1997) with little modifications.

Reagents

(i) 60 µM riboflavin

(ii) 17 mM methionine

(iii) 0.5 mM nitrobluetetrazolium (NBT)

(iv) 1.5 M Na_2CO_3

(v) 3mM EDTA

Procedure

The reaction mixture contained 0, 0.2, 0.3, 0.4, 0.5 and 1 ml of enzyme extract (D=5x) in separate sets and to these added 0.5 ml of each of methionine, NBT, EDTA and Na_2CO_3 and the total volume of 2.5 ml was made with buffer in each set adjusting the pH at 10.2 then 0.5 ml of riboflavin was added to each set in the last. The tubes were shaken and placed 30 cm from light source (8 x 20 W fluorescent lamps). The reaction was allowed to run for 10 minutes and then reaction was stopped by switching off the light. The tubes were immediately covered with a black cloth. The absorbance was recorded at 560 nm. A non-irradiated reaction mixture, which did not develop colour, served as control. However, in the presence of SOD the reaction was inhibited and the amount of inhibited was used to quantify the enzyme.

Log A560 was plotted as a function of enzyme extract used in reaction mixture. Form the resultant graph, volume of enzyme extract corresponding to 50% inhibition of the photochemical reaction was obtained and considered as one enzyme unit expressed as units mg^{-1} protein min^{-1}.

3.2.4.5.2 Catalase (EC 1.11.1.6)

Catalase (CAT) activity was estimated by the UV method of Aebi, (1984)

Reagents

(i) 0.1M H_2O_2

(ii) 0.05M potassium phosphate buffer (pH 7.0)

Procedure: Five hundred µl of enzyme extract was taken and to this added 0.2 ml of 100 m M H_2O_2 and 1.5 ml of 50 mM potassium phosphate buffer. The enzyme sample was added immediately at the time of taking the absorbance and incubated for 3 minutes. The change in absorbance was recorded at 240 nm at an interval of 15 seconds for 1.5 minute. The enzyme activity was expressed as unit mg^{-1} protein min^{-1}.

3.2.4.5.3 Peroxidase (EC 1.11.1.7)

Peroxidase **(**POX) activity was estimated by the method of Shannon *et al.* (1996).

Reagents

(i) 0.2 M H_2O_2

(ii) 0.05 M sodium acetate buffer (pH=4.5)

(iii) O-dianisidine (0.25% in methanol)

Procedure:

0.2 ml enzyme extract was taken (D=5x) and to this added 1.8 ml of 0.05 M sodium acetate buffer, 0.5 ml of O-dianisidine and 0.1 ml of 0.2 M H_2O_2 An equivalent volume of buffer substituted for H_2O_2 in the reference. The absorbance was recorded immediately after adding H_2O_2. The change in absorbance was recorded at 470 nm at an interval of 10 seconds upto 2 minutes. Change of 0.01absorbance has been taken as unit.

The enzyme activity was expressed as unit mg^{-1} proteins min^{-1}.

3.2.5 Reproductive parameters

At anther dehiscence stage pollen viability *was* recorded.

3.2.5.1 Pollen viability

Viability of freshly released pollen grains was assessed by 2,3,5 triphenyl tetrazoliumchloride (TTC) test (Hauser and Morrison, 1964).

Reagents

(i) TTC solution: 0.5% TTC (w/v) in 15% sucrose

(ii) Sodium succinate crystals

Procedure

To the TTC solution taken in a test tube and to it few crystals of sodium succinate were added and shaken till a clear solution is formed. A drop of this solution was put on a clean and dry micro-slide and small amount of pollen was sprinkled over TTC drop and cover slip was applied immediately. It was incubated at 35-40 °C for 5 minutes in dark. At the end of the incubation period, preparations were scored for percentage of viable pollen grains (bright red) under a light microscope. Ten observations per replicate and three replicates per treatment were taken for this test. Percentage pollen viability was computed from this data.

Viability (%) = [No. of viable pollen / (No. of viable pollen + No. of non-viable pollen)] x 100

3.2.6 Yield and yield attributes

3.2.6.1 Pods plant^{-1} (no)

The total number of pods obtained from five randomly selected plants was recorded and expressed as number of pods plant$^{-1.}$

3.2.6.2 Pod weight plant^{-1} (g)

Total weight of pods of five plants were counted at harvest and average was recorded.

3.2.6.3 Seeds pod^{-1} (no)

The number of seeds pod^{-1} was averaged from randomly taken ten pods on five randomly selected plants at maturity.

3.2.6.4 Test weight (g)

100 seed were counted randomly from each genotypes and the test weight (g) was recorded.

3.2.6.5 Biological yield plant^{-1} (g)

The completely matured plants were uprooted carefully along with roots and were dried completely. The weight of dried plant along with pods was recorded as biological yield (g plant^{-1}).

3.2.6.6 Seed yield plant^{-1} (g)

The seed weight in grams from each plant was recorded.

3.2.6.7 Harvest index (%)

Harvest index is represented in terms of percentage. The harvest index for each plot was calculated by dividing the economic (grain) yield by the biological yield (seed + stover yield) of the same net plot and multiplied by 100 as given below:

$$HI = (\text{Seed yield} / \text{Biological yield}) \times 100$$

3.2.7 Statistical analysis

For each parameter, three plots having three plants per plot were sampled at a time which comprises of three replicates. Data were analysed using Randomised Block Design (RBD) for two factors. Treatments were compared using critical difference (CD) at 5% level of significance. Data were subjected to analysis of (ANOVA) using Online Statistical Analysis Package (OPSTAT) Computer Section, CCS Haryana Agricultural University, Hisar. Correlation of various parameters with seed yield were determined by using Pearson's correlation coefficient.

CHAPTER – IV

RESULTS

The present investigations were conducted on six chickpea (*Cicer arietinum* L.) genotypes namely ICCV 88506 (Tolerant, National check), HC1, H03-56, H07-120, H08-71 and H09- 96 (differing in their sensitivity to cold) to study morphological, physiological, biochemical, reproductive and yield traits in relation to low temperature tolerance. The plants were raised in the field area of Pulses Section, Department of Genetics and Plant Breeding, Choudhary Charan Singh Haryana Agricultural University, Hisar. The experimental plot was 1.2 m x 4 m in size. The field observation was recorded 4 days after exposure to low temperature below 5^0C (chilling stress) and above 5^0C in the range 5-10 ^{0}C (control). The results obtained are described below:

4.1 Phenology

4.1.1 Days to 50% flowering

There was a significant variation in days to 50% flowering among the six genotypes. Minimum number of days taken for 50% flowering was recorded in H03-56 (78) followed by H07-120(80) and maximum in ICCV-88506 (87) (Table 4.1).

4.1.2 Days to 50% podding

There was significant variation in the days to 50% pod formation in all the six chickpea genotypes. The minimum days taken for 50% pod formation was in H03-56 (94) followed by HC-1 (113) and maximum in ICCV-88506 (133) among various chickpea genotypes studied (Table 4.1).

4.1.3 Days to physiological maturity

The days to maturity among different genotypes is sown in Table. 4.1. The genotypes also showed significant differences for days to maturity in early sown condition. The minimum days taken for physiological maturity were in H03-56 (155) and maximum days taken in ICCV-88506 (172).

Table 4.1: Variation in phenological traits of chickpea genotypes at different days after sowing (DAS)

Genotypes	DAS		
	50%Flowering	50%Podding	Physiological Maturity
HC-1	85	113	166
H03-56	78	94	155
H07-120	80	129	169
H08-71	86	127	169
H09-96	85	127	170
ICCV-88506	87	133	172
CD at 5%	3.3	6.0	7.1

4.2 Growth parameters at different growth stages

4.2.1 Plant height (cm)

The plant height (cm) had significant variation among the six chickpea genotypes. It increased gradually with days after sowing. Maximum plant height 90.6 cm was observed in chickpea genotype H03-56, followed by 87.3 cm in ICCV88506 and minimum 63.3 cm in H07-120 at 120 DAS (Table 4.2).

Table 4.2: Variation in plant height (cm) of chickpea genotypes at different days after sowing (DAS)

Genotypes	DAS			
	30	60	90	120
HC-1	9.0	27.0	40.6	67.6
H03-56	13.3	37.3	55.6	90.6
H07-120	11.0	30.6	41.0	63.3
H08-71	11.6	33.3	54.0	80.3
H09-96	14.3	36.0	56.6	85.6
ICCV-88506	13.6	36.3	58.0	87.3
CD at 5%	1.5	4.4	4.2	9.8

4.2.2 Number of branches plant^{-1}

The number of branches plant^{-1}had significant variation among the six chickpea genotypes. It increased gradually with days after sowing. It was observed maximum in chickpea genotype HC-1, followed by H07-120 and minimum in H03-56 at 120 DAS (Table 4.3).

Table 4.3: Variation in number of branches plant^{-1} of chickpea genotypes at different days after sowing (DAS)

Genotypes	DAS			
	30	60	90	120
HC-1	3.86	4.98	5.66	5.66
H03-56	2.81	3.72	4.06	4.06
H07-120	3.52	4.83	5.40	5.40
H08-71	2.85	3.89	4.33	4.33
H09-96	2.94	4.13	4.95	4.95
ICCV-88506	3.14	4.77	5.25	5.25
CD at 5%	0.07	0.21	0.29	0.24

4.2.3 Dry weight of leaves (g plant^{-1})

All the six chickpea genotypes showed significant variation in dry weight of leaf at different growth stages (Table 4.4). Maximum dry weight of leaves was observed in the chickpea genotype HC-1, followed by H07-120 and minimum in H03-56 at 120 DAS.

Table 4.4: Variation in dry weight (gplant^{-1}) of leaves of chickpea genotypes at different days after sowing (DAS)

Genotypes	DAS			
	30	60	90	120
HC-1	0.098	0.830	4.320	5.120
H03-56	0.045	0.510	3.020	1.930
H07-120	0.089	0.780	4.210	4.860
H08-71	0.056	0.530	3.230	2.570
H09-96	0.069	0.590	3.670	3.660
ICCV-88506	0.081	0.720	4.090	4.340
CD at 5%	0.004	0.037	0.198	0.176

4.2.4 Dry weight of stem (g plant^{-1})

Dry weight of stem had significant variation among the six chickpea genotypes at different growth stages. Maximum dry weight of stem was observed in the chickpea genotype HC-1, followed by H07-120 and minimum in H03-56 at 120 DAS (days after sowing) (Table 4.5).

