STATUS OF ENDOGENOUS ANTIOXIDANTS DEFENSE IN PRECONDITIONED CONTRASTING CHICKPEA(*Cicer arietinum* L.)SEEDLINGS

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For the Award of

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In

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BY

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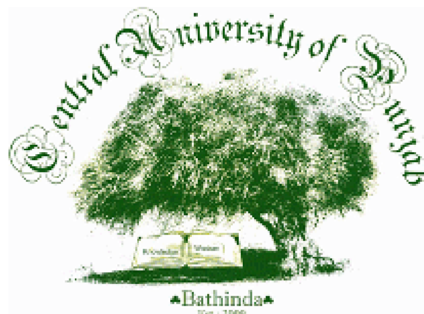

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November, 2014

CERTIFICATE

I declare that the dissertation entitled "STATUS OF ENDOGENOUS ANTIOXIDANTS DEFENSE IN PRECONDITIONED CONTRASTING CHICKPEA(*Cicer arietinum* L.)SEEDLINGS." has been prepared by me under the guidance of Dr. Sanjeev K. Thakur, Assistant Professor, Centre for Biosciences, School of Basic and Applied Sciences, Central University of Punjab. No part of this dissertation has formed the basis for the award of any degree or fellowship previously.

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ABSTRACT

“STATUS OF ENDOGENOUS ANTIOXIDANTS DEFENSE IN PRECONDITIONED CONTRASTING CHICKPEA(*Cicer arietinum* L.)SEEDLINGS”

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Plants experience various abiotic stresses of low or elevated temperature, drought and heavy metals. Chickpea (*Cicerarietinum*L.) is one of the important crop belongs to the family Leguminosae and it is the second most important grain legume plant in the world after common bean. Chickpea has evolved as a spring crop in warmer climate of West Asia. Therefore, it is prone to chilling injury as in north India; chickpea is cultivated in winter season. To protect the plant from chilling injury, preconditioning the plants with mild drought stress might triggers the signalling pathways that will prepare the plants to combat lethal cold stress. This is determined by monitoring the various cell responses at different phases. Therefore antioxidative enzyme activity of the SOD, APX, GPOX, LOX, CAT enzymes was done. In our study, both the varieties, PDG3 and GPF2 showed equalvariation towards SOD, CAT, GPOX, and APX activity. Enhanced SOD, CAT & GPOX activity in both the varieties showed increased tolerance towards cold stress. Less LOX activity showed preconditioning decreased membrane damage in preconditioned seedlings. Thus, preconditioning with mild drought stress has enhanced tolerance capacity of the plant during the cold stress.

(Ankur Jairath) (Dr. Sanjeev K. Thakur)

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LIST OF ABBREVIATIONS

Sr.No.	Full form	Abbreviation
1	Micron	µ
2	Microliter	µl
3	Ascorbate peroxidase	APX
4	Ascorbate	ASc
5	*Bacillus thuringiensis*	*Bt*
6	Celsius	C
7	Catalase	CAT
8	Centimetre	Cm
9	Cold regulated	COR
10	Cold-regulated	*Cor*
11	Copper	Cu
12	Dehydroascorbate	DHA
13	Dehydroascorbate reductase	DHAR
14	Deoxyribonucleic acid	DNA
15	Drought responsive element/C-repeat	DRE/CRT
16	Ethylenediaminetetraacetic acid	EDTA
17	Early responsive to dehydration	*Erd*
18	Iron	Fe
19	Fresh mass	FM
20	Fresh weight	FW
21	Gram	G
22	Guaiacol peroxidase	GPOX
23	Glutathione peroxidase	GPX
24	Glutathione reductase	GR
25	Reduced glutathione	GSH
26	Glutathione	GSSG
27	Hydrogen peroxide	H_2O_2
28	Hydrochloric acid	HCl
29	Hours	Hrs
30	Cold-inducable	*Kin*

31	Linoleic acid	LA
32	α-linoleic acid	LeA
33	Lipoxygenase	LOX
34	Low temperature induced	*Lti*
35	Mitogen activated protein	MAP
36	Megabase pair	Mb
37	Monodehydroascorbate	MDHA
38	Monodehydroascorbate reductase	MDHAR
39	Milligram	mg
40	Minute	min
41	Millilitre	ml
42	Millimolar	mM
43	Manganese	Mn
44	Mega Pascal	MPa
45	Reduced nicotinamide adenine dinucleotide phosphate	NADPH
46	Nitrobluetetrazoliumsalt	NBT
47	Nanometre	nm
48	Non-preconditioning	NP
49	Preconditioning	P
50	Polyethylene glycol	PEG
51	Phenylmethylsulfonylfluoride	PMSF
52	Peroxidase	POX
53	Photosystem	PS
54	Polyunsaturated fatty acid	PUFA
55	polyvinylpyrrolidone	PVP
56	Root	R
57	Responsive to dehydration	*Rd*
58	Reactive oxygen species	ROS
59	Shoot	S
60	Superoxide dismutase	SOD
61	Unit	U
62	Volume/Volume	v/v

63	Weight/Volume	w/v
64	Zinc	Zn

INTRODUCTION

CHAPTER 1

INTRODUCTION

1.1. Origin of the problem

In world, population is expected to touch six billion by 2050 (Mahajan Tuteja, 2005). Due to this rapid increase in population, there will be big gap between demand and supply of agricultural products. To feed such a large population, there is a demand of sustainable and alternate agriculture, which is only possible by elevating the production of important crops like cereals and legumes.

In agriculture plants faces major threat from biotic as well as abiotic stress. Biotic stress can be combated with the help of transgenic approach such as *Bt* Cotton and *Bt* Brinjal. With the changing environmental conditions, majority of crops are suffering from multiple abiotic stresses i.e, low temperature, high temperature, drought stress, salinity and heavy metal stress.

As legumes and cereals are major source of nutrition for population around the world, we need a strategy to increase the production of these plants. But majority of cereals and legume crops are prone to either low or high temperature stress. For example, wheat (*Triticum aestivum*) is prone to terminal heat stress whereas, chickpea (*Cicer arietinum* L.) experiences both cold and terminal heat stress at reproductive stage.

Chickpea (*Cicer arietinum* L.) is the second most major food legume cultivated in winter season in Northern India and some parts of Australia.Other major producers are Pakistan, Turkey, Iran, Myanmar and Canada. Ethiopia and Kenya are the leading producers in Sub-Saharan Africa (Chohan *et al.*, 2011). Chickpea can be broadly classified into two types, Kabuli (white flower, cream-colored seeds) and Desi type (purple flowers and small, dark seeds). Both varieties are grown in temperate and semiarid tropical regions of the world (Gangola *et al.*, 2013). Chickpea fulfils a noticeable nutritional requirement of population of developing countries as it is a rich source of carbohydrates (40-59%), proteins (13.5-31.7%), vitamins, minerals etc. (Gangola *et al.*, 2013).

Chickpea originated as a spring season crop but it is cultivated as winter crop in northern parts of India; where it experience chilling stress at reproductive stage. Optimum temperature for growth of chickpea is 23-30˚C but seedlings are

prone to temperature range of -1.5˚C to 15˚C (Thakur *et al.*, 2010). The most malicious effect of cold stress on chickpea is dehydration of tissue leading to membrane damage by alteration of its composition in terms of fatty acids. Change in fluidity of plasma membrane is one of the major indicators of cold stress. Cold stress leads to the formation of ice crystals in tissues which further leads to the formation of reactive oxygen species and eventually connects the cold stress to oxidative damage. This oxidative stress further causes damage at cellular level ultimately reducing the rate of metabolic activities in plants. To protect the plant from the oxidative damage, plants activate their antioxidative defense system.Antioxidative defense system includes antioxidative enzymes and antioxidants. Antioxidative enzymes include Superoxide dismutase (SOD), Catalase (CAT), Ascorbate peroxidase (APX), Glutathione reductase (GR), Guaiacol peroxidase (GPOX), Glutathione peroxidase (GPX) etc. These enzymes are induced during the stress conditions that will activate a free radical quenching mechanism and this will make the plant stress tolerant against the oxidative damage.

1.2. Hypothesis

The basis of the present study is quite simple in terms of feasibility and cost effectiveness under field conditions. Chickpea experiences cold stress throughout its life cycle which reduces the crop yield by severely damaging the reproductive development. Preconditioning at vegetative stage with mild drought stress may enhance the tolerance to incoming lethal cold stress during the reproductive stage. Our hypothesis is to expose the chickpea seedlings to non–lethal stress (drought) and see the extent of tolerance to the low temperature.

1.3. Outcome

There is a dedicated endogenous enzymes machinery to control the oxidative molecules/free radicals, but excess of oxidative/free radical is only restricted with well activated/induced endogenous antioxidants. There are individual as well as group of specialized enzymes and non-enzymatic molecules which get induced during the excess production of free radicals.

REVIEW OF LITERATURE

CHAPTER 2
REVIEW OF LITERATURE

Chickpea (*Cicer arietinum* L.) is one of the most consumed legume crop after bean and soyabean (Katerji *et al.*, 2001), belongs to the family of *Leguminosae / Fabaceae (*Varshney *et al., 2009)* which is grown in arid and semi-arid areas around the world. Chickpea is diploid (2n=2x=16), self-pollinated annual crop with a genome size of about 740Mb (Garg *et al.*, 2011). Chickpea is not only a major source of protein for human food in semi-arid tropical regions but it also plays an important role in maintaining the soil fertility, particularly in the dry, rainfed areas.

2.1. Area and Production

The total world production of chickpea is approximately 11.62 million tonnes and the majority of it is grown in South Asia. India is the largest producer in the world with an estimated average production of 8.832 million tonnes (FAOSTAT, 2013), but there is a big gap between demand and supply because there is a loss in productivity due to various abiotic and biotic stresses. Global loss in annual production due to abiotic stresses alone are estimated to be around 3.7 million tonnes, which accounts for 30-40% average loss (Varshney *et al.*, 2009).

