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# The Characterisation of the Freezing Damage Response during Flowering in European and Middle Eastern Wheat Cultivars (*Triticum aestivum* L)

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University of Plymouth

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**The characterisation of the freezing damage  
response during flowering in European and Middle  
Eastern wheat cultivars (*Triticum aestivum L*)**

*by*

***Mohammed Hamdan Eden Al-Issawi***

A thesis submitted to the University of Plymouth in partial fulfilment for  
the degree of

**DOCTOR OF PHILOSOPHY**

School of Biomedical and Biological Sciences  
Faculty of Science and Technology

2013

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## **The characterisation of the freezing damage response during flowering in European and Middle Eastern wheat cultivars (*Triticum aestivum L*)**

**Mohammed Hamdan Eden Al-Issawi**

Wheat (*Triticum aestivum L.*) is occasionally exposed to low temperature during flowering and huge economic losses can occur especially in some key production countries such as Australia. Although it is generally predicted that there will be a rise in global temperature there are still predicted to be risks associated with low temperature for temperate crops. Post head emergence frost damage remains a major constraint to increasing wheat production.

Five Iraqi varieties (Abu-Ghariab, Fatah, Sham6, IPA95 and IPA99) were screened for their frost hardiness ( $LT_{50}$ ). Abu-Ghariab was chosen for further investigations along with the European cv. Claire because the acclimated frost hardiness level in these two varieties ( $LT_{50}$  -8.07 and -8.01°C for Claire and Abu-Ghariab respectively) was found to be significantly lower than the other varieties in this study.

Several techniques were employed including REC%, IR thermography and molecular analysis of cold acclimation in order to characterise the frost resistance of those two varieties. REC% revealed that both Claire and Abu-Ghariab could tolerate some freezing when the spikes were just visible (ZCK 51-60) with an acclimation shift of the  $LT_{50}$  of -1.6 and -2.11°C respectively but this was not apparent at later growth stages. Based on molecular analysis, cold acclimation was shown to be activated at ZCK 51-60 in both varieties. *Cbf14* was expressed after 8 hours exposure to acclimating temperatures (4°C) and then declined to a low, but still up-regulated level in both varieties and this led to expression of the COR15a protein. These molecular changes correlated with the frost tolerance recorded at ZCK 51-60. It was concluded that the possibility existed to up-regulate cold acclimation after spike emergence if there was enough environmental stimulus.

Molybdenum (Mo) was demonstrated to work synergistically with low temperature in increasing the expression of *Cbf14* and COR15a. The European wheat cv. Claire showed a higher capacity (-8.14°C) to be acclimated than Iraqi wheat (-7.40°C) under the effect of both Mo and acclimation temperatures. Mo alone increased the expression of *Cbf14* in both varieties but did not increase the frost tolerance.

Observations of ice nucleation using an infrared thermography (IR) revealed that supercooling is highly likely in spikes and some spikes avoided frost damage even when the temperature fell to -12°C. It was observed that the proportion of frozen spikes was 22.8% while the remainder supercooled. Spraying plants with distilled water was not effective in facilitating ice nucleation in wheat spikes. Observations also revealed that spikes that did freeze started freezing at temperatures of -4 to -5 °C close to temperature of the putative constitutive frost hardiness of un-acclimated wheat and it is suggested that this may reflect that many laboratory freezing experiments may not actually freeze until -5°C. The anthesis stage of wheat was found to be the most vulnerable stage and it needs to be given more attention in terms of research to up-regulate cold acclimation. Frost damage to wheat during flowering continues to be a serious problem in certain production areas and therefore continued effort in characterising and finding suitable solutions to this are imperative.

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## **Dedication**

*I dedicated this thesis to*

*My father whose memory will never leave my mind and his presence is still sensible in every single second for me*

*My mother whose prayers are a backing to my life and entity*

*My cherished wife who always has accompanied me in every stage of my life*

***Mohammed Al-Issawi 2013***

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*Mohammed Al-Issawi 2013*



## **Author's Declaration**

At no time during the registration for the degree of Doctor of philosophy has the author been registered for any other University award.

I declare that the work submitted in this thesis is the results of my own investigations except where reference is made to published literature and where assistance where acknowledged.

This study was sponsored by the Iraqi Ministry of Higher Education.

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**Word account of main body of thesis: 46,127 words, total 54,200 words**

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- 21- Genstat software, 25/10/2010.

## Public output from this work

### - Publications

- 1- **Al-Issawi**, M., Rihan, H. Z., El-sarkassy, N. & Fuller, M. P. 2012. Frost Hardiness Expression and Characterisation in Wheat at Ear Emergence. *Journal of Agronomy and Crop Science*, 1-9.
- 2- **Al-Issawi**, M., Rihan, H. Z., Woldie, W. A., Burchett, S. & Fuller, M. P. 2013. Exogenous application of molybdenum affects the expression of CBF14 and the development of frost tolerance in wheat. *Plant Physiology and Biochemistry*, 63, 77-81.

### - Conferences attended

#### 1- Oral presentations:

- a. **Al-Issawi, M.** The characterisation of the freezing damage response during flowering in European and Middle Eastern wheat cultivars. Oral presentation of PhD project in plant physiology symposium in Plymouth university 09/03/2010
- b. Michael P. Fuller, **Mohammed Al-Issawi**, Nasser El-Sarkassy, Jack Christopher & Troy Fredericks. Frost damage to wheat during flowering. UK workshop on abiotic stress in cereals, Broom Barn, UK 23<sup>rd</sup> March 2010 (Oral presentation).
- c. **Al-Issawi, M.**, Rihan, H. Z, Burchett, S. & Fuller, M. P. Cross-protection of wheat to abiotic stress. SCI Bio Resources young Researchers (Science and technology for innovation in agriculture)-Reading University 8<sup>th</sup> July 2011.
- d. **Al-Issawi, M.**, Rihan, H. Z Burchett, S. Al-Swedi, F. & Fuller, M. P. Frost resistance and CBF expression in wheat at ear emergence. First annual symposium of Plant Environmental Physiology (PEP) 20<sup>th</sup> Dec 2011.
- e. **Al-Issawi, M.**, Rihan, H. Z, Burchett, S. & Fuller, M. P .The expression of CBF and physiological effect of frost in wheat during ear emergence. Plant and Microbial Adaptation to Cold conference (PMAC 2012) – Sapporo, Japan.
- f. **Al-Issawi, M.** The characterisation of the freezing damage response during flowering in European and Middle Eastern wheat cultivars. School of Biomedical Sciences Seminar for PhD students 11<sup>th</sup> January 2013.

#### 2- Posters presentations

- a. **Al-Issawi, M.**, Fuller, M.P., Fredrick, T and Burchett, S. Frost resistance of wheat during ear emergence. International conference organised by the Society for Experimental Biology (SEB) 30<sup>th</sup> June-3<sup>rd</sup> July 2010-Prague, Czech Republic, pp312-313.
- b. **Al-Issawi, M.**, Rihan, H. Z., Fadhel, F., Burchett, S. & Fuller, M. P. The effect of Molybdenum of frost tolerance to wheat