Table 4.5: Variation in dry weight of stem (g plant^{-1}) of chickpea genotypes at different days after sowing (DAS)

Genotypes	DAS			
	30	60	90	120
HC-1	0.067	0.78	14.79	16.85
H03-56	0.031	0.49	8.47	9.67
H07-120	0.059	0.75	13.96	16.02
H08-71	0.034	0.51	9.41	10.92
H09-96	0.048	0.54	10.17	13.06
ICCV-88506	0.055	0.67	12.61	14.57
CD at 5%	0.002	0.03	0.48	0.81

4.2.5 Crop Growth Rate (g m^{-2}day^{-1})

There was wide variation in crop growth rate of six chickpea genotypes at various growth stages shown in table 4.6. It was observed maximum in chickpea genotype HC-1, followed by H07-120 and minimum in H03-56 genotype at 120 DAS.

Table 4.6: Variation in crop growth rate (g $m^{-2}day^{-1}$) of various chickpea genotypes at different days after sowing (DAS)

Genotypes	DAS			
	0-30	31-60	61-90	91-120
HC-1	0.009	0.061	0.597	0.778
H03-56	0.005	0.039	0.540	0.689
H07-120	0.009	0.060	0.595	0.763
H08-71	0.006	0.044	0.557	0.704
H09-96	0.008	0.053	0.586	0.723
ICCV-88506	0.008	0.058	0.591	0.751
CD at 5%	0.001	0.003	0.029	0.030

4.2.6 Relative growth rate (g g^{-1} day^{-1})

There was significant variation in relative growth rate of various chickpea genotypes at different growth stages. It was observed maximum in chickpea genotype HC-1, followed by H07-120 and minimum in H03-56 genotype at 120 DAS (Table 4.7).

Table 4.7: Variation in relative growth rate (g g^{-1} day^{-1}) of chickpea genotypes at different days after sowing (DAS)

Genotypes	DAS			
	0-30	31-60	61-90	91-120
HC-1	0.031	0.036	0.039	0.009
H03-56	0.016	0.024	0.031	0.002
H07-120	0.026	0.032	0.038	0.008
H08-71	0.017	0.026	0.032	0.003
H09-96	0.019	0.027	0.034	0.004
ICCV-88506	0.022	0.029	0.036	0.006
CD at 5%	0.001	0.001	0.001	0.001

4.3 Physiological parameters

4.3.1 Water potential (ψ_w)

There was decline in ψ_w (-MPa) of leaves in all the six chickpea genotypes after 4 days of exposure to low temperature (<5°C) as compared with the control (>5°C) plants. The water potential ranged from 0.45 to 0.70 and 0.65 to 0.85 in control (>5°C) and stress (<5°C) plants, respectively in different chickpea genotypes (Table 4.8).The chickpea genotype H03-56 showed more negative values of ψ_w *i.e.* -0.85 as compared to HC-1 -0.65 after 4 days exposure to low temperature (<5°C) among different chickpea genotypes. Results with respect to genotypes and temperature levels were statistically significant but the interaction of genotypes and temperature was non significant.

Table 4.8: Effect of low temperature (<5 °C) on water potential (ψ_w, -Mpa) of leaves in chickpea genotypes

Genotypes	Temperature		
	Control (>5 °C)	Stress (<5 °C)	Mean
HC-1	0.45	0.65	0.55
H03-56	0.70	0.85	0.78
H07-120	0.50	0.70	0.60
H08-71	0.65	0.84	0.75
H09-96	0.60	0.80	0.70
ICCV-88506	0.55	0.75	0.65
Mean	0.58	0.76	-
CD at 5 %	Genotypes = 0.08; Temperature =0.07; Genotypes X Temperature =NS		

4.3.2 Osmotic potential (ψ_s)

There was a significant decline in ψ_s (-MPa) of leaves in all the six chickpea genotypes after 4 days of exposure to low temperature (<5 °C) as compared with the control (>5 °C) plants. The osmotic potential ranged from 0.59 to 0.84 and 0.81 to 1.04 in control (>5 °C) and stress (<5 °C) plants, respectively in different chickpea genotypes (Table 4.9). The chickpea genotype H03-56 showed more negative values of ψ_s *i.e.* -1.04 as compared to HC-1 -0.81 after 4 days exposure to low temperature (<5 °C) among different chickpea genotypes. Results with respect to genotypes and temperature levels were statistically significant but the interaction of genotypes and temperature was non significant.

Table 4.9: Effect of low temperature (<5 °C) on osmotic potential (ψ_s, -Mpa) of leaves in chickpea genotypes

Genotypes	Temperature		
	Control (>5 °C)	Stress (<5 °C)	Mean
HC-1	0.59	0.81	0.70
H03-56	0.84	1.04	0.94
H07-120	0.67	0.87	0.77
H08-71	0.78	0.94	0.86
H09-96	0.76	0.92	0.84
ICCV-88506	0.71	0.90	0.81
Mean	0.73	0.91	-
CD at 5 %	Genotypes =0.02 ; Temperature =0.01; Genotypes XTemperature =NS		

4.3.3 Relative water content (RWC %)

Data presented in Table 4.10 that RWC in control (>5 °C) plants varied between 67.9 and 82.3 %. In chilling stressed (<5°C) plants the RWC began to decrease and varied from

52.9 to 76.5 % after 4 DAE. Maximum RWC was noticed in HC-1 (76.5%) and minimum in H03-56 (52.9%) in the low temperature (<5 °C) condition. The overall interaction for RWC between genotypes and temperature was significant.

Table 4.10: Effect of low temperature (<5 °C) on relative water content (RWC %) of leaves in chickpea genotypes

Genotypes	Temperature		
	Control (>5 °C)	Stress (<5 °C)	Mean
HC-1	82.3	76.5	79.4
H03-56	67.9	52.99	60.4
H07-120	75.3	65.2	70.2
H08-71	68.3	57.2	62.8
H09-96	70.5	61.3	65.9
ICCV-88506	72.9	63.7	68.3
Mean	72.8	62.8	-
CD at 5 %	Genotypes =2.4; Temperature =1.4; Genotypes X Temperature =3.3		

4.3.4 Photochemical efficiency/quantum yield of PS-II

There was wide variation in quantum yield with 4 DAE to low temperature (<5 °C) of chickpea genotypes (Table 4.11). The mean value of quantum yield (Fv/Fm) significantly declined from 0.705 to 0.642 with the increasing DAE to low temperature (<5 °C) as compared to control (>5 °C). The maximum Fv/Fm was observed in chickpea genotype HC-1 (0.664), followed by H07-120 (0.650) and minimum in H03-56 (0.628) at 4 DAE to low temperature. Result with respect to genotypes, temperature levels and their interactions were statistically significant.

Table 4.11: Effect of low temperature (<5 °C) on quantum yield (Fv/Fm) of leaves in chickpea genotypes

Genotypes	Temperature		
	Control (>5 °C)	Stress (<5 °C)	Mean
HC-1	0.736	0.664	0.700
H03-56	0.686	0.628	0.657
H07-120	0.715	0.650	0.683
H08-71	0.692	0.631	0.662
H09-96	0.698	0.636	0.667
ICCV-88506	0.704	0.643	0.674
Mean	0.705	0.642	-
CD at 5 %	Genotypes =0.004 ; Temperature =0.002; Genotypes X Temperature =0.005		

4.3.5 Relative stress injury (RSI %)

Significant disturbance in membrane stability was observed in leaves of various chickpea genotypes. The effect of low temperature (<5 °C) on leaves membrane stability of chickpea genotypes is shown in the Fig. 4.1. Stress injury was evaluated in terms of increase in electrolyte leakage. Mean values of RSI increased significantly after 4 days exposure to low temperature in all genotypes *i.e.* from 6.41 to 13.84%. The maximum RSI% was observed in H03-56 (15.63%) and minimum was noticed in HC-1 (10.87%) in stress condition (<5 °C) (Fig. 4.1). There were significant differences among genotypes, temperature and their interaction.

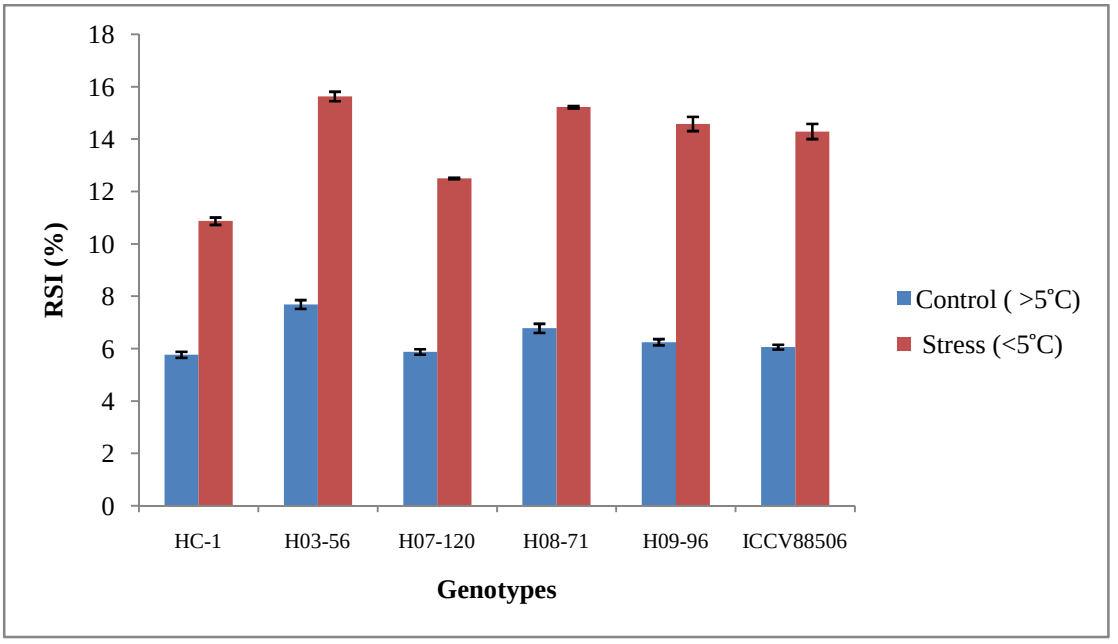


Fig.4.1: Changes in relative stress injury (RSI %) in leaves of chickpea genotypes under low temperature (<5 °C) condition [*Vertical bars indicate ± SE for each treatment*]

4.3.6 Chlorophyll

4.3.6.1 Chlorophyll 'a' content

The chlorophyll 'a' in leaves reduced significantly after 4 days exposure to low temperature in all the six chickpea genotypes. The mean value of Chlorophyll 'a' content decreased from 12.67 to 11.24 mg g^{-1} dry weight after 4 DAE to low temperature. The genotype HC-1 showed less reduction in the pigment concentration as compared to H03-56 among all the six chickpea genotypes in cold stress condition (Table 4.12). The statistical data revealed that the interaction between genotypes and temperature was significant.