Interestingly, there is a major shift in chickpea area from Northern India (cooler, long season environments) to Southern India (warmer, short season environments) during the past four decades. This shift in pattern of chickpea cultivation is made to avail the better use of moisture in Mediterranean climate and to avoid the loss of crop due to terminal drought and cold stress. A lot of efforts are constantly being done to generate tolerant chickpea varieties to major stresses experienced by crop such as, drought, cold, salinity etc. (Singh *et al.*, 1994).

2.2. Chilling injury

The average temperature for growth of chickpea is 23-27°C, but temperature lower than the favourable can lead to deformities in growth and lowers the average productivity of the crop. As chickpea cultivation is done during winters in North India, it experiences temperature lower than 15°C which leads to adverse effects on different stages of growth. Temperature below 15°C have been

demonstrate to cause detrimental effects at the seeding stage of the crop causing poor growth and even may lead to death sometimes. At the vegetative stage, chilling stress may lead to noticeable effect on dry matter production and overall growth of plant (Croser *et al.*, 2003). But there are much more damaging effects of low temperature stress especially at reproductive stage, which can lead to bud abortion, poor pollen germination, impaired ovule development, and finally poor pod and seed set, which is reflected in poor productivity (Kumar *et al.*, 2010).

Damage to chloroplast have also been observed due to low temperature stress on plants which further affects PS II due to imbalance between energy harvested and energy expanded by the plants leading to the generation of reactive oxygen species (ROS) (Miura *et al.*, 2013).

2.3. Cold stress tolerance mechanism in plants

The response of plant towards any abiotic stress signal is mediated by a cascade of events, collectively termed as signal transduction. Abiotic stresses trigger a number of physiological events at the molecular level. The responses are complicated and highly regulated, resulting in the activation of signalling pathways and gene encoding proteins that help in coping with adverse environmental conditions (Pirzadah *et al.*, 2013).

2.3.1. Antioxidant system

Major chilling injury to the plants is caused by ROS at cellular level which results in damage of various biomolecules inside the cells(Suzuki *et al.*, 2006). Higher plants have active oxygen scavenging systems consisting of several antioxidant enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), glutathione reductase (GR) (Thakur *et al.*, 2013). The chloroplast is the cellular compartment associated with photosynthetic electron transport system and is the generous provider of oxygen, which is a rich source of ROS. A common feature of ROS is their ability to cause oxidative damage to proteins, lipids and DNA (Ceron-Garcia *et al.*, 2012).

2.3.2. Scavenging ROS: the antioxidant system of plants

Since ROS are constantly produced during normal cell metabolism, it is important that their basal levels are tightly controlled. Furthermore, plant cells need to be able to scavenge cytotoxic levels of ROS during stress, but also finely modulate lower levels of ROS for signalling purposes. This is achieved by a complex battery of antioxidants, both enzymatic and non-enzymatic (summarised below in Table 2.1).

Table 2.1: Examples of antioxidants in plants

Scavenging Mechanism	Localisation	Main ROS scavenged	Reference
Enzymatic:			
Superoxide Dismutase	Chloroplast, cytosol, mitochondria, peroxisomes, apoplast	$O_2^{\cdot -}$	(Bowler *et al.*, 1992)
Catalase	Peroxisome	H_2O_2	(Willekens *et al.*, 1997)
Glutathione peroxidase	Cytosol	H_2O_2	(Milla *et al.*, 2003)
Ascorbate Peroxidase	Chloroplast, cytosol, mitochondria, peroxisomes, apoplast	H_2O_2	(Asada, 1999)
Non-enzymatic:			
Ascorbate	Chloroplast, cytosol, mitochondria, peroxisomes, apoplast	H_2O_2, $O_2^{\cdot -}$	(Asada, 1999; Noctor *et al.*, 1998)
Glutathione	Chloroplast, cytosol, mitochondria, peroxisomes, apoplast	H_2O_2	(Asada, 1999; Noctor *et al.*, 1998)
α-Tocopherol	Thylakoid membranes	$^1O_2$	(Noctor *et al.*, 1998)
Carotenoids	Chloroplast	$^1O_2$	(Holt *et al.*, 2005)

2.3.3. Superoxide dismutase (EC 1.15.1.1)

Within a cell, the superoxide dismutase (SOD) forms the first line of defense against ROS. $O_2^{\cdot -}$ is produced at a location where an electron transport chain is operated. Different forms of SODs are present at different sites of $O_2^{\cdot -}$ production(Takahashi *et al.*, 1983).

Based on the metal cofactor used by the enzyme, SODs are of three types *viz.*, iron SOD (Fe-SOD), manganese SOD (Mn-SOD) and Copper-zinc SOD (Cu-Zn-SOD), and these SODs are located in different compartments of the cell. Fe-SODs are located in the chloroplast, Mn-SODs in the mitochondrion and the peroxisomes whereas, Cu-Zn-SODs are mainly located in the chloroplast, cytosol and possibly in the extracellular space as shown in figure 2.1 (Alscher *et al.*, 2002).

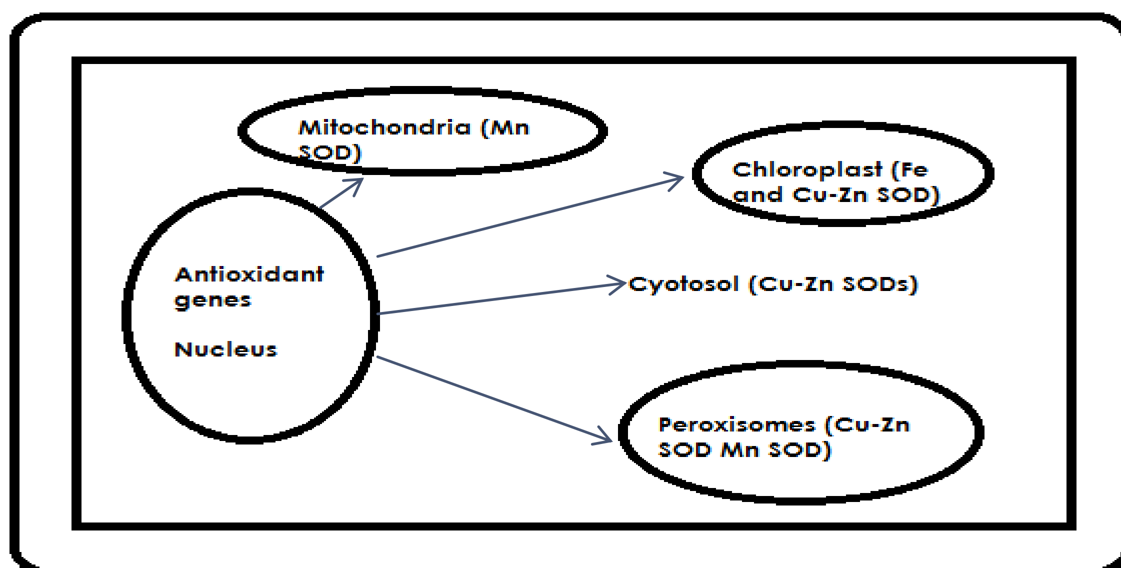
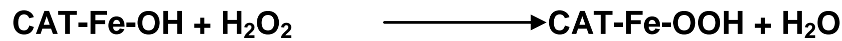


Figure 2.1: Locations of SODs throughout the plant cell

2.3.4. Catalase (EC 1.11.1.6)

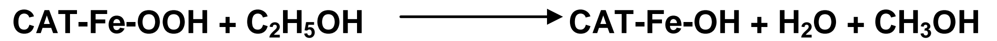
The enzyme catalyses the decomposition of hydrogen peroxide to water (Chelikani *et al.*, 2004). Catalase is mainly present in the peroxisomes of nearly all aerobic cells, which serves to protect the cell from the toxic effects of hydrogen peroxide by catalysing the decomposition into molecular oxygen and water without the production of free radicals (Boon *et al.*, 2007). CAT is one of the most active naturally occurring enzymes which have a catalytic centre activity of about 10^7/min. Depending upon the concentration of H_2O_2 it shows dual function in the form of peroxidatic and catalytic activity. Both peroxidatic and catalytic activity

follow two step mechanisms in which the first step is the formation of oxygen rich peroxide (Intermediate iron peroxide).



$$\textbf{CAT-Fe-OH} + H_2O_2 \longrightarrow \textbf{CAT-Fe-OOH} + H_2O$$

- **Peroxidatic activity -:** At low H_2O_2 concentration iron peroxide is reduced by hydrogen donor (e.g. ethanol).



$$\textbf{CAT-Fe-OOH} + C_2H_5OH \longrightarrow \textbf{CAT-Fe-OH} + H_2O + CH_3OH$$

- **Catalytic activity -:** At high H_2O_2 concentration iron peroxide reacts with second H_2O_2 to produce H_2O and O_2.

The overall reaction is as follows:

$$2H_2O_2 \longrightarrow 2H_2O + O_2$$

2.3.5. Ascorbate peroxidase (EC 1.11.1.11)

Ascorbate peroxidase (APX) belongs to the class I heme-peroxidases which are found in higher plants, chlorophytes, red algae and members of the protist kingdom. It is a key enzyme regulating ROS levels acting in different subcellular compartments (figure 2.2) (Caverzan *et al.*, 2012).

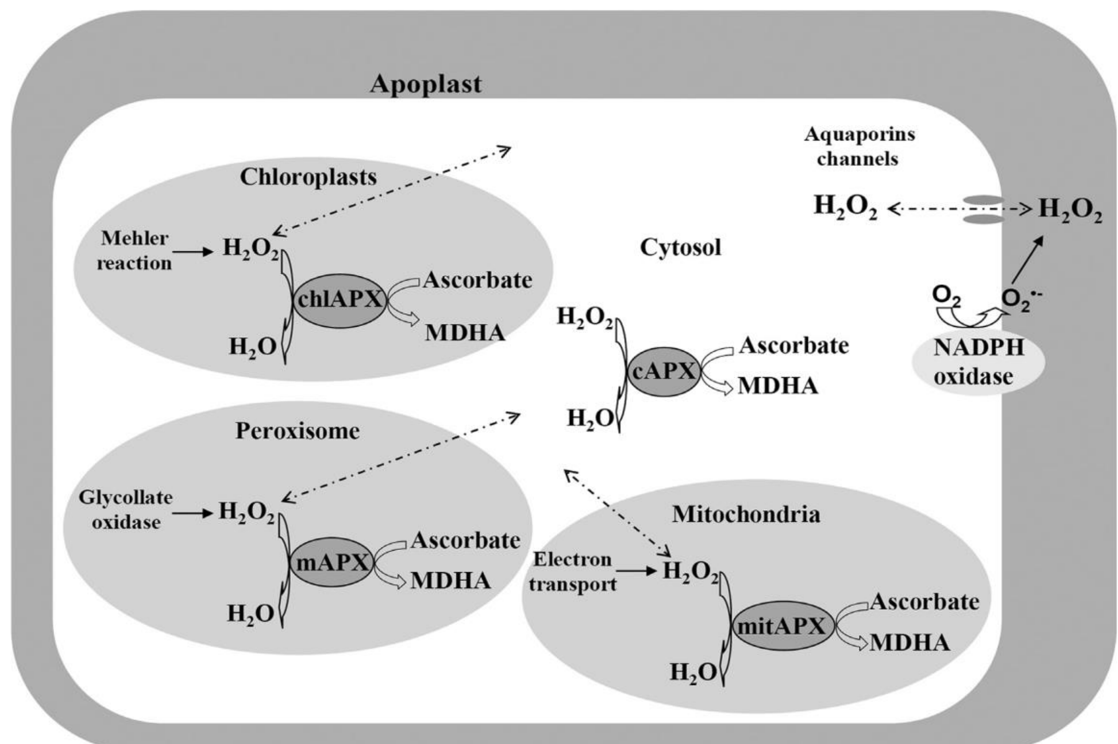


Figure 2.2: APX enzyme and the elimination of ROS in different subcellular compartments of the cell (Caverzan *et al.*, 2012)

In plant cells, the APX can be detected in all intracellular compartments such as cytosol, chloroplasts, mitochondria and plant cell thylakoids. APX reduces

H_2O_2 to H_2O in the presence of ascorbate, which acts as an electron donor. The enzyme is highly specific to ascorbate and loses its activity in absence of it. The overall reaction is as follows:

$$\mathbf{H_2O_2 + 2Asc \longrightarrow 2MDA + 2H_2O}$$

$$\mathbf{2MDA + NAD(P)H + H^+ \longrightarrow 2Asc + NAD(P)^+}$$

2.3.6. Glutathione reductase (EC 1.6.4.2)

The enzyme glutathione reductase (GR), converts the oxidised form of glutathione (GSSG) to its reduced form (GSH), using NADPH as cofactor. It is a key enzyme regulating ROS levels in different subcellular compartments. GR accerlates the H_2O_2 scavenging pathway in plants, particularly under stress conditions (Anjum *et al.*, 2010). GR catalyses the following reaction:

$$\mathbf{GSSG + NADPH + H^+ \longrightarrow 2GSH + NADP^+}$$

2.3.6.1. Interdependence of Ascorbate and Glutathione

The stable oxidised form of ascorbate is DHA. Ascorbate undergoes univalent oxidation to give MDHA and therefore; spontaneous MDHA dismutation produces the stable form of ascorbate i.e., DHA. MDHA and DHA regenerate ascorbate through the recycling reactions catalysed by MDHAR and DHAR in the Foyer-Halliwell-Asda cycle, also known as the Ascorbate-Glutathione pathway (figure 2.3) (Locato *et al.*, 2013). In this pathway, GSH regenerate ascorbate by reducing DHA, either chemically or via DHARs enzyme (Foyer *et al.*, 2011).

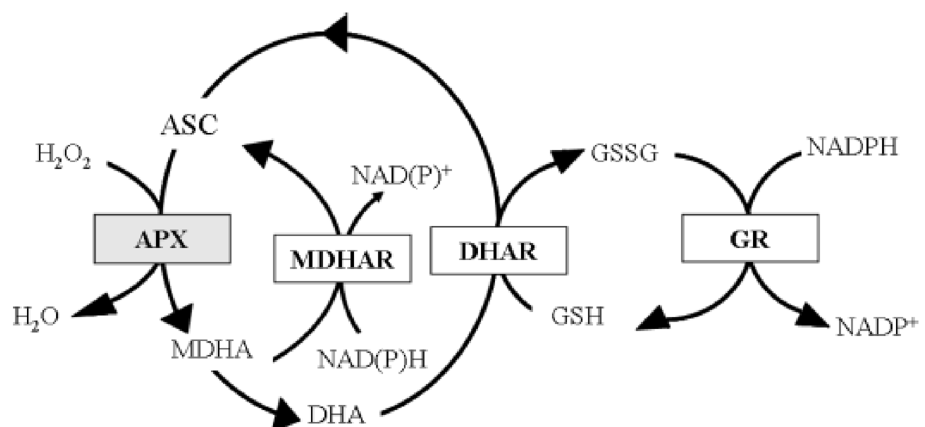
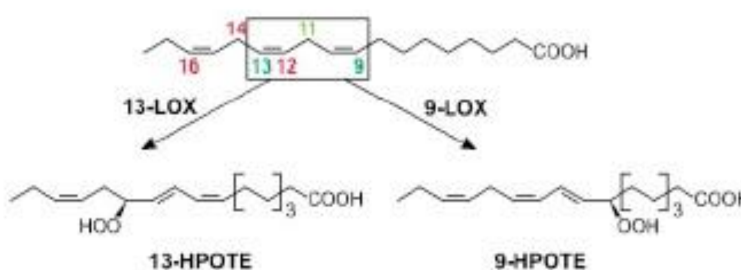


Figure 2.3: Ascorbate-Glutathione pathway (Locato *et al.*, 2013)

2.3.7. Lipoxygenase (EC 1.13.11.12)

Lipoxygenase (LOX) constitute a large gene family of non-heme iron containing fatty acid deoxygenases which are present in plants and animals. LOX catalyse the regio- and stereo-specific deoxygenation of PUFAs containing a (1Z, 4Z)-pentadiene system e.g., linoleic acid (LA), α-linoleic acid (LeA) or archidonic acid (AA) (figure 2.4) (Liavonchanka *et al.*, 2006). As shown in reaction, the major reaction products are fatty acid hydroperoxides, but LOX mediated formation of $^1O_2$ and $O_2^{\cdot -}$ has also been observed (Zuo *et al.*, 2004).



<u>Figure 2.4</u>: LOX reaction and positional specificity (Feussner *et al.*, 2002)

2.4. Acclimatization of plants

Cold acclimatization is preparing the plants in advance by slowly exposing the plants to stress. Plant exhibit cold tolerance due to their pre-exposure to low temperature due to cold acclimatization (Thomashow, 1999). There are numerous studies conducted on acclimatization where the plants combat the severe stress. Cold acclimatization is associated with number of changes in the plants in response to low temperature which include changes in gene expression (Pearce, 1999), accumulation of cryo-protectants such as proline, sugars, and antioxidants such as, SOD, CAT and POX (Kang *et al.*, 2001).

It has been well understood that acclimatized chickpea are better adapted to low temperature stress than non-acclimatized chickpea seedlings by activating the defense pathways against onset of lethal cold stress in advance (Nayyar *et al.*, 2005). It has been noticed that there is a complex array of gene networks, which

is activated in cold acclimatization phase which lead to cold tolerance in plants (Chinnusamy *et al.*, 2007).

2.5. Preconditioning of plants against abiotic stress

Preconditioning is a different term from acclimatization as some alternate abiotic stress is used to make the plants tolerant to lethal abiotic stress. There has been very limited number of studies which are conducted on preconditioning the crop with some abiotic stress. The concept is only applied in some grasses. Preconditioning of crop plants with prior applications of hormones, antioxidants and ABA has already been tested (Nayyar *et al.*, 2005). Preconditioning of crops can be a popular way to avoid abiotic stresses. Different abiotic stresses may activate similar kind of defense mechanisms e.g. dehydration and low temperature stresses can induce the reactions which are involved in defense against both dehydration and low temperature stress e.g. DRE/CRT elements, MAP kinase and COR genes (Shinozaki *et al.*, 2000). Both stresses can induce activation of similar genes and transcription factors in *Arabidopsis thaliana*. In *Arabidopsis*, these genes include *rd (responsive to dehydration), erd (early responsive to dehydration), cor (cold-regulated), lti (low temperature induced)* and *kin (cold-inducable)*(Shinozaki *et al.*, 2000).

MATERIALS AND METHODS

CHAPTER 3
MATERIALS AND METHODS

Chickpea (*Cicer arietinum* L.), released varieties of PDG3 and GPF2 were employed to study the preconditioning response on cold affected antioxidative defence system. The germplasm consisting of two released varieties GPF2 and PDG3 was procured from Punjab Agricultural University, Ludhiana.

3.1. Experimental design

To achieve the objective Completely Randomized Block Design was followed.