Table 4.12: Variation in chlorophyll 'a' content (mg g^{-1}DW) in leaves of chickpea genotypes after exposure to low temperature (<5 °C)

Genotypes	Temperature		
	Control (>5 °C)	Stress (<5 °C)	Mean
HC-1	15.04	14.83	14.94
H03-56	10.16	7.84	9.00
H07-120	13.12	12.56	12.84
H08-71	12.26	9.52	10.89
H09-96	12.56	10.75	11.66
ICCV-88506	12.89	11.93	12.41
Mean	12.67	11.24	
CD at 5 %	Genotypes =0.81 ; Temperature =0.71; Genotypes X Temperature =0.31		

4.3.6.2 Chlorophyll 'b' content

Table 4.13 show the chlorophyll 'b' content in leaves decreased significantly after 4 days exposure to low temperature. The mean value of Chlorophyll 'b' content decreased from 4.17 to 3.67 mg g^{-1}dry weight after 4 DAE to low temperature(<5 °C) with respect to control (>5 °C). The genotype HC-1 showed less reduction in the pigment concentration in cold stress as compared to H03-56 among all the six chickpea genotypes. The interaction genotypes and temperature was significant.

Table 4.13: Variation in chlorophyll 'b' content (mg g^{-1} DW) in leaves of chickpea genotypes after exposure to low temperature (<5 °C)

Genotypes	Temperature		
	Control (>5 °C)	Stress (<5 °C)	Mean
HC-1	5.03	4.86	4.95
H03-56	3.47	2.32	2.90
H07-120	4.28	4.04	4.16
H08-71	3.95	3.14	3.55
H09-96	4.09	3.68	3.89
ICCV-88506	4.21	3.98	4.10
Mean	4.17	3.67	-
CD at 5 %	Genotypes =2.32 ; Temperature =1.04; Genotypes X Temperature =1.14		

4.3.6.3 Chlorophyll stability index (CSI %)

Chlorophyll stability index (%) decreased after 4 days exposure to low temperature (<5 °C) in all the six genotypes and the mean value decreased from 69.07 to 65.44 with respect to control (>5 °C). It was highest in HC-1 (73.22) and lowest in H03-56 (61.00) after 4 DAE to low temperature (<5°C) as compared to control (>5°C) (Table 4.14). CSI follows

the similar trend as that of the total chlorophyll content. The statistical data revealed that the significant differences among genotypes, temperature levels and their interaction was found to be non significant.

Table 4.14: Variation in chlorophyll stability index (CSI %) of leaves in chickpea genotypes after exposure to low temperature (<5 °C)

Genotypes	Temperature		
	Control (>5 °C)	Stress (<5 °C)	Mean
HC-1	77.33	73.22	75.28
H03-56	62.66	61.00	61.83
H07-120	72.48	68.01	70.25
H08-71	65.91	61.83	63.87
H09-96	67.01	63.33	65.17
ICCV-88506	69.00	65.22	67.11
Mean	69.07	65.44	-
CD at 5 %	Genotypes =2.56 ; Temperature =1.47; Genotypes X Temperature =NS		

4.4 Biochemical parameters

4.4.1 Lipid peroxidation

Lipid peroxidation was measured in terms of malondialdehyde (µ mol MDA g^{-1} DW) content. The mean values of MDA content in chilling stressed (<5°C) plants showed marked increase (20.26) over the control (>5°C) plants (14.23) in all the chickpea genotypes (Fig. 4.2). Maximum MDA content was measured in H03-56 (24.18) and minimum in HC-1 (17.34) in chilling stressed plants. There were significant differences among genotypes, temperature and their interaction.

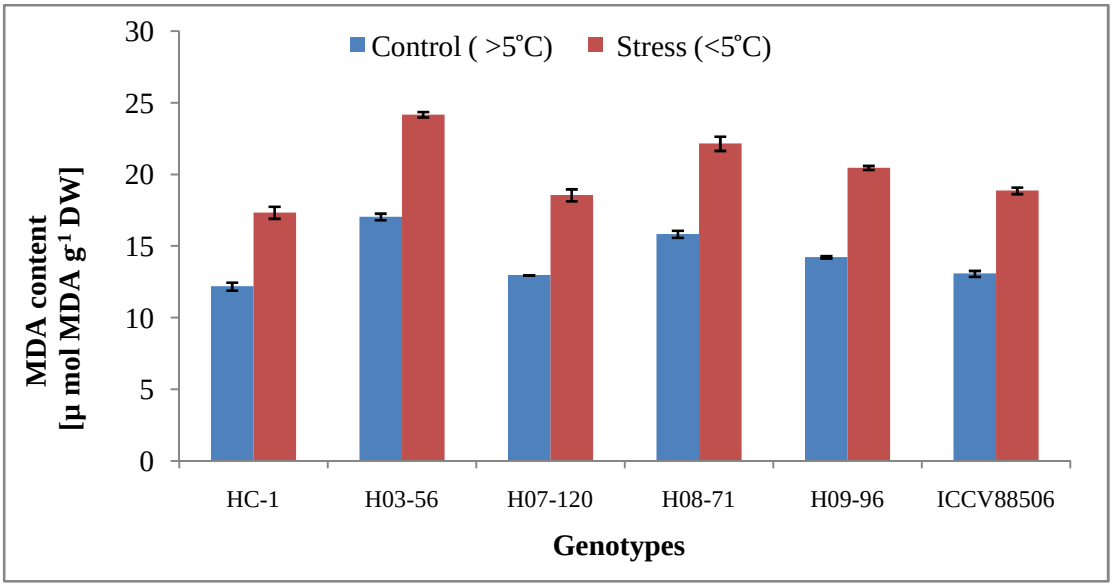


Fig.4.2: Changes in malondialdehyde (MDA) content in leaves of chickpea genotypes under low temperature (<5 °C) condition [*Vertical bars indicate ± SE for each treatment*]

4.4.2 Hydrogen peroxide (H_2O_2) content

It is obvious from the data presented in Fig.4.3 that the hydrogen peroxide content (µ moles g-1 DW) increased after 4 DAE to low temperature (<5 °C) in all the chickpea genotypes over the control (>5 °C) plants. The H_2O_2 content observed maximum in H03-56 (0.636) and minimum in HC-1 (0.556) in stress (<5 °C) condition. The overall interaction between genotypes and temperature was statistically significant.

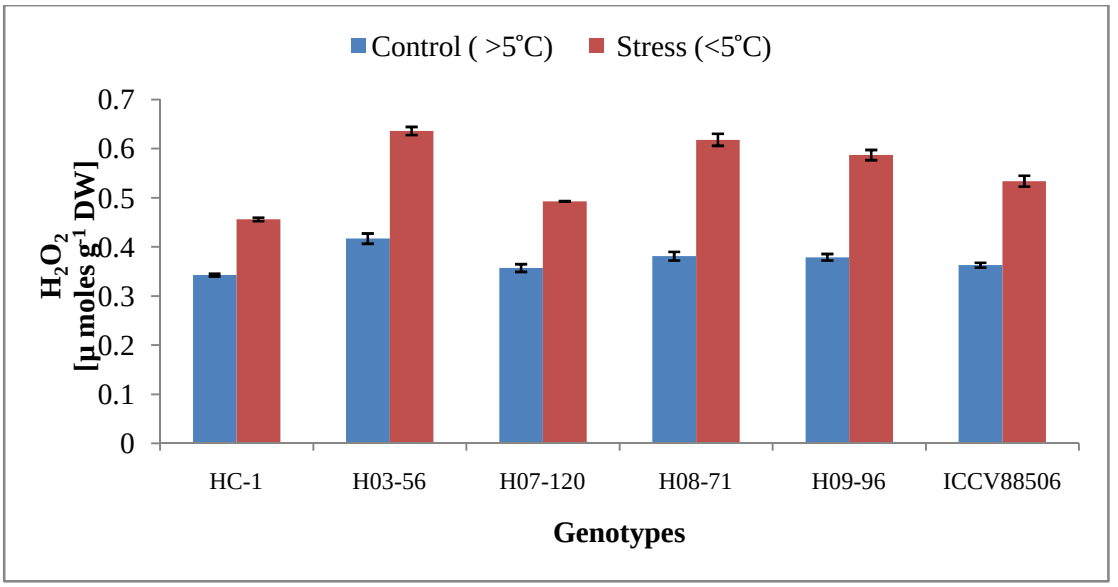


Fig.4.3: Changes in hydrogen peroxide (H_2O_2) content in leaves of chickpea genotypes under low temperature (<5 °C) condition [*Vertical bars indicate ± SE for each treatment*]

4.4.3 Proline content

The changes in proline content (mg g^{-1} DW) after 4 days exposure to low temperature (<5 °C) in leaves of chickpea genotypes were represented in Fig.4.4. There was significant differences in proline content of leaves and it increased from 0.691 to 0.850 with increasing 4 DAE to low temperature (<5 °C) as compared to control (>5 °C). The highest proline content was observed in HC-1 (0.847) and lowest in H03-56 (0.702) in stress (<5 °C) as compared to control (>5 °C) plants. There were significant differences among genotypes, temperature and their interaction was non significant.

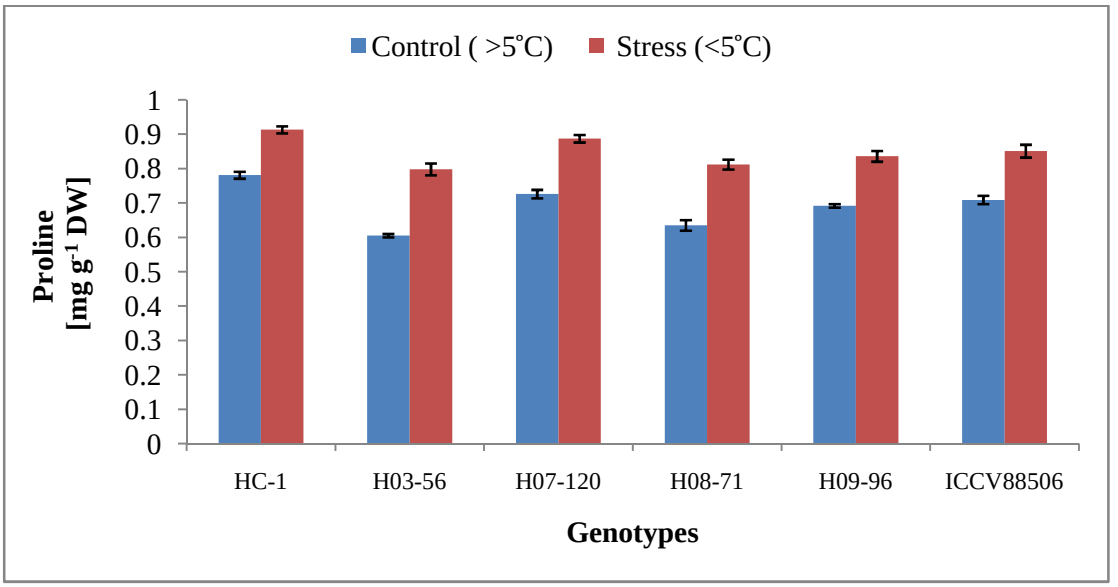


Fig.4.4: Changes in proline content in leaves of chickpea genotypes under low temperature (<5 °C) condition [*Vertical bars indicate ± SE for each treatment*]

4.4.4 Total soluble carbohydrates (TSC)

The change in the level of TSC content after 4days exposure to low temperature (<5°C) in leaves of chickpea genotypes in shown in Fig. 4.5. The TSC increased significantly 4 days after exposure to low temperature (<5°C) as compared to control (>5 °C). The genotype HC 1 maintained higher (20.55) TSC and lowest in H03-56 (18.26) in stress condition (<5 °C).The interaction of genotypes and temperature was found to be significant.