3.1.1. Experimental setup: (Raising seedling and preconditioning treatments)

Initially, healthy seeds were picked, surface sterilized with 10% sodium hypochlorite. Petri-plates (15 inches) were used for keeping the seeds for germination, covered with lid to avoid light as seeds are negatively photo-blastic. After germination (48 hours), the lids were removed and the seeds were exposed to 16/8 hrs (light/dark) photoperiod. The optimum conditions (temperature 27˚C) were maintained in a climate controlled seed germinator (Macro Scientific Works Pvt. Ltd.). Seedlings were grown using Hoagland medium for eleven days to have enough biomass. On 11th day, a part of seedlings (preconditioned) were exposed to mild water stress conditions with PEG-6000 (-0.1 MPa). The preconditioned seedlings were kept for three days under stressed conditions and on 14th day preconditioned samples were washed with water, put into the recovery by shifting it to normal conditions for another five days. On 19th day, non-preconditioned and preconditioned seedlings were exposed to lethal cold stress at 5˚C for another three days. After three days of lethal exposure the experiment was terminated and status of different parameters was checked by harvesting samples (figure 3.1).

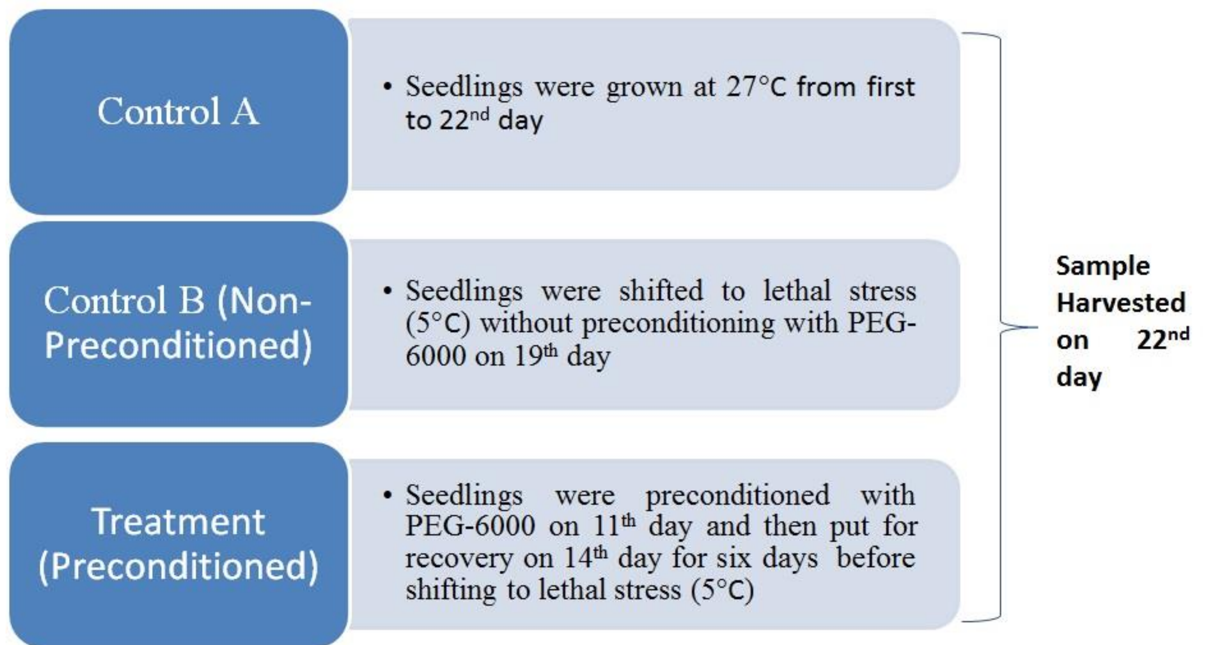


Figure 3.1: Experimental setup

3.2. Percent seed germination of var. GPF2 and PDG3

The surface sterilized seeds were kept on petri-plates and monitored for seed germination patterns under optimum conditions. 100% germination was recorded to check the seed viability of two genotypes.

The sample harvested both root and shoot were processed for fresh matter analysis using various parameters (figure 3.2).

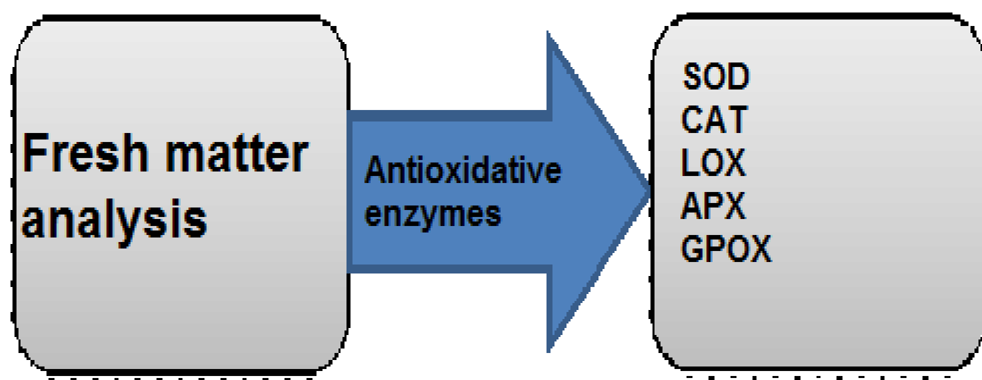


Figure 3.2: Parameters studied in fresh matter analysis

3.3. Sample harvesting

Fresh root and shoot samples were harvested on 22nd day (lethal stress) were analysed for following parameters.

3.3.1. Lipoxygenase assay

The protocol was modified in which 50mg FM of leaves were homogenized at 4˚C in 50 mM potassium phosphate buffer (pH 6.5) containing 0.5% (v/v) Triton X-100, 1% (v/v) Tween-20 and 20% (w/v) PVP. The homogenate was centrifuged at 15,000 x g for 15 min at 4˚C. The supernatant was collected as plant extract. The assay mixture contained 200µl of 0.2 mM linoleic acid, 790µl of 100 mM potassium phosphate buffer (pH 6.5) and 10µl of plant extract. For blank assay mixture contained 800µl of 100mM potassium phosphate buffer (pH 6.5) and 200µl of 1 mM linoleic acid (final concentration of linoleic acid in 1 ml reaction mixture was 0.2mM). Lipoxygenase activity (LOX, EC 1.13.11.12) was determined spectrophotometrically at 234 nm (Shimadzu UV-VIS spectrophotometer UV-2458) and change in absorbance was recorded for one minute. The lipoxygenase activity was calculated and expressed as $\Delta A_{234} min^{-1} mg^{-1}$ FW where $\Delta A_{234} = A_{234}$0 min. – A_{234}1 min. (Palma *et al.*, 2013).

3.3.2. Antioxidative enzyme assay

3.3.2.1. Preparation of enzyme extract

For enzyme extract preparation 50 mg FM were homogenized in 2ml of plant extraction buffer [50mM phosphate buffer (pH 7.8), 0.1mM EDTA, 1mM phenylmethylsulfonyl fluoride (PMSF) and 2% PVP]. For APX activity, extraction buffer also contains 5mM ascorbic acid. Homogenized samples were centrifuged at 14,000xg for 30 minutes at 4˚C and supernatant was used for the determination of protein content and enzyme activities. Enzyme extracts were kept at 4˚C for all the assays and were stored at -20˚C for long period (Ceylan *et al.*, 2013).

3.3.2.2. Superoxide dismutase (SOD)

For SOD assay the reaction mixture (Solution A) was prepared by mixing 25 mL of 50 mM potassium phosphate buffer (pH 7.8), 35 mg of L-methionine (9.9 mM), 7.5µl of Triton X- 100 (0.025%) and 4 mg NBT (57µmol/L). Aliquots (1 mL) of this Solution A were delivered into small glass tubes, followed by 20µL of enzyme extract and 10µL of Riboflavin (4.4 mg/100 ml). The cocktail was mixed and then illuminated for 10 min under 20W florescent tubes at 25˚C. A control tube in which the sample was replaced by 20µL of buffer was run in parallel and the A_{560} was

measured in all tubes. The test tubes containing the reaction mixture were exposed to light immersing the glass tubes in a cylindrical glass container three fourth filled with clean water and maintained at 25˚C. The increase in absorbance due to formazan formation was read at 560 nm. Under the described conditions, the increase in absorbance without the enzyme extract was taken as 100% and the enzyme activity was calculated by determining the percentage inhibition per min. Fifty percent of inhibition was taken as equivalent to 1unit of SOD activity (Chakrabarty *et al.*, 2009).

3.3.2.3. Catalase (CAT)

Catalase activity was performed as follows. 1ml reaction mixture contained 50 mM potassium phosphate (pH 7.0), 0.1 mM H_2O_2 and 40µl enzyme extract, and the initial linear rate of decrease in absorbance at 240 nm was used to calculate the activity. The activity of catalase was calculated by using extinction co-efficient 43.6 M^{-1} cm^{-1} and expressed as µM min^{-1} mg^{-1} FW (Chakrabarty *et al.*, 2009).

3.3.2.4. Ascorbate peroxidase (APX)

Reaction mixture in a total volume of 1 ml contained 50 mM K-phosphate buffer (pH 7.0), 0.2 mM ascorbic acid, 0.2 mM EDTA, 20 mM H_2O_2 and 10 µl enzyme extract, and the initial linear rate of decrease in absorbance at 240 nm was used to calculate the activity. The specific activity of ascorbate peroxidase was calculated by using extinction co-efficient 2.8 $mM^{-1}cm^{-1}$ and expressed as µM min^{-1} mg^{-1} FW (Verma *et al.*, 2003)

3.3.2.5. Guaiacol peroxidase (GPOX)

Assay mixture in a total volume of 1 ml contained 50 mM K-phosphate buffer (pH 6.1), 2mM H_2O_2, 9 mM guaiacol and 20 µl enzyme. Increase in absorbance was measured at 420 nm (extinction coefficient of GPOX is 26.6 mM^{-1} cm^{-1}) for 1 min. Enzyme specific activity was expressed as µM min^{-1} mg^{-1} FW (Verma *et al.*, 2003).

RESULTS

CHAPTER 4
RESULTS

4.1. Superoxide dismutase activity (SOD)

Superoxide dismutase (SOD) forms the first line of defense against ROS. $O_2^{\cdot -}$ is produced at location where an electron transport chain is operated.

The data collected for SOD in the form of 50% inhibition was analysed by two way ANOVA with all pairwise multiple comparison procedure using Tukey test. Both genotypes, PDG3 and GPF2 showed significant difference ($p < 0.05$) in terms of sensitivity to the stress as the level of SOD activity was more in GPF2 (34.622Ug^{-1}FW) as compared to PDG3 (30.019Ug^{-1}FW).

In PDG3 shoot, significant increase in SOD activity was observedinpreconditioned seedlings (24.841Ug^{-1}FW) in comparison to control seedlings (7.955Ug^{-1}FW) (figure 4.1A). Also between non-preconditioned and preconditioned seedlings there was significant increase in preconditioned over non-preconditioned seedlings (12.008Ug^{-1} FW).

In PDG3 root, no marked difference was observed among the three treatments*viz*., control (16.736Ug^{-1} FW), preconditioned (12.908Ug^{-1} FW) and non-preconditioned seedlings (15.610Ug^{-1} FW) respectively (figure 4.1B).

In GPF2 shoot, the SOD activity was significantly more in preconditioned seedlings (32.270Ug^{-1}FW) over non-preconditioned (20.338Ug^{-1} FW) and control seedlings (12.608Ug^{-1}FW). As shown in figure 4.1C no significant difference was observed between control seedlings and non-preconditioned seedlings.

Whereas, in GPF2 root, SOD activity was significantly decreased in preconditioned seedlings (-0.450Ug^{-1}FW) as compared to control (19.437Ug^{-1} FW) and non-preconditioned treatments (19.662Ug^{-1} FW) (figure 4.1D). But there was no difference betweencontroland non-preconditioned seedlings.

Therefore, it can be inferred that preconditioning enhances the SOD activity in tissue shoot of both varieties. But, there was no effect of preconditioning in roots of GPF2 and SOD activity was decreased in PDG3 root on preconditioning.

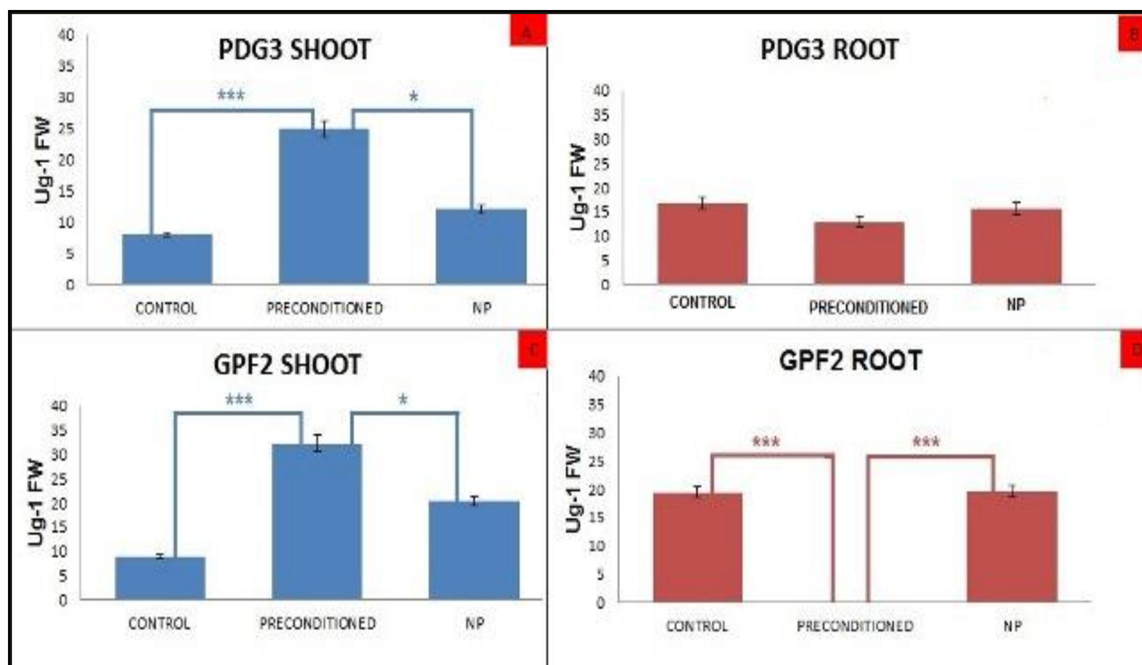


Figure 4.1: SOD activity after preconditioning in PDG3 and GPF2 in both shoot and root tissue.(A) Graph showing SOD activity of PDG3 shoot in different treatments. (B) Graph showing SOD activity of PDG3 root in different treatments. (C) Graph showing SOD activity of GPF2 shoot in different treatments. (D) Graph showing SOD activity of GPF2 root in different treatments. The level of significance was measured at p<0.05 using multiple comparison using Tukey test.

4.2. Catalase activity

Catalase is a tetrameric heme containing enzyme found in all aerobic organisms that helps in the rapid degradation of H_2O_2. The overall reaction is as follows:

$$2H_2O_2 \longrightarrow 2H_2O + O_2$$

The data collected for CAT in the form of CAT activity was analysed by two way ANOVA with all pairwise multiple comparison procedure using Tukey test. Both genotypes, GPF2 and PDG3 showed significant difference ($p < 0.05$) in terms of sensitivity to the stress as the level of CAT activity was more in PDG3 (6.821μMol/min/g FW) as compared to GPF2 (1.552μMol/min/g FW). But on tissue, both root and shoot levels were equally sensitive, no significant difference was recorded between them.

In PDG3 shoot, no significant difference was found among control (1.703μMol/min/g FW), preconditioned (4.690μMol/min/g FW) and non-preconditioned seedlings (1.411μMol/min/g FW) (figure 4.2A).

In PDG3 root, significant increase in CAT activity was observed in preconditioned seedlings (6.086μMol/min/g FW) over non-preconditioned seedlings (2.231μMol/min/g FW). But no significant difference was found when

control (4.346µMol/min/g FW) seedlings were compared with preconditioned and non-preconditioned seedlings, respectively (figure 4.2B).

In GPF2 shoot [control (0.115µMol/min/g FW), preconditioned (2.288µMol/min/g FW) and non-preconditioned seedlings (0.258µMol/min/g FW)], (figure 4.2C) as well as for GPF2 root [control (0.390µMol/min/g FW), preconditioned (1.109µMol/min/g FW) and non-preconditioned seedlings (0.497µMol/min/g FW)], (figure 4.2D), no significant difference was found among the three treatments.

It can be inferred that preconditioning induced CAT activity to a significant level especially in PDG3. On the other hand it could not influence in case of GPF2 seedlings.

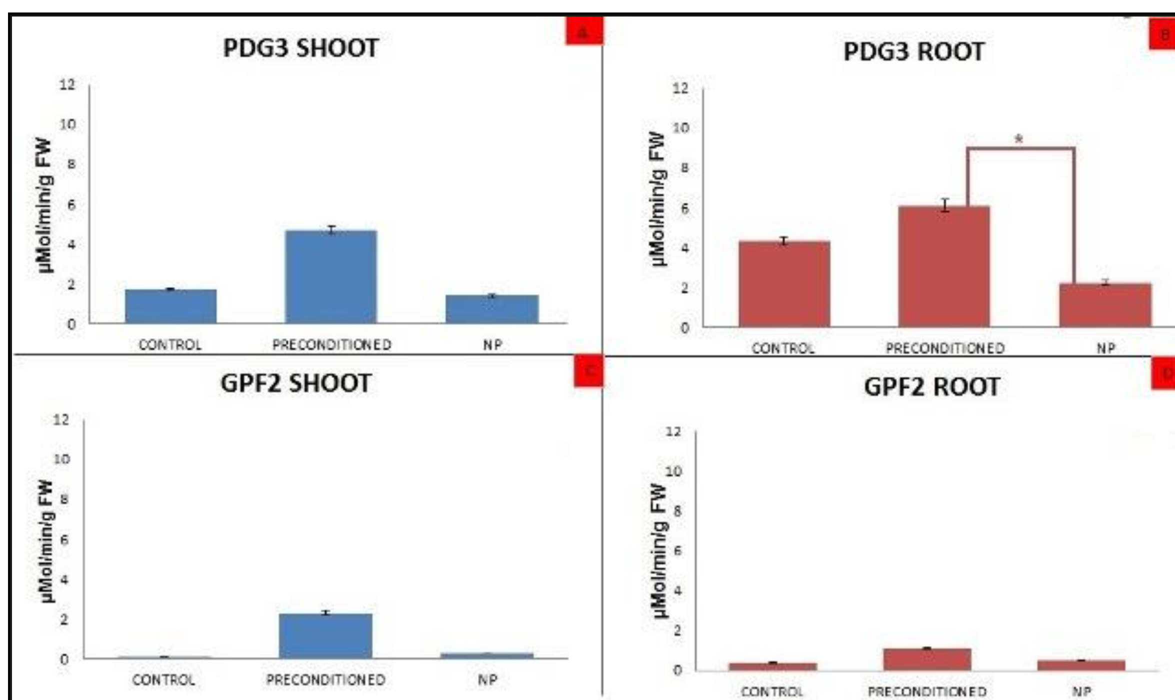


Figure 4.2: CAT activity after preconditioning in PDG3 and GPF2 in both shoot and root tissue.(A) Graph showing CAT activity of PDG3 shoot in different treatments. (B) Graph showing CAT activity of PDG3 root in different treatments. (C) Graph showing CAT activity of GPF2 shoot in different treatments. (D) Graph showing CAT activity of GPF2 root in different treatments. The level of significance was measured at $p<0.05$ using multiple comparison using Tukey test.