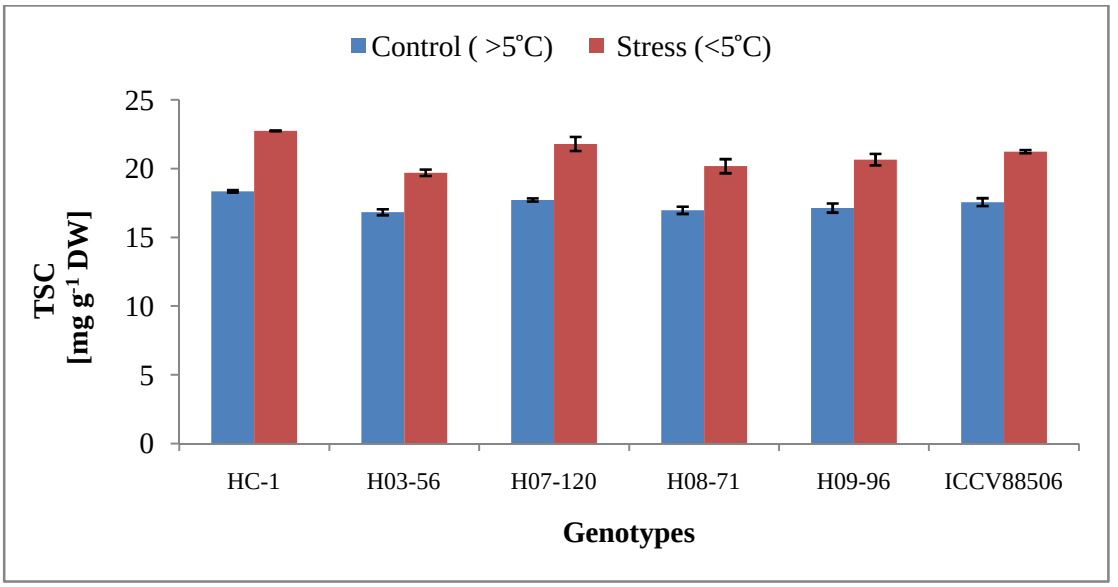


Fig. 4.5: Changes in total soluble carbohydrates (TSC) content in leaves of chickpea genotypes under low temperature (<5 °C) condition [*Vertical bars indicate ± SE for each treatment*]

4.4.5 Antioxidant enzymes

4.4.5.1 Superoxide dismutase (SOD)

After 4 days exposure to low temperature (<5 ^{O}C) the specific activity of SOD (mg^{-1} protein min^{-1}) in leaves was found to be increased as compared to control (>5 ^{O}C) temperature condition in all the six genotypes. HC-1observed maximum (16.50 units mg^{-1} protein min^{-1}) and H03-56minimum (11.87 units mg^{-1} protein min^{-1})specific activity of SOD in stress (<5 ^{O}C) as shown in Fig. 4.6.

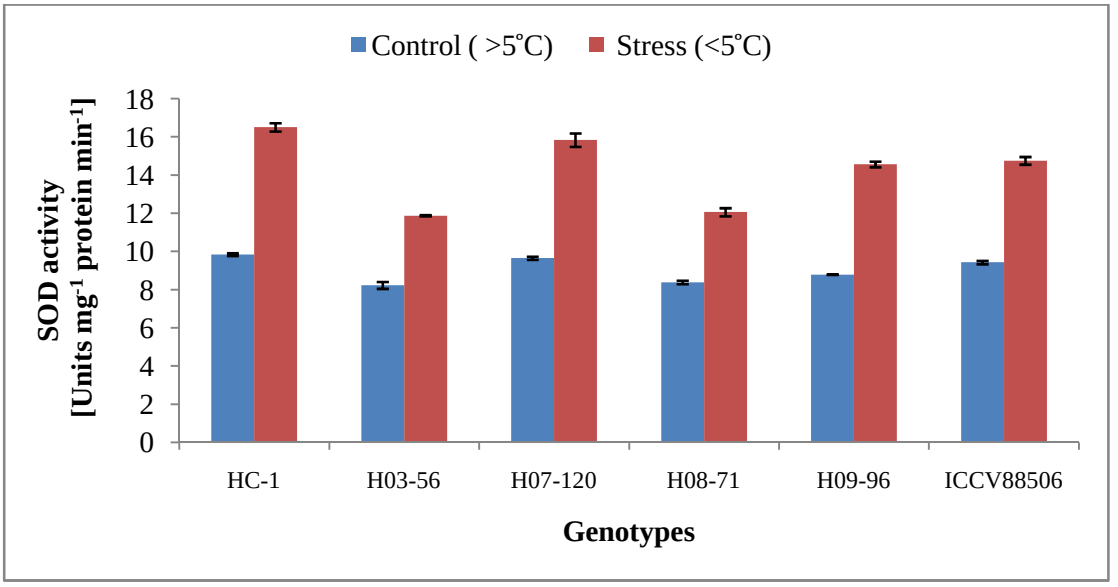


Fig. 4.6: Changes in specific activity of superoxide dismutase (SOD) in leaves of chickpea genotypes under low temperature (<5 °C) condition [*Vertical bars indicate ± SE for each treatment*]

4.4.5.2 Catalase (CAT)

The specific activity of catalase [mg^{-1} (protein) min^{-1}] in leaves of six genotypes after 4 days exposure to low temperature (<5°C) is represented in Fig.4.7. The specific activity of CAT increased significantly after 4 days after exposure to low temperature (<5 °C) of all six genotypes as compared to control (>5°C). The activity of CAT was highest in HC 1(7.65) and lowest in H03-56(5.82) genotype. The statistical data revealed that the significant differences among genotypes, temperature levels and their interaction were found to be significant.

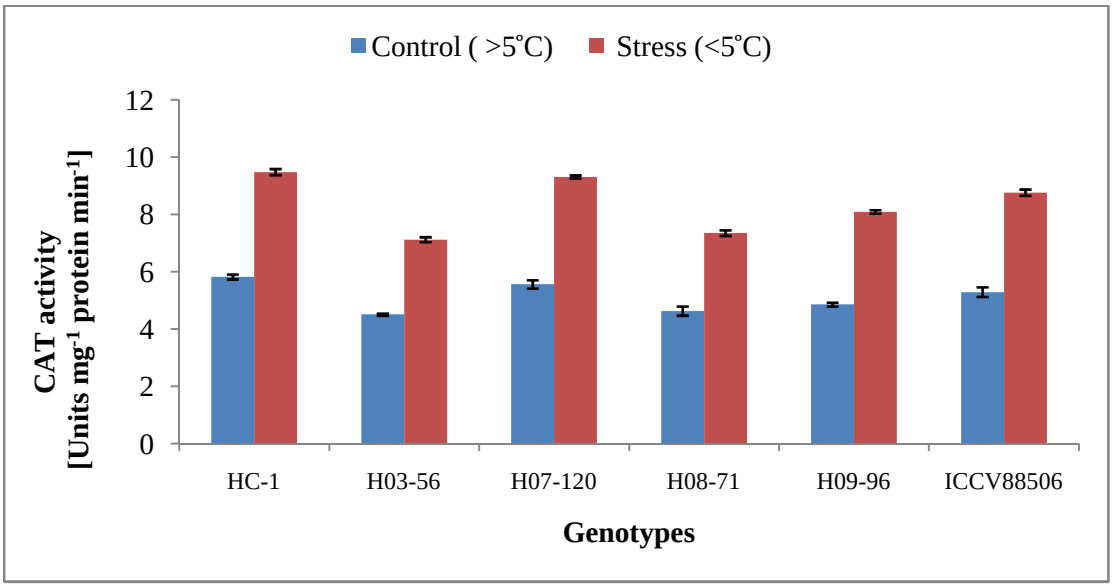


Fig. 4.7: Changes in specific activity of catalase (CAT) in leaves of chickpea genotypes under low temperature (<5 °C) condition [*Vertical bars indicate ± SE for each treatment*]

4.4.5.3 Peroxidase (POX)

The specific activity of POX measured as units [mg^{-1} (protein) min^{-1}] in leaves significantly among various chickpea genotypes. Fig. 4.8 show that low temperature stress resulted in an increase in the specific activity POX in leaves of all six genotypes. It was highest in HC-1 (0.130) and lowest in H03-56 (0.083) under low temperature (<5°C) stress conditions. The interaction between genotype and temperature was found significant.

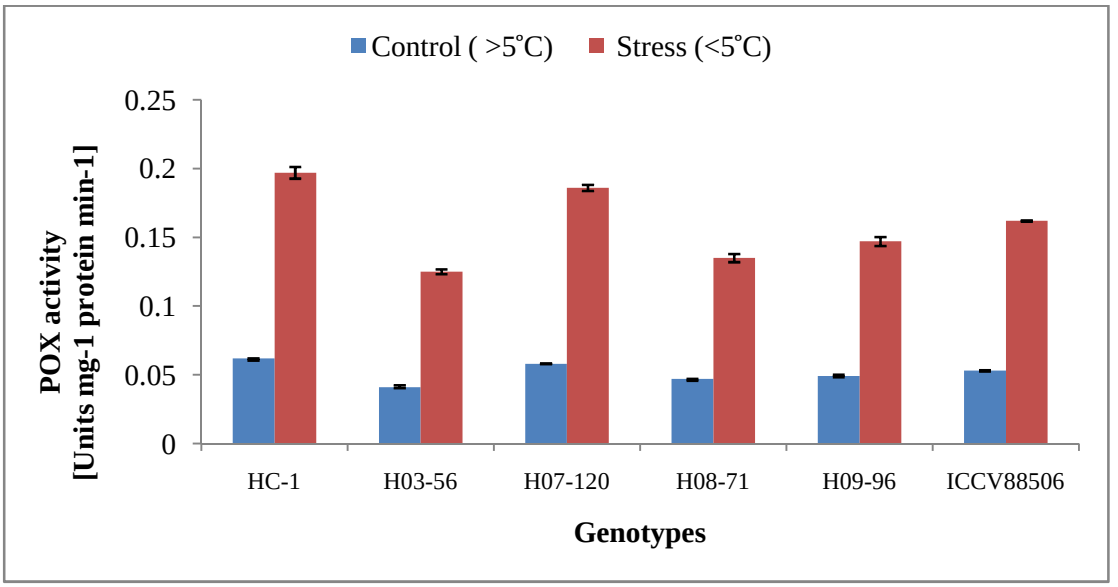


Fig. 4.8: Changes in specific activity of peroxidase (POX) in leaves of chickpea genotypes under low temperature (<5 °C) condition [*Vertical bars indicate ± SE for each treatment*]

4.5 Reproductive studies

4.5.1 Pollen viability

The pollen viability (%) was tested by using TTC solution. The loss of purple stain indicated decrease in pollen viability. The viability decreased in all the six genotypes with increased days to exposure to low temperature (<5 °C) as compared with control (>5 °C) as assessed by loss of purple stain (Table. 4.15). The HC-1 showed significantly higher pollen viability (77.6%) followed by H07-120 (76.3%) and low in H03-56 (61.6%) with 4 days after exposure to low temperature (<5 °C). Stigma receptivity was less in H03-56 compared to other genotypes to increasing exposure to low temperature (<5 °C) than control (>5 °C). Results with respect to genotypes and temperature levels were statistically significant but their interaction was non significant.