4.3. Guaiacol peroxidase activity (GPOX)

Guaiacol peroxidase (GPOX) reduces the toxic levels of H_2O_2, by reducing it to H_2O, in the presence of guaiacol which acts as a reducing agent. The overall reaction is as follows:

Guaiacol + H_2O_2 **H_2O + O_2**

The data collected for GPOX activity was analysed by two way ANOVA with all pairwise multiple comparison procedure using Tukey test. Both genotypes, PDG3 and GPF2 showed significant difference ($p < 0.05$) in terms of sensitivity to the stress as the level of GPOX activity was slightly more in GPF2 (17.415µMol/min/g FW) as compared to PDG3 (17.162µMol/min/g FW). But both root and shoot tissues were equally sensitive as no significant difference was recorded between them.

In PDG3 shoot, significant increase in GPOX activity was observed in case of preconditioned seedlings (9.522µMol/min/g FW) over the control (3.055µMol/min/g FW) and non-preconditioned seedlings (6.266µMol/min/g FW) (figure 4.3A).

In PDG3 root, significant increase in GPOX activity was observed in case of preconditioned seedlings (13.158µMol/min/g FW) over non-preconditioned (9.950µMol/min/g FW) and control seedlings (9.536µMol/min/g FW). As shown in figure 4.3B, no significant difference was observed between non-preconditioned and control seedlings.

In GPF2 shoot, GPOX activity wassignificantly increased in both preconditioned (8.158µMol/min/g FW) and non-preconditioned seedlings (7.005µMol/min/g FW) when compared with the control seedlings (2.995µMol/min/g FW). But no significant difference was observed between preconditioned and non-preconditioned seedlings (figure 4.3C).

In GPF2 root, significant increase in GPOX activity was observed in preconditioned seedlings (16.930µMol/min/g FW) over the control (7.832µMol/min/g FW) and non-preconditioned (9.323µMol/min/g FW) seedlings (figure 4.3D). But no significant difference was observed when control and non-preconditioned seedlings were compared to each other.

Overall, preconditioning enhances the GPOX activity in PDG3 and GPF2 seedlings.

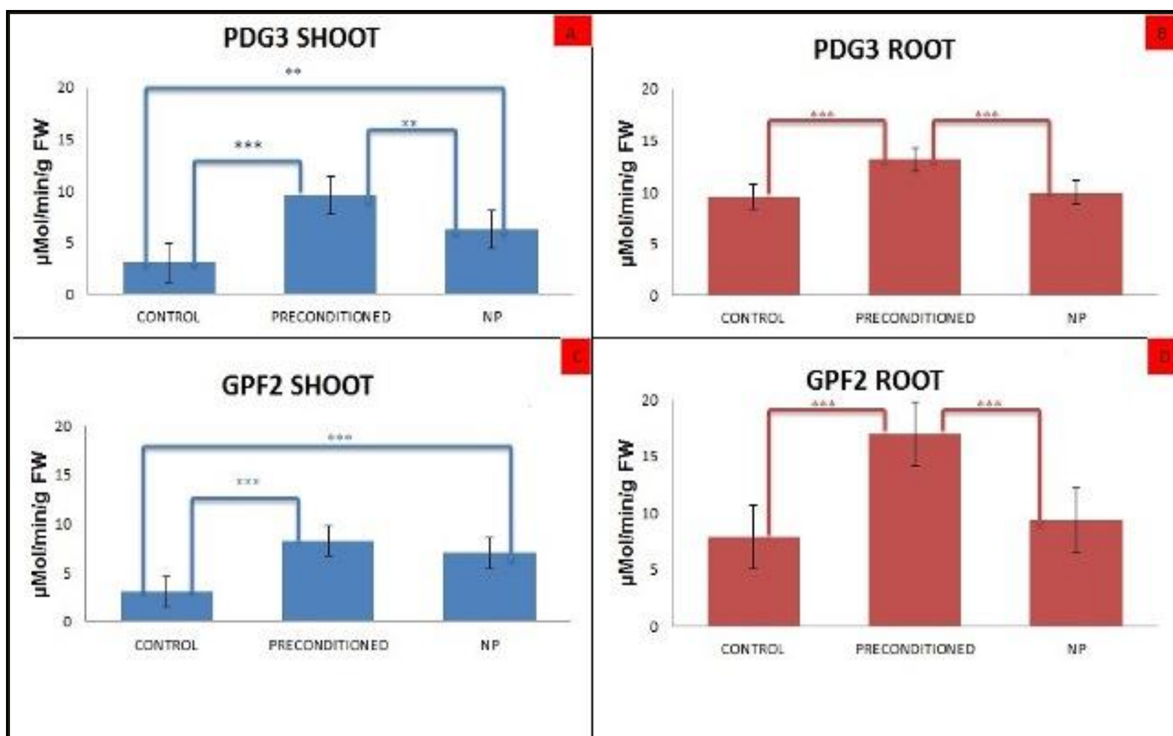


Figure 4.3: GPOX activity after preconditioning in PDG3 and GPF2 in both shoot and root tissue.(A) Graph showing GPOX activity of PDG3 shoot in different treatments. (B) Graph showing GPOX activity of PDG3 root in different treatments. (C) Graph showing GPOX activity of GPF2 shoot in different treatments. (D) Graph showing GPOX activity of GPF2 root in different treatments. The level of significance was measured at p<0.05 using multiple comparison using Tukey test.

4.4. Ascorbate Peroxidase activity (APX)

In plants, APX acts as the reductant of H_2O_2 and thus helps in reducing H_2O_2 detoxification in photosynthetic organisms. The main electron donor of APX is the ascorbate. Ascorbate reduces the H_2O_2 to H_2O with the generation of monodehydroascorbate (MDHA). Then MDHA is spontaneously broken into ascorbate and dehydroascorbate (DHA). MDHA can also be directly reduced to ascorbate by the action of NADPH dependent MDHAR which utilizes glutathione (GSH) to reduce DHA to generate ascorbate. Thus APX in combination with ASc-GSH cycle helps in reducing the toxic levels of H_2O_2.

The data collected for APX in the form of APX activity was analysed by two way ANOVA with all pairwise multiple comparison procedure using Tukey test. Both genotypes, GPF2 and PDG3 showed significant difference (p value < 0.05) in terms of sensitivity to the stress as the level of APX activity was more in GPF2 (40.803μMol/min/g FW) as compared to PDG3 (10.415μMol/min/g FW). But shoot

and root tissues were equally sensitive as there was no significant difference between them.

PDG3 did not respond to preconditioning treatment as far as APX activity is concerned. As shown in figure 4.4A and 4.4B, no significant difference was observed in APX activity when three different treatments *viz.*, control, precondition and non-precondition were observed for shoot and root level respectively.

In GPF2 shoot, no significant increase in APX activity was observed among three different treatments (figure 4.4C) *viz.*, control (14.167µMol/min/g FW), preconditioned (25.625µMol/min/g FW) and non-preconditioned seedlings (18.929µMol/min/g FW) respectively.

In GPF2 root, significant decrease in APX activity was observed in non-preconditioned (15.119µMol/min/g FW) seedlings when compared with control seedlings (29.643µMol/min/g FW) (figure 4.4D). But no significant difference in APX activity was found when preconditioned seedlings (18.929µMol/min/g FW) were compared with non-preconditioned seedlings (15.119µMol/min/g FW).

In general, no significant difference was found when all the three treatments were compared with each other, but overall greater activity of APX was found in GPF2 as compared to PDG3 variety which means that GPF2 is a better scavenger of H_2O_2 and helps in preventing the accumulation of toxic levels of H_2O_2 when compared with the PDG3 variety.

4.5. Lipoxygenase activity (LOX)

LOX catalyse the hydroperoxidation of polyunsaturated fatty acids hence forming the first step in the synthesis of fatty acid metabolites in plants. Products of the LOX pathway have multiple functions as growth regulators, antimicrobial compounds, flavours, odours as well as signal molecules.

The data collected for LOX in the form of LOX activity was analysed by two way ANOVA with all pairwise multiple comparison procedure using Tukey test. Both genotypes, GPF2 and PDG3 showed significant difference ($p < 0.05$) in terms of sensitivity to the stress as the level of LOX activity was more in PDG3 (22.48µMol/min/g FW) as compared to GPF2 (7.6µMol/min/g FW). But both root and shoot tissue was equally sensitive, no significant difference was recorded between them.

In PDG3 shoot, significant increase in LOX activity was observed in non-preconditioned seedlings (21.380μMol/min/g FW) and preconditioned seedlings (6.200μMol/min/g FW) than control seedlings (1.300μMol/min/g FW) as shown in figure 4.5A. Between preconditioned and non-preconditioned, LOX activity was significantly more in non-preconditioned seedlings.

In PDG3 root, no significant difference was observed between preconditioned seedlings (6.467μMol/min/g FW) and control seedlings (10.013μMol/min/g FW) in terms of LOX activity. But LOX activity increased significantly in non-preconditioned (22.080μMol/min/g FW) plants than control and preconditioned plants (figure 4.5B).

In GPF2 shoot, no significant difference in LOX activity was observed among the three treatments *viz.*, control (4.660μMol/min/g FW), preconditioned (4.320μMol/min/g FW) and non-preconditioned seedlings (1.640μMol/min/g FW) (figure 4.5C).

In GPF2 root, the LOX activity was decreased in both non-preconditioned (1.080μMol/min/g FW) and preconditioned seedlings (1.380μMol/min/g FW) than control seedlings (9.720μMol/min/g FW). But there was no significant difference between preconditioned and non-preconditioned seedlings (figure 4.5D).

LOX activity in preconditioned seedlings was at par with control but less than non-preconditioned seedlings showed, preconditioning decreases the activity of LOX in both shoot and root tissue of PDG3.

In roots of GPF2, less LOX activity was observed in both non-preconditioned and preconditioned seedlings, but there was no significant difference was observed between them. Also, there was no significant difference was observed in shoots of GPF2 indicating, preconditioning does not play any role in shoots.

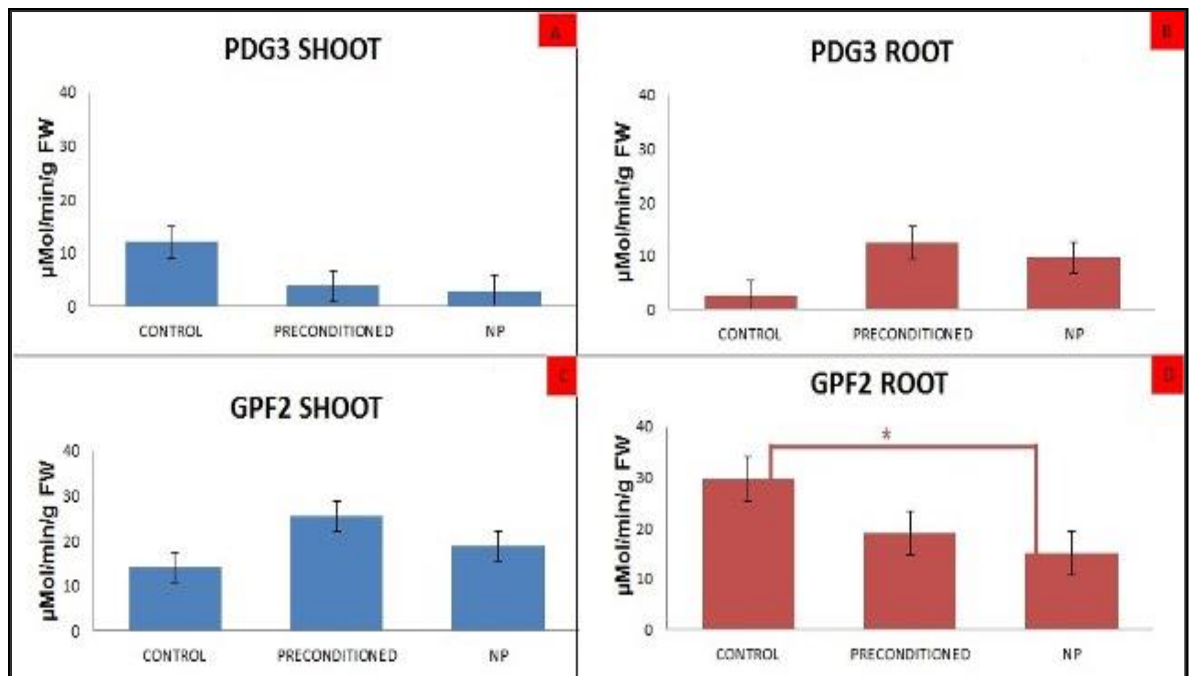


Figure 4.4: APX activity after preconditioning in PDG3 and GPF2 in both shoot and root tissue.(A) Graph showing APX activity of PDG3 shoot in different treatments. (B) Graph showing APX activity of PDG3 root in different treatments. (C) Graph showing APX activity of GPF2 shoot in different treatments. (D) Graph showing APX activity of GPF2 root in different treatments. The level of significance was measured at $p<0.05$ using multiple comparison using Tukey test.

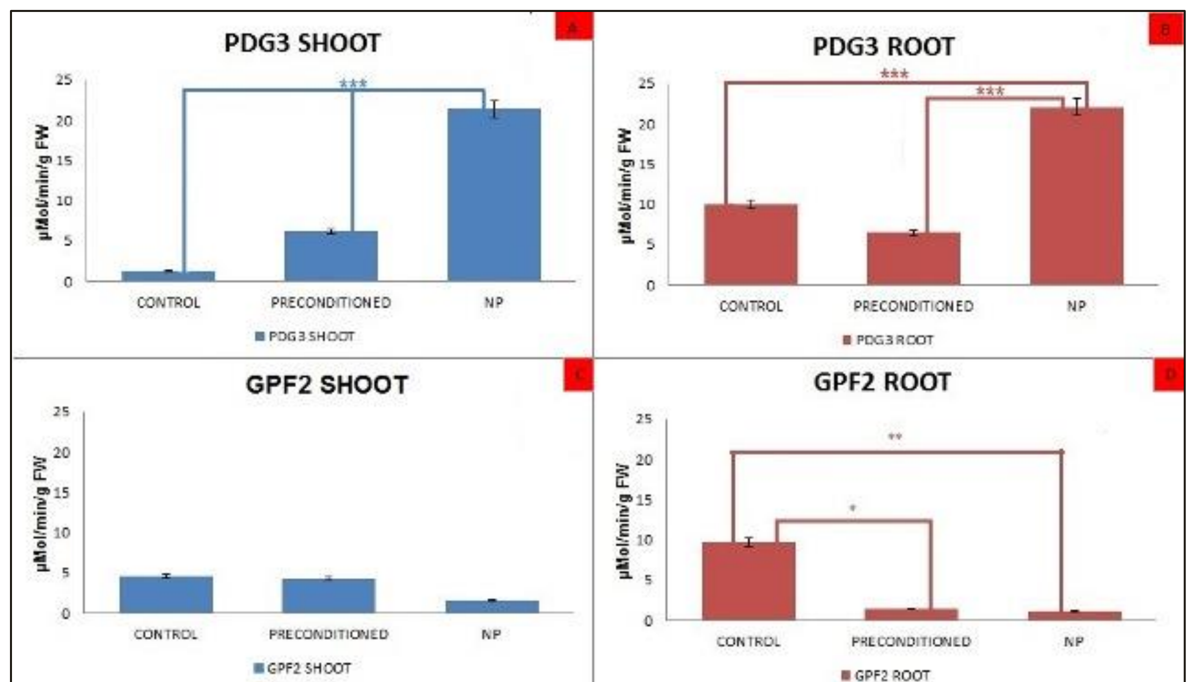


Figure 4.5: LOX activity after preconditioning in PDG3 and GPF2 in both shoot and root tissue.(A) Graph showing LOX activity of PDG3 shoot in different treatments. (B) Graph showing LOX activity of PDG3 root in different treatments. (C) Graph showing LOX activity of GPF2 shoot in different treatments. (D) Graph showing LOX activity of GPF2 root in different treatments. The level of significance was measured at $p<0.05$ using multiple comparison using Tukey test.

DISCUSSION

CHAPTER 5

DISCUSSION

Chickpea (*Cicer arietinum* L.) is grown as a winter season crop in many regions of Northern India. It experiences chilling stress at reproductive stage which leads to poor yield due to alterations in the metabolic activities and gene networks. It has already been proved that acclimation of chickpea seedlings with some non–lethal stress improves the tolerance against the cold stress by improving antioxidative defence mechanism. In present course of study; we have tried to counteract the chilling injury by using preconditioning of chickpea seedlings with non–lethal water stress.

In present study, role of different antioxidative enzymes for evaluating the extent of tolerance induced by mild stress was checked in root as well as shoot in the chickpea seedlings. These parameters were checked on 22nd day when recovered seedlings (preconditioned) were put under lethal low temperature stress.

5.1. Antioxidative Enzyme Activity:

The extent of oxidative damage was measured by evaluating the activity of different antioxidative enzymes in both tissues in different treatments (control, preconditioned and non-preconditioned).

5.1.1. Superoxide dismutase activity:

We found that tolerance towards oxidative stress improved due to preconditioning as the SOD activity was more in preconditioned seedlings indicating greater dismutation of $O_2^{\cdot -}$ radicals than the non-preconditioned ones, showing greater damage in case of non-preconditioned seedlings. But overall control seedlings showed less damage than the preconditioned and non-preconditioned seedlings i.e. chilling stress acclimatised seedlings showed higher SOD activity than non-acclimatised seedlings. Similar results were reported when cucumber leaves were acclimatised to low temperature stress (Lee *et al.*, 2000).

5.1.2. Catalase activity:

Catalase activity is another marker to check the oxidative damage caused by stress in plants. CAT activity also showed similar results as that of the SOD i.e., higher conversion of toxic products into non-toxic forms was observed in preconditioned seedlings over non-preconditioned one, showing less damage in preconditioned seedlings, therefore; it can be inferred that direct exposure to cold stress without giving any preconditioning may have decreased the activity of CAT in non-preconditioned seedlings because low temperature stress causes catalase degradation due to impair repair synthesis at low temperature (Gechev *et al.*, 2003). Similar results were obtained when tobacco was exposed to chilling stress (Gechev *et al.*, 2003).

5.1.3. Guaicol peroxidase activity:

The present study stated that in both the varieties i.e., PDG3 and GPF2, the GPOX activity was more in roots as compared to shoots this may be due to the fact that roots have stronger capability of scavenging ROS. Similar results were obtained when tobacco seedlings were exposed to chilling stress (Xu *et al.*, 2010). Overall significant increase in GPOX activity was observed in preconditioned seedlings over non-preconditioned ones, which means preconditioning of seedlings can generate a better defence mechanism to cope with incoming lethal low temperature stress.

5.1.4. Ascorbate peroxidase activity:

For APX activity, our results showed no significant difference among the three treatments, this may be due to the fact that GPOX and APX are competitors of each other working over the same substrate i.e., H_2O_2 and at the same site in the cell. As previously discussed, GPOX showed higher activity in both the genotypes, therefore; we can conclude that higher GPOX activity have supressed the activity of APX enzyme (Cordoba Pedregosa *et al.*, 1996; Demirevska Kepova *et al.*, 1976).

5.1.5. Lipoxygenase activity:

The formation of ROS due to peroxidation of cell lipid membranes is one of the most damaging processes for membranes. LOX directly attacks unsaturated

fatty acids and increased ROS production have been observed in a variety of plant tissues (Arafat, 2005). In present studies, less membrane damage was observed in preconditioned seedlings when compared with non-preconditioned seedlings. Previous studies suggests the same kind of membrane response in cold acclimated and non-acclimated chickpea seedlings (Shahandashti *et al.*, 2013). The similar outcome was seen in present study when we replaced acclimation with induced water stress preconditioning in seedlings.