Table 4.15: Variation in pollen viability (%) of leaves in chickpea genotypes after exposure to low temperature (<5 °C)

Genotypes	Temperature		
	Control (>5 °C)	Stress (<5 °C)	Mean
HC-1	82.6	77.6	80.1
H03-56	77.6	61.6	69.6
H07-120	82.3	76.3	79.3
H08-71	75.6	65.3	70.5
H09-96	79.0	69.3	74.1
ICCV-88506	80.3	72.6	76.5
Mean	79.6	70.5	-
CD at 5 %	Genotypes =3.9 ; Temperature =2.3; Genotypes X Temperature =NS		

4.6 Yield and its attributes

4.6.1 Pods plant^{-1} (no.)

The early sown conditions had significantly affected the number of pods plant^{-1} in all the six genotypes. Maximum numbers of pods were observed in H07-120 (126) and minimum was in H03-56 (109) (Table 4.16).There was significant variation in the number of pods plant^{-1}.

4.6.2 Seeds pod^{-1} (no.)

Table 4.16 depicts the number seeds pod^{-1}in the six chickpea genotypes and the differences were significant. The highest number of seeds per pod were in H09-96 (1.8) and lowest were observed in H07-120(1.0)

4.6.3 100 seed weight (g)

Significant variation was also noticed on 100 seed weight (g) in all the six chickpea genotypes. The maximum 100 seed weight was in HC-1(18.15); followed by ICCV-88506 (17.12) and minimum in H03-56 (14.93) among various chickpea genotypes studied (Table 4.16).

4.6.4 Biological yield plant^{-1} (g)

The chickpea genotypes showed significant variation for biological yield plant^{-1} (dry weight basis).The highest biological yield was observed in HC-1 (68.4 g), followed by H07-120 (65.6 g) and lowest in H03-56 (52.8 g) (Table 4.16).

4.6.5 Seed yield plant^{-1} (g)

The genotypes showed significant differences for seed yield in the six chickpea genotypes. HC-1 genotype observed maximum seed yield plant^{-1} (20.3 g); followed by H07-120 (19.1 g) and minimum in H03-56 (14.5 g) (Table 4.16).

4.6.6 Harvest Index (HI)

A significant variation was observed in harvest index (HI %) among the six chickpea genotypes. The high value of HI was found in HC-1 (29.7), followed by H07-120 (29.1) and the lowest in H03-56 (25.6).

Table 4.16: Yield and its attributes of various chickpea genotypes

Genotypes	Plant height (cm)	Pods plant^{-1} (no.)	Seeds pod^{-1} (no.)	100 Seed weight (g)	Biological yield plant^{-1} (g)	Seed yield plant^{-1} (g)	HI (%)
HC-1	68.6	121	1.40	18.15	68.4	20.3	29.7
H03-56	91.6	109	1.60	14.93	52.8	14.5	25.6
H07-120	63.3	126	1.00	16.18	65.6	19.1	29.1
H08-71	80.3	110	1.40	15.03	55.3	16.3	27.7
H09-96	86.6	112	1.80	15.19	59.5	16.9	28.4
ICCV-88506	87.3	119	1.40	17.12	64.8	18.6	28.7
Mean	79.1	116	1.40	16.10	61.1	17.6	28.2
CD at 5%	9.8	7.3	0.07	0.87	2.9	0.6	1.5

4.7 Correlation analysis of seed yield with growth indices, physiological and biochemical traits

4.7.1 Growth indices

The correlation between different growth parameters during different growth stages with seed yield are shown in Table. 4.17. Seed yield exhibited significant positive correlation with all growth stages of CGR and RGR.

Table 4.17: Correlation coefficients for different growth indices with seed yield in chickpea genotypes

Traits	GS	CGR (g m^{-2} day^{-1})				RGR (g g^{-1} day^{-1})			
		0-30	31-60	61-90	91-120	0-30	31-60	61-90	91-120
CGR (g m^{-2} day^{-1})	0-30	-							
	31-60	0.984**							
	61-90	0.988**	0.987**						
	91-120	0.940**	0.970**	0.925**					
RGR (g g^{-1} day^{-1})	0-30	0.853*	0.870*	0.806^{NS}	0.957**				
	31-60	0.861*	0.873*	0.817*	0.956**	0.996**			
	61-90	0.944**	0.962**	0.915*	0.995**	0.966**	0.962**		
	91-120	0.911*	0.933**	0.875*	0.989**	0.979**	0.976**	0.995**	
SY	-	0.930**	0.960**	0.923**	0.989**	0.942**	0.955**	0.977**	0.974**

Abbreviations: CGR= Crop growth rate, RGR= Relative growth rate, SY= Seed yield, GS=Growth stages at different days after sowing
Here * and ** significant at 5% and 1%, respectively.

4.7.2 Physiological Traits

The correlations between different physiological parameters with seed yield are shown in Table. 4.18. Seed yield was significant and positively correlated with RWC, CSI and pollen viability and negatively with water potential, osmotic potential and RSI.

Table 4.18: Correlation coefficients for different physiological traits with seed yield in chickpea genotypes

Trait	WP	OP	RWC	Fv/Fm	RSI	CSI	PV
OP	0.911*						
RWC	-0.930**	-0.963**					
Fv/Fm	-0.977**	-0.898*	0.899*				
RSI	0.973**	0.900*	-0.867*	-0.986**			
CSI	-0.978**	-0.903*	0.874*	0.980**	-0.992**		
PV	-0.976**	-0.944**	0.984**	0.939**	-0.919**	0.924**	
SY	-0.960**	-0.972**	0.981**	0.913*	-0.909*	0.923**	0.987**

Abbreviations: WP= Water potential, OP=Osmotic potential, RWC=Relative water content, Fv/Fm=Quantum Yield, RSI=Relative stress injury, CSI=Chlorophyll stability index, PV=Pollen viability, SY=Seed yield
Here * and ** significant at 5% and 1%, respectively.

4.7.3 Biochemical Traits

Significant positive correlations between proline content, total soluble carbohydrates, superoxide dismutase, catalase, peroxidase and negative between MDA content and hydrogen peroxide with seed yield (Table 4.19) were observed.

Table 4.19: Correlation coefficients for different biochemical traits with seed yield in chickpea genotypes

Trait	*MDA*	*H_2O_2*	*PC*	*TSC*	*SOD*	*CAT*	*POX*
H_2O_2	0.952**						
PC	-0.942**	-0.991**					
TSC	-0.953**	-0.990**	0.993**				
SOD	-0.956**	-0.911*	0.912*	0.911*			
CAT	-0.970**	-0.985**	0.972**	0.965**	0.949**		
POX	-0.945**	-0.995**	0.996**	0.988**	0.903*	0.981**	
SY	-0.989**	-0.974**	0.962**	0.975**	0.917*	0.970**	0.967**

Abbreviations: MDA=Malondialdehyde, H_2O_2=Hydrogen peroxide, PC= Proline content, TSC=Total soluble carbohydrates, SOD= Superoxide dismutase, CAT= Catalase, POX= Peroxidase, SY= Seed yield

Here * and ** significant at 5% and 1%, respectively.

4.7.4 Seed yield and its attributes

The correlations between yield attributes with seed yield are shown in Table. 4.20. Significant positive correlations were observed between 100 seed weight and biological yield with seed yield and negative with plant height, and pods plant^{-1}.

Table 4.20: Correlation coefficients for different yield attributes with seed yield in chickpea genotypes

Trait	PHT	PP	HSW	BY
PP	-0.386			
HSW	-0.501	-0.127		
BY	-0.402	-0.105	0.976**	
SY	-0.749	0.127	0.890*	0.884*

Abbreviations: PHT= Plant height, PP=Pods per plant, HSW=Hundred seed weight, BY=Biological yield, SY= Seed yield

Here * and ** significant at 5% and 1%, respectively.

4.8 Visual observations:

Visual symptoms of chilling injury were evident in the form of tip burning, chlorosis, anthocyanin pigmentation on leaves and stem rachis and necrosis on the leaflets of some chickpea genotypes (Plate 3, 4 and 5).

Cold injury below 5°C

HC-1 H03-56 ICCV88506

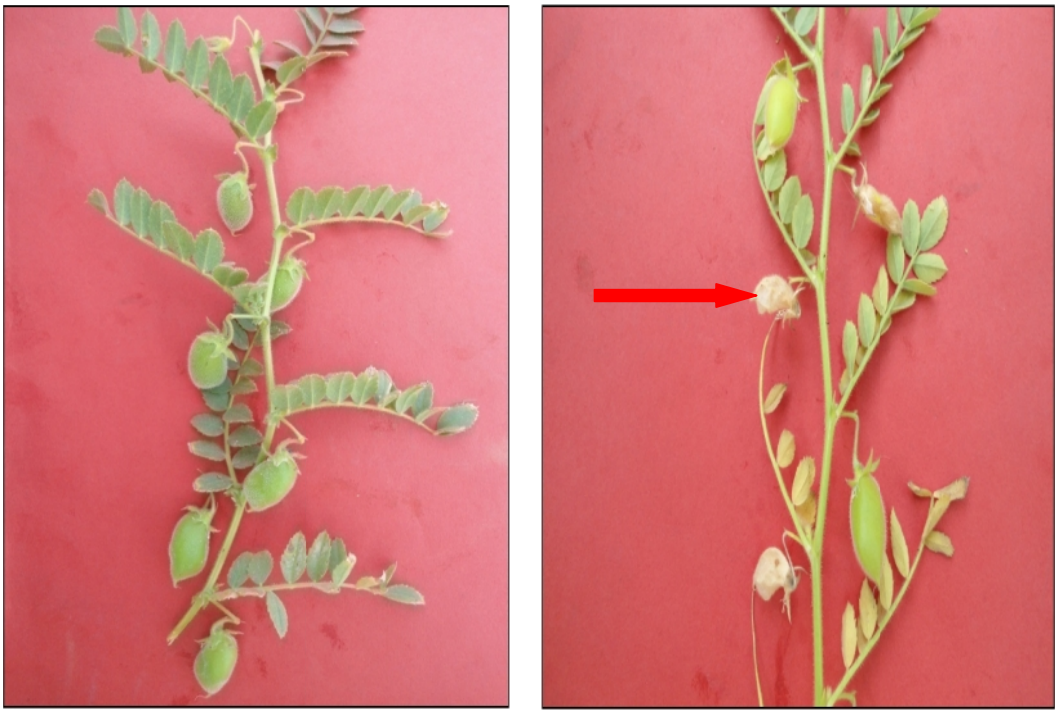
H08-71 H07-120 H09-96

Plate 3: Cold injury in chickpea genotypes as affected by low temperature (<5 °C)

Flower abortion below 5 °C

H07-120 **H03-56**

Pod abortion below 5 °C



H07-120 **H03-56**

Plate 4: Flower and pod abortion in chickpea genotypes as affected by low temperature (<5 °C)

H03-56

H08-71

H09-96

H09-96

Plate 5: Anthocyanin pigmentation in chickpea genotypes as affected by low temperature (<5 °C),.