Overall, preconditioning of seedlings has improved the tolerance against different ROS over the non-preconditioned seedlings. Similar results were obtained when cucumber seedlings were pretreated with methyl jasmonate (Li *et al.*, 2012) and cucumber leaves were preconditioned with silicon (Liu *et al.*, 2009).

It can be inferred from the present study that preconditioning improves the antioxidative defense system which mainly constitute SOD, CAT, GPOX and lowering of LOX activity. Lowering of APX activity is resultant of better GPOX activity in the preconditioned plants.

SUMMARY

CHAPTER 6

SUMMARY

Chickpea (*Cicer arietinum* L.) is the second most important legume crop sown as a winter crop in northern parts of India which experiences a major yield loss due to lethal low temperature stress at reproductive stage. The main factors associated with tolerance are antioxidative enzyme activity, membrane fluidity and integrity, cellular respiration ability, etc. To counteract the chilling stress, the present strategy employed is feasible especially in field conditions, which may induce tolerance mechanism in chickpea.

Already, a lot of work has been done in field and *in vitro* conditions on acclimation of chickpea against cold stress using the exposure of chickpea seedlings and plants to non-lethal stress for a certain period of time before it experiences lethal chilling stress. It has already been established that during acclimation, defense mechanism activates due to alterations at biochemical and molecular level.

In present course of study, we used the preconditioning of seedlings with mild water stress which is different from acclimation i.e., alternate stress pre-treatment before chilling exposure. After preconditioning with water stress, we checked the changes in metabolism by monitoring and comparing antioxidative enzyme activities in treated and non-treated plants.

It can be summarized from the results obtained that preconditioning improved the SOD activity in case of shoots only, in both PDG3 and GPF2 shoots. In case of roots, both PDG3 and GPF2 varieties do not show any significant difference between control, preconditioned and non-preconditioned. Overall, the SOD activity was more in preconditioned seedlings indicating greater dismutation of $O_2^{\cdot}$- radicals than the non-preconditioned ones.

Both the varieties PDG3 and GPF2 were equally sensitive for CAT activity. In both the varieties preconditioning of seedlings played its role and increased CAT activity was observed at both the tissue (root and shoot) level when compared with the control and non-preconditioned seedlings.

GPOX showed greater activity in roots of both the genotypes with preconditioning treatment. Only PDG3 showed greater GPOX activity in shoots at

the preconditioned stage over the control and non-preconditioned seedlings whereas, no significant difference was observed in case of GPF2.

In case of PDG3 more membrane damage due to greater LOX activity was observed in non-preconditioned seedlings over preconditioned and control seedlings which means that preconditioning has played its role in generating the defense mechanism. In case of GPF2 less membrane damage was observedafter preconditioning the plant with mild water stress.

To conclude the whole study, results obtained clearly indicate that preconditioning with mild water stress has the ability to improve chilling tolerance as it improved the membrane integrity in terms of reduced membrane damage and less peroxidation of chickpea seedlings, which further can be correlated with increased activity of different antioxidative enzymes, results in the conversion of toxic ROS into non-toxic forms. It can also be concluded that both PDG3 and GPF2 were equally sensitive towards different antioxidative enzyme activities (Table 6.1).

This study can be easily done in fields by withholding the water given to the crop before onset of period of chilling injury to chickpea. This study can be further elaborated to molecular level to identify up regulation or down regulation of particular genes during preconditioning and important targets can be identified and can be used for creating improved varieties.

Table 6.1:Comparison of enzymes in preconditioning over non-preconditioning in shoot and root tissue of both the varieties

		PDG3			GPF2		
Enzyme	**Tissue**	**P**	**NP**	**Remarks***	**P**	**NP**	**Remarks***
SOD (Ug^{-1} FW)	**S**	24.841	12.008	2.07 fold ↑	32.27	20.338	1.59 fold ↑
	R	12.908	15.61	1.2 fold ↓	-0.45	19.662	43.69 fold ↓
CAT (µMol/min/g FW)	**S**	4.69	1.411	3.33 fold ↑	2.288	0.258	8.87 fold ↑
	R	6.086	2.231	2.73 fold ↑	1.109	0.497	2.23 fold ↑
APX (µMol/min/g FW)	**S**	3.81	2.857	1.34 fold ↑	25.625	18.929	1.35 fold ↑
	R	12.411	9.643	1.29 fold ↑	18.929	15.119	1.25 fold ↑
GPOX (µMol/min/g FW)	**S**	9.522	6.266	1.52 fold ↑	8.158	7.005	1.26 fold ↑
	R	13.158	9.95	1.32 fold ↑	16.93	9.323	1.82 fold ↑
LOX (µMol/min/g FW)	**S**	6.2	21.38	3.41 fold ↓	4.32	1.64	2.63 fold ↑
	R	6.47	22.08	3.41 fold ↓	1.38	1.08	1.28 fold ↑

- **All the comparison of enzyme activity is done in preconditioning over non-preconditioning**

The level of significance was measured at $p<0.05$ using multiple comparison using Tukey test.

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APPENDICES

APPENDIX A

Preparation of Potassium phosphate buffers

To make 0.1M potassium phosphate buffer, prepare the following solutions:

1. Solution A: 27.2 g KH_2PO_4 per litre (0.2 M final) in water.
2. Solution B: 34.8 g K_2HPO_4 per litre (0.2 M final) in water.

Refer Table A for desired pH, mix the indicated volumes of solutions A and B, then dilute with water to 200 ml. Filter sterilize if necessary. To prepare buffers with pH intermediate between the points listed in Table A, prepare closest higher pH, then titrate with solution A.

Table A.: Preparation of 0.1M Potassium phosphate buffer solution at different pH

Desired pH	Solution A (ml)	Solution B (ml)	Desired pH	Solution A (ml)	Solution B (ml)
5.7	93.5	6.5	6.9	45.0	55.0
5.8	92.0	8.0	7.0	39.0	61.0
5.9	90.0	10.0	7.1	33.0	67.0
6.0	87.7	12.3	7.2	28.0	72.0
6.1	85.0	15.0	7.3	23.0	77.0
6.2	81.5	18.5	7.4	19.0	81.0
6.3	77.5	22.5	7.5	16.0	84.0
6.4	73.5	26.5	7.6	13.0	87.0
6.5	68.5	31.5	7.7	10.5	90.5
6.6	62.5	37.5	7.8	8.5	91.5
6.7	56.5	43.5	7.9	7.0	93.0
6.8	51.0	49.0	8.0	5.3	94.7

APPENDIX B

Preparation of solutions for various experimental analysis

Sr. No.	Stock solutions	Method
1.	0.1 M Na_2EDTA	Dissolve 3.727g of Na_2EDTA in distilled water to make 100ml solution
2.	100mM PMSF (phenylmethylsulfonyl fluoride)	Dissolve 0.174g of PMSF in 10ml of 100% isopropanol. Note: PMSF is light sensitive. Store it at -20^{0}C in dark. PMSF is toxic.
3.	0.1M Ascorbic acid	Dissolve 1.76g in distilled water to make 100ml solution. Note: Ascorbic acid is light sensitive. Store it in dark at 4^{0}C.
4.	1.5M Tris-Cl (pH8.8)	Dissolve 9.085 g of Tris base in 20ml distilled water. Adjust the pH using conc. HCl. Make final volume 50ml using distilled water.
5.	0.5M Tris-Cl (pH6.8)	Dissolve 3.02 g of Tris base in 20ml distilled water. Adjust the pH using conc. HCl. Make final volume 50ml using distilled water.
6.	10mM H_2O_2	Dissolve 39.3µl of 30% H_2O_2in distilled water to make 50ml solution. Note: H_2O_2 is light sensitive. Keep it in dark.
7.	1mM Riboflavin	Weigh 4.78g of riboflavin and dissolve it in distilled water to make final volume of 10ml
8.	1N NaOH	Weigh 2g NaOH dissolve in distilled water to make 50ml solution.
9.	0.6% TTC (2,3,5-triphenyl tetrazolium chloride)	Dissolve 0.6g of TTC in 0.1M potassium phosphate buffer pH 7.6 to make 100ml solution. Note: Store the solution in dark
10.	0.1% TCA (trichloroacetic acid)	Dissolve 0.1 g of TCA in distilled water to make final volume of 100ml. Note: Wear gloves while handling TCA

Sr. No.	Stock solutions	Method
11.	20% TCA	Dissolve 20g of TCA in distilled water to make 100ml solution
12.	0.5% TBA (thiobarbituric acid)	0.5g of TBA was dissolved in 100ml of 20% TCA. Note: Wear gloves while handling TBA solution.
13.	Bradford's Reagent	Dissolve 100mg CoomassieBrillant Blue G-250 in 50ml of 95% ethanol, add 100ml of 85% (w/v) ortho-phosphoric acid. Dilute to 1lt with distilled water when the dye has been completely dissolved and filter through Whatman #1 paper just before use. Note: Bradford's reagent is light sensitive. Store it in dark at 4^0C.
14.	100mM Guaiacol	111.48μl guaiacol dissolved in isopropanol to make final volume of 10ml
15.	PBS (phosphate buffer saline)	Dissolve 8g NaOH, 0.2g KCl, 0.24 KH_2PO_4 in distilled water to make final volume of 1litre.
16.	1mM NADPH	Weigh 8.33mg and dissolve in 10ml PBS. Note: NADPH is highly unstable must be dissolved in PBS and stored at -20^0C in small aliquots. Once thawed solution should not be used again
17.	1mM Linoleic acid	Dissolve 3.15 μl in 5ml ethanol. Mix thoroughly. Make the final volume of 10ml with distilled water. Note: Store linoleic acid in dark at 4^0C.