CHAPTER – V

DISCUSSION

Chickpea is the third major cool season grain legume crop in the world after dry bean and field pea. Chilling and freezing range temperatures in many of its production regions adversely affect the chickpea production (Croser *et al*., 2003). The genotypes, duration of exposure and physiological process had their effect of chilling injury due to sub-optimal temperature (Hogewoning and Harbinson, 2007). The modification of membrane lipid components has a crucial role in cell activities upon exposure to cold stress. Response mechanisms to chilling (<15°C) and freezing (<0°C) (Clarke and Siddique, 2004) are considered at the morpho-physiological and biochemical levels. This capacity of plants to withstand chilling stress is not constant but increases noticeably upon exposure to progressively lower temperatures (<5°C) (Shahandashti *et al.,* 2012). Low temperature in plants exhibit physiological and biochemical dysfunctions and limits yield (Rymen *et al*., 2007).

The genetics of tolerance to freezing in chickpea are outlined. Sources of resistance to chilling within the cultivated *Cicer* genepools are compared with normal conditions and novel breeding technologies for the improvement of tolerance in chickpea are suggested. We also suggest future research be directed toward understanding the mechanisms involved in cold tolerance of chickpea at the morpho-physiological and biochemical level. Further screening of *Cicer* species is required in order to identify superior sources of tolerance, especially to chilling at the reproductive stages (Nayyar *et al.*, 2005).

5.1 Phenology

Crop phenology (flowering and maturity) contributes a key role in increasing seed yield of chickpea. Chickpea represents a valuable source of genetic diversity that is anticipated to be highly useful for direct and future breeding programmes. The evaluated genotypes differed significantly for all studied traits. Early flowering trait is useful in crops with indeterminate growth habit such as chickpea, in which vegetative growth, flowering, podding and pod filling period occur concurrently. Earliness enables the genotypes to escape from biotic and abiotic stresses that occur late in the growing season (Mallu *et al.,* 2014). In the present investigation minimum days for 50 % flowering was observed in H03-56 (78) and maximum in genotype HC-1 (85) (Table 4.1). In chickpea, earlier studies (Khan *et al.,* 2011; Gul *et al.,* 2013) have reported significant genetic variability for days to 50 % flowering. Breeding for earliness is one of the prime breeding objectives of chickpea as most end users and farmers usually seek for early maturing varieties in order to enable the crop to mature within the rainy season and utilize the available moisture and nutrients. In addition early

maturity could give sequential merit like excess nitrogen fixation and enhancement of soil organic matter.

These findings were in agreement with results reported by Khan *et al.* (2011) in chickpea, Oladejo *et al*. (2011) in cowpea and Imani *et al*. (2013) in lentil. Similarly in the present study the minimum days taken for physiological maturity were in H03-56 (155) and maximum days taken in ICCV-88506 (172) (Table 4.1). The recorded great variation for days to maturity could be attributed to pod filling duration and pod size because early flowered genotype might not be early matured while in some genotypes early flowering trait is correlated to early maturity. Overall, the main reasons for significant great variation among evaluated genotypes could be due to genetic, environment and genetic makeup combined with environmental factors. Thus early genotypes along with those medium reproductive duration and reasonable yield traits can be candidates for potential breeding material in future improvement of chickpea in various regions.

5.2 Growth parameters

The plant indices and its measurement are often used to monitor the comparison between different genotypes continued to increase with advancement in crop age. The results of present study showed the significant variations in plant height (Table 4.2), number of branches (Table 4.3) and dry weight of leaves (Table 4.4) and stem (Table 4.5) under early sown conditions. In our investigation maximum dry weights of leaves and stem were observed in chickpea genotype HC-1 and H07-120 at 120 DAS.

The sudden increase in plant height in chilling sensitive genotypes at later stages could be ascribed to avoiding the chilling temperature at vegetative stage. Kumar *et al.* (2005) also reported reduction in plant height as well as delayed vegetative and reproductive growth under low temperature stress. Crosser *et al.* (2003) observed that chilling range temperatures had pronounced negative effect on plant growth parameters at the vegetative stage. Singh *et al.* (1995) observed that cold susceptibility was minimal at the seedling stage but increased substantially as plants progressed from early vegetative to late vegetative stage. Yadav *et al.* (1998) also recorded reduction in number of leaves, relative growth rate and biomass production at lower temperatures which is also observed in present study.

During different days after sowing (DAS) the growth indices such as crop growth rate (CGR) and relative growth rate (RGR) were recorded. There was significant variation in CGR and RGR among chickpea genotypes at different days after sowing (DAS). In the present investigation maximum CGR and RGR was recorded in chickpea genotype HC-1, followed by H07-120 and minimum in H03-56 at 120 DAS (Table 4.6 and 4.7). Our results are in par with those of Srivastava and Singh (1980) and Gosh and Singh (1998). The maximum and minimum values of growth indices indicate of yield parameters. These findings are in agreement with that of Ahad (1986), Gosh and Singh (1998) and Sun *et al*. (1999).

5.3 Plant water relations

The water deficit is caused by a reduction in the root water uptake greater than in the leaf transpiration rate during chilling temperatures (Aroca *et al*., 2003). At low temperature, the disruption of stomatal control of leaf transpiration and the reduced water supply from the roots induced a marked water deficit (Pardossi *et al*., 1992). Leaf water potential (Ψ_w), osmotic potential (Ψ_s), relative water content (RWC) and accumulation of solutes are good indicators of plant water stress and are well associated with different plant functions (Nayyar and Walia, 2003). Our results showed that water status was affected by exposure to low temperature below 5 °C as manifested by decrease in water relations in stress (<5 °C) as compared to control (>5 °C) conditions in all the chickpea genotypes. Among six genotypes H03-56 showed more negative values of ψ_w as compared to HC-1 and H07-120 and were less affected after 4 days exposure to low temperature (<5 °C) (Table 4.8, 4.9 and 4.10). The differences in water status of these genotypes may be due to their genetic make-up or resistance capacity to avoid chilling stress; accordingly these are able to absorb water from rhizosphere.

5.4 Chlorophyll contents

Chilling temperature has a direct impact on the photosynthetic apparatus, essentially by disrupting all the major components of photosynthesis (Zhou *et al.*, 2007), including the thylakoid membranes and subsequently the chlorophyll pigments (Hola *et al.*, 2007). Likewise, low temperatures (<5 °C) caused a decrease in chlorophyll a, b and chlorophyll stability index of all the six genotypes at in stress (<5 °C) as compare to control (>5 °C). However, genotypes H03-56 was lowest chlorophyll a, b and total chlorophyll content as compared to HC-1 and H071-20 at 4 DAE to low temperature (<5 °C) (Table 4.12, 4.13 and 4.14). Reduced content of chlorophyll could be due to reduced chlorophyll synthesis or its faster degradation or both in response to low temperature have also been reported in chickpea (Mafakheri *et al.*, 2010).

5.5 Photosynthetic quantum yield (Fv/Fm)

Photosynthesis is susceptible to chilling, especially when low temperatures are combined with exposure to physiologically significant levels of irradiance (Powles *et al*., 1983; Allen and Ort, 2001), although the effects of chilling on photosynthesis, including photosystem II (PSII) efficiency, are well known (e.g. Bodner and Larcher, 1989; Tijskens *et al*., 1994; Allen and Ort, 2001). In general, chilling can produce damage to both PSI (e.g. Havaux and Davaud, 1994; Terashima *et al.*, 1998) and PSII, and, therefore, a loss of the photochemical efficiency measured under strictly light-limiting conditions (i.e. the maximum quantum yield for electron transport). In our results quantum yield (Fv/Fm) was affected after 4 days exposure to low temperature below 5 °C.

The photosynthetic quantum yield was decreased significantly in stress (<5 °C) as compare to control (>5 °C) condition. Among genotypes the maximum Fv/Fm was observed in HC-1, followed by H07-120 and minimum was noticed in H03-56 in stress (<5 °C) condition (Table 4.11).

In most cases, chilling in the dark produces a loss of photosynthetic capacity without any loss of light-limited efficiency, at least in the short term (e.g. Kingston Smith *et al.,* 1997; Allen *et al.,* 2000). Nonetheless, the quantum yield for PSII electron transport measured by chlorophyll fluorescence has been reported as being sensitive to chilling temperature to the extent that it has been used as a general indicator of chilling injury (e.g. van Kooten *et al.,* 1992), in much the same way as electrolyte leakage.

5.6 Membrane stability

Lipid peroxidation (LP) is the symptom most easily ascribed to oxidative damage. It is an attack upon polyunsaturated fatty acids of the membrane (Heath and Packer, 1968) which lead to the breakdown of lipid and impairment of membrane function. It most frequently measured in terms of malondialdehyde (MDA) content and is most easily ascribed as an indicator of oxidative damage (Mandhania *et al.,* 2006). Wery *et al.* (1994) observed that altered membrane functions during chilling stress by lipid peroxidation and leakage of electrolytes in the chickpea. In our results membrane stability was affected after 4 days exposure to low temperature below 5 °C. The damage of membrane was reflected in the lipid peroxidation and relative stress injury as these were increased at 4 DAE to low temperature. Among six genotypes studied the maximum RSI % and MDA content was observed in H03-56 and minimum was noticed in HC-1 in stress (<5 °C) (Fig.4.1, 4.2). Chilling temperature causes the loss of membrane integrity due to lipid peroxidation (Kuk *et al.*, 2003) and MDA is a common product of lipid peroxidation that reflects the extent of oxidative injury (Tambussi *et al.,* 2004).

5.7 Hydrogen peroxides

Hydrogen peroxides is a toxic compound produced as a result of the dismutation of the superoxide radical, and a higher concentration is injurious to the cell and the plant, resulting in lipid peroxidation and membrane injury. Our results also showed an increased H_2O_2 levels in leaves after 4 days exposure to low temperature (stress) condition in all the chickpea genotypes. However, the H_2O_2 accumulation increased was more in leaves of H03-56 than those in HC-1 and H07-120 (Figure 4.3). Increased in H_2O_2 production under low, high temperature and salinity stress has been reported in chickpea (Kukreja *et al.,* 2005; Manohar, 2013, Bhaskar, 2012). A progressive increase in H_2O_2 content with increasing the NaCl concentration was observed in *Brassica juncea* (Verma and Mishra, 2005). The higher H_2O_2 content was observed in the salt sensitive as compared salt tolerant cultivars of *Oryza sativa* under salt stress (Vaidyanathan *et al.,* 2003).

5.8 Proline content

Proline is an amino acid with potent role in osmotic regulation under abiotic stresses. It also acts as an antioxidant under oxidative stress. Proline is considered to be a compatible molecule that has the ability to stabilize subcellular structures and scavenge radicals. Our investigation revealed that enhanced proline accumulation in leaves in stress (<5 °C) plants of six chickpea genotypes (Fig. 4.4). Among six, proline was more accumulated in HC-1 and H07-120 as compared to others with increasing exposure to low temperature (<5 °C). Varietal evaluation for proline content was conducted at temperature below 5˚C and above 5˚C. A relationship was found between levels of proline and chilling resistance (Purvis, 1981). It has been assumed that the accumulation of organic salts including proline is involved in protection mechanisms such as restoring cell volume and turgor, reduction in cell damage by free radicals etc (Timasheff and Arakawa, 1990) which is in accordance with our results.

5.9 Total soluble carbohydrates

Carbohydrates such as sugars (glucose, fructose, sucrose and fructans) accumulate under salt stress, also playing a leading role in osmoprotection, osmotic adjustment, carbon storage and radical scavenging. The osmoprotectants (total soluble carbohydrates) protect plants from stress by (1) osmotic adjustment which helps in turgor maintenance, (2) detoxification of reactive oxygen species and (3) stabilization of the quaternary structure of proteins (Yancey *et al.,* 1982; Bohnert and Jensen, 1996). At low temperature stress, total soluble carbohydrates content increased in early maturing chickpea genotypes (Nayyar, 2005). Our study corroborated these results. In the present investigation total soluble carbohydrates also increased in stress plants compared to their respective controls in all the six chickpea genotypes (Fig. 4.5). The accumulation of total soluble carbohydrates was more in HC-1 than other genotypes in the present study. Taffouo *et al.* (2009) also reported increase in TSC accumulation in stressed plants compared to control in *Vigna unguiculata* L. In cold stress, pea leaves accumulated high quantities of sucrose, glucose-6-phosphate, fructose-6-phosphate and mannose-6-phosphate (Streb *et al.*,2003) and a close relationship was found between soluble sugars concentration of leaves just before the frost and the degree of freezing tolerance obtained by the different genotypes (Bourion *et al.*, 2003). Hughes and Dunn (1996) suggested that cellular and metabolic changes that occur during cold acclimation include increased levels of sugars, soluble proteins, proline and organic acids as well as the appearance of new isoforms of proteins and altered lipid membrane composition.

5.10 Antioxidants defense system

Chilling temperatures lead to oxidative stress which occurs due to the overproduction of reactive oxygen species (ROSs), [such as superoxide (O^{-}_{2}), singlet oxygen ($^{1}O_{2}$), hydrogen peroxide (H_2O_2) and hydroxyl redicals (^{-}OH)] that cause peroxidations of membrane lipids, denaturation and aggregation of proteins, DNA fractures and inactivation of enzymes (Bowler

et al., 1992; Gill and Tuteja, 2010). It was suggested that oxidative stress may be a significant factor in relation to chilling injury in *Arabidopsis thaliana* and rice (Fadzillah *et al.,* 1996, O'Kane *et al.,* 1996). Chilling induced oxidative stress has also been observed in maize and coffee (Prasad *et al.,* 1994; Queiroz *et al.,* 1998). Higher plants have defense system to scavenge reactive oxygen species (ROS).

The activity of SOD, which is responsible for scavenging O^{-2} to produce H_2O_2, increased under cold stress. This increased activity may reflect both the enhanced amount of O^{-2} production and the scavenging ability of SOD in response to cold stress and could be an important protective mechanism for the photosynthetic apparatus. The specific activity of SOD was also increased in leaves of all the six chickpea genotypes after 4 days exposure to low temperature (>5 °C), which was found to be higher in genotype HC-1, followed by H07-120 and minimum in H03-56 in response to cold stress (>5 °C) (Fig. 4.6) during present investigation.

CAT is the principal H_2O_2-scavenging enzyme located in peroxisomes and scavenges photorespiratory H_2O_2 without consuming reducing power. Our data showed that the specific activity of CAT in leaves was increased with the cold stress (<5 °C) as compared to control (>5 °C) condition. The specific activity of CAT was higher in HC-1 followed by H07-120 and minimum in H03-56 in cold stress (<5 °C). The cold stress enhanced higher specific activity of CAT in HC-1 and H07-120 may suggest its effective scavenging mechanism to remove H_2O_2 (Fig. 4.7) and imparting tolerance against cold stress. The capacity of plants to withstand cold stress is not constant but increases noticeably upon exposure to progressive cold stress (Gill and Tuteja, 2010).

An important enzyme regulating intracellular H_2O_2 levels is peroxidase (POX). Peroxidases, besides their main function in H_2O_2 elimination, can also catalyse O_2 and H_2O_2 formation by a complex reaction in which NADH is oxidized using trace amounts of H_2O_2 (Blokhina *et al*., 2003). In the present investigation, the specific activity of POX in leaves increased after 4 days exposure to low temperature (<5 °C), which was found to be higher in HC-1 followed by H07-120 and minimum in H03-56 genotypes (Fig. 4.8). In creating cold tolerance, plant cells induce cascades of alterations in metabolic pathways including the activity of antioxidant enzymes (Maali-Amiri *et al.,* 2007). In acclimated plants, antioxidants and antioxidant enzymes such as SOD, CAT, POX had an important role in creating greater cold tolerance. In our study we hypnotized that an increased activity of these antioxidant enzymes (SOD, CAT and POX) contributes to the protection of chickpea genotypes.

5.11 Pollen Viability (%)

Flowers acclimatize to low temperature resulted in loss of pollen viability. A wide variation in the germination of pollens was observed among the genotypes tested for cold tolerance. Low temperature at flowering is a major constraint to improved yield of chickpea.

Clarke and Siddique (2004) also reported that low temperature (<15 °C) affected the pollens and fertilization. In the present study, pollen grains in control (>5 °C) were more viable than in chilling (<5 °C) temperature and chickpea genotype HC-1 and H07-120 recorded highest pollen viability (%) over all other six genotypes at low temperature (Table 4.15). The results are in conformity with those of Srinivasan *et al.* (1999) who found that pollen in tolerant genotypes were more viable (90%) as compared to susceptible genotypes (60%). A study by Clarke (2001) indicated that pollen germination was effected by chilling temperature in chickpea. Clarke and Siddique (2004) found that low temperature (<15 ºC) affects both development and function of reproductive structures in the chickpea flower. The results are also supported by Leport *et al.* (1999) who suggested that early flowering would benefit yield if flowers were fertile, leading to early pod development and seed filling and thus, avoiding terminal soil moisture stress in case of chilling tolerant genotypes. As chickpea is sensitive to cold stress (<8 ºC) at its reproductive phase that results in flower abortion, poor pod set and thus reduced yield (Nayyar, 2005). Early maturing genotypes are especially more sensitive as observed in our study. In this crop, the metabolic causes underlying cold injury that are imperative to induce cold tolerance are not known. Srinivasan *et al*. (1999) have reported that pollen development was largely unaffected by cold stress under field conditions in most chickpea cultivars. Viability of pollen, however, was reduced in some cultivars because of a higher sensitivity of one or more stages in meiosis to cold stress and/or failure of pollen to mature at low temperature.

5.12 Yield and its attributes

This experiment was conducted under early sown conditions. There was significant variation in seed yield and its components among six chickpea genotypes under early sown conditions. Genotypes varied with respect to number of pods plant^{-1}, seeds pod^{-1}, hundred seed weight, biological yield plant^{-1}, seed yield plant^{-1} and harvest index (Table 4.16). Yield is a quantitative character, the result of various physiological and biochemical processes. Yield and yield contributing traits could have dynamic correlation with environmental effects. In our study we found that maximum seed yield plant^{-1} was in HC-1 followed by H07-120 and minimum in H03-56. The presence of significant variation among evaluated genotypes for seed yield plant^{-1} could be due to genetic, environment and genetic makeup combined with environmental effect. Best performance and high seed yield plant^{-1} is one of the basic criteria for identifying and selecting superior varieties for end users and farmers. Besides, the presence of wide variation for seed yield plant^{-1} could be attributed to high number of pods plant^{-1}, high biomass yield enables to converted final seed yield and heavier 100 seed weight. Significantly high variation for seed yield per plant indicated the potential of the germplasm to determine the best genotypes for specific and broad adaptation across environments. In chickpea germplasm, previous studies have reported substantial variation for seed yield

(Farshadfar and Farshadfar, 2008; Malik *et al.*, 2009). In other legumes, earlier studies (Roy *et al.*, 2013; Hegde and Mishra, 2009; Furat and Uzun, 2010) have reported substantial variation for seed yield in lentil, cowpea and sesame germplasm respectively. Low temperature often causes flower abortion, pollen and ovule infertility, breakdown of fertilization, poor seed filling, decreases in seed setting which ultimately reduce the grain yield (Thakur *et al.*, 2010). Similar results have been reported by many researchers (Qureshi *et al*., 2004; Malik *et al.*, 2010; Gul *et al*., 2013) in chickpea germplasm, (Hegde and Mishra, 2009) in cowpea, (Latief *et al*., 2011) in lentil germplasm.

CHAPTER – VI

SUMMARY AND CONCLUSION

The investigations were carried out on six chickpea (*Cicer arietinum* L.) genotypes *i.e.* ICCV 88506 (Tolerant, National check), HC1, H03-56, H07-120, H08-71 and H09- 96 to study differing in their physiological, biochemical, yield traits and in relation to low temperature tolerance. The crop was raised in the field under early sown conditions; the experimental plot was 1.2 m × 4 m in size. Low temperature stress was given by manipulation of sowing dates *i.e.* early sown (15th October, 2015) *rabi* season during the year 2015-16. The sampling was done after 4 days exposure to low temperature <5 °C (stress) and >5 °C (control) conditions.

Under early sown conditions, the genotype H03-56 took minimum 78 and 155 days for 50 % flowering and physiological maturity, respectively. Low temperature stress adversely effected plant water status of all the chickpea genotypes. Substantial variations were noticed between six genotypes for water potential (Ψw), osmotic potential (Ψs) and relative water content (RWC %). The Ψw and Ψs of leaves became more negative in HC-1 and H07-120 with the increasing days after exposure to low temperature (<5°C). The relative water content (RWC %) was significantly decreased in all the chickpea genotypes in stress (<5 °C) condition, the decline being maximum in leaves of H03-56 and least in HC-1 and H07-120 over their respective control.

Exposure to low temperature (<5°C) significantly decreased chlorophyll a, chlorophyll b contents and chlorophyll stability index (CSI %) in all the chickpea genotypes. Under stress (<5 °C) condition, the decline in all photosynthetic pigments was maximum in H03-56 and minimum in HC-1 and H07-120. Maximum quantum yield (Fv/Fm) in HC-1, H07-120 and minimum in H03-56 was recorded. The exposure to low temperature (<5°C) has less damaging affect on photosynthetic apparatus in HC-1 and H07-120.

Our investigation showed higher accumulations of proline and total soluble carbohydrate (TSC) in leaves in stress (<5°C) condition. The chickpea genotype HC-1, H07-120 showed significantly higher accumulation in proline and TSC contents than other chickpea genotypes. These osmolytes contributed better osmoregulation in HC-1 and H07-120 in comparison to all others.

Exposure to low temperature (<5°C) induced increased in production of H_2O_2 and consequently the lipid peroxidation and membrane injury. Overall, the H_2O_2 accumulation increase was more in leaves of H03-56 than those in HC-1 and H07-120 in stress (<5 °C) which confirms that chilling stress lead to excessive ROS production in H03-56. The degree of accumulation of MDA content was higher in H03-56 than in HC-1 and H07-120 indicating

higher rate of lipid peroxidation in H03-56 due to chilling stress. This indicated that H03-56 is chilling sensitive as compared to HC-1 and H07-120. The leakage of ions which is measured as relative stress injury (RSI %) increased significantly with increasing days to exposure to low temperature (<5°C) in all the six genotypes. Relative stress injury per cent was found to be more in H03-56 as compared to HC-1 and H07-120 in leaves under stress (<5°C).

The specific activities of reactive oxygen species (ROS) scavenging enzyme i.e. SOD, CAT and POX increased significantly in all the chickpea genotypes at 4 DAE to low temperature (<5°C) as compared to their respective control (>5 °C). The genotypes HC-1 and H07-120 showed higher activity of antioxidant enzymes as compared to all other genotypes. Higher specific activity of SOD, CAT, POX, lower accumulation H_2O_2 and MDA content in HC-1 and H07-120 than all other genotypes indicated that these enzymes play a better key role in removal of ROS in HC-1 and H07-120 and thus minimizing the cellular damage caused by ROS under low temperature (<5°C).

There was significant reduction in pollen viability (%) among chickpea genotypes and was found to be highest in HC-1 and H07-120 and lowest in H03-56 under low temperature (<5°C) stress conditions.

There was wide variation for seed yield and its attributes among chickpea genotypes under early sown conditions. The chickpea genotypes HC-1 and H07-120 performed better than other genotypes under early sown conditions as far as seed yield and its attributes are concerned.

Results obtained from association among traits indicated that seed yield had positive and significant correlation with CGR , RGR, RWC, Fv/Fm, CSI, TSC, proline, SOD, CAT, POX, 100 seed weight and biological yield and negative with water potential, osmotic potential, RSI, MDA and H_2O_2 content

More negative values of Ψw, Ψs of leaves and better accumulation of osmotically active solutes, i.e. proline and TSC in leaves, helped in maintaining the higher RWC % of these organs of HC-1 and H07-120 than noticed in other chickpea genotypes in stress (<5°C) condition. Higher activities of antioxidant enzymes SOD, CAT, POX, lower accumulation H_2O_2 and MDA in HC-1 and H07-120 than in other chickpea genotypes indicated those enzymes play a key role in removal of ROS in these chickpea genotypes, thus minimizing the cellular damage caused by ROS under low temperature stress. Pollen viability (%) was also adversely affected with low temperature; the effect being less pronounced in HC-1 and H07-120 than other chickpea genotypes. The chickpea genotypes HC-1 and H07-120 performed better than other genotypes under early sown conditions as far as seed yield and its attributes are

concerned. The chickpea genotypes HC-1 and H07-120 were found to be relative tolerant to low temperature stress than other genotypes based upon various morpho-physiological, biochemical and seed yield attributes studied. These chickpea genotypes can be used in further crop improvement programme of chickpea for cold tolerance.

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ABSTRACT

Title of thesis	:	**Morpho-physiological and biochemical traits for cold tolerance of chickpea (*Cicer arietinum* L.) genotypes.**
Name of the degree holder	:	**Mahesh Kumar**
Title of the degree	:	**Master of Science**
Admission No.	:	2014BS34M
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Degree awarding University/Institute	:	CCS HAU, Hisar-125004 (Haryana) India.
Year of award of degree	:	2016
Major subject	:	Plant Physiology
Total number of pages in the thesis	:	51+ xiii
Number of words in the abstract	:	538

Key words: Osmotic potential, membrane injury, proline content, antioxidants and seed yield

The present investigation was carried out with six chickpea genotypes viz. ICCV 88506 (Tolerant, National check), HC-1, H03-56, H07-120, H08-71 and H09- 96 differing in their cold sensitivity, to study the low temperature (<5°C) induced changes in morphological, physiological, biochemical, yield and its attributing character under field conditions. Sampling was done at 30, 60, 90 and 120 days after sowing (DAS) for growth parameters and 4 day exposure to below (stress) and above (control) 5 °C temperature for physiological and biochemical parameters. The water potential (Ψ_w) and osmotic potential (Ψ_s) of leaves decreased in all the genotypes after 4 days exposure (DAE) to low temperature (<5 °C). H03-56 showed more negative values Ψ_w of leaves i.e. from -0.70 to -0.85 MPa as compared to -0.45 to -0.65 MPa in HC-1, respectively in control (>5 °C) and stress (<5 °C) conditions. Likewise, the Ψ_s of leaves decreased from -0.84 to -1.04 MPa in H03-56 and -0.59 MPa to -0.81 MPa in HC-1. With exposure to low temperature (<5 °C), RWC (%) of leaves also declined in all the six genotypes. RWC (%) was higher in HC-1, followed by H07-120 and lowest in H03-56 in stress (<5 °C) conditions. The proline content of leaves increased significantly from 0.781 to 0.913 and 0.605 to 0.798 mg g^{-1} dry weight, respectively in HC-1 and H03-56 genotypes in stress (<5°C) conditions. Likewise, the total soluble carbohydrate (TSC) content of leaves also increased from 18.35 to 22.74 and 16.83 to 19.69 mg g^{-1} dry weight in HC-1 and H03-56, respectively. A marked increase in hydrogen peroxide H_2O_2, lipid peroxidation (MDA content) and relative stress injury (RSI %) was noticed in leaves which was much highest in H03-56 than all other six chickpea genotypes in stress (<5 °C) condition. More negative values of Ψ_w of leaves, Ψ_s of leaves and better accumulation of osmotically active solutes, i.e. proline, TSC of HC-1 and H07-120, helped in maintaining the higher RWC % of these organs than noticed in other genotypes in stress conditions. The specific activities of ROS scavenging enzymes such as SOD, CAT and POX also increased in leaves of all the six chickpea genotypes, in stress (<5 °C) condition. The increase was more in HC-1 and H07-120 as compared to other chickpea genotypes. Higher activities of antioxidant enzymes, lower accumulation H_2O_2 and MDA content in HC-1 and H07-120 than all others indicated that these enzymes play a key role in removal of ROS better in these genotypes than all other six chickpea genotypes, thus minimizing the cellular damage caused by ROS under chilling stress. Pollen viability (%) was also adversely affected in stress (<5 °C) condition; the effect being most pronounced in H03-56. The yield parameters like number of branches $plant^{-1}$, number of pods $plant^{-1}$, number of seeds $plant^{-1}$, 100 seed weight and seed yield $plant^{-1}$ were more in HC-1 and H07-120 than others in early sown condition.Seed yield had significantly positive correlation with RWC, pollen viability, proline content, total soluble carbohydrates, SOD, CAT, POX and negative with water potential, osmotic potential, H_2O_2 and RSI. Hence, the mechanism of cold tolerance was better in HC-1 and H07-120 than other six chickpea genotypes as found from physiological, biochemical, reproductive behavior, yield and its attributing traits studied.

MAJOR ADVISOR **SIGNATURE OF STUDENT**

HEAD OF THE DEPARTMENT

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j) Academic Qualifications:

Degree	University/Board	Year of Passing	Percentage of Marks	Subjects
10th	Board of School Education Haryana	2005	82.33	Hindi, English, Sanskrit, Mathematics, Science, Social Science
10+2 th	Board of School Education Haryana	2007	63.80	Hindi, English, Physics, Chemistry, Biology
B.Sc. (Hons.) Agriculture	CCS HAU, Hisar	2014	63.00	All Agricultural subjects
M.Sc. (Plant Physiology)	CCS HAU, Hisar	2016	68.50	Major: Plant Physiology Minor: Soil Sciences

k) Co-curricular Activities

- Actively participated in Quiz competitions
- Member of Mountaineering Club – CCS HAU, Hisar
- Regular Blood Donor
- Participitation in atheletics meet in university

List of Publications: 3 (Abstract)

1. **Mahesh Kumar**, Neeraj Kumar, Parveen Kumar, Sarita Devi and Krishan Kumar (2016). Physiological responses of chickpea genotypes for cold tolerance as evaluated by quantum yield, relative stress injury, pollen viability and seed yield. Abstract in National Seminar on **"Technical advances in Botanical Sciences"**. Organized by Department of Botany, Kumari Vidyavati Anand DAV College for Women, Karnal. pp. 28.
2. Parveen Kumar**,** Neeraj Kumar, **Mahesh Kumar,** Sarita Devi and Krishan Kumar (2016). Evaluation of chickpea genotypes for heat tolerance. Abstract in National Seminar on **"Technical advances in Botanical Sciences"**. Organized by Department of Botany, Kumari Vidyavati Anand DAV College for Women, Karnal. pp. 23.
3. S. Devi, **M. Kumar**, N. Kumar, Dharmvir and R. Prakash (2016). Physiological responses of some weeds to salinity stress tolerance. Abstact in National Conference of Plant Physiology. **Challenges in Crop Physiology Research: From Molecular to Whole Plant**. Organized by Department of Crop Physiology, University of Agriculture, GKVK, Bengaluru & Indian Society for Plant Physiology, New Delhi. pp 35.

Mahesh Kumar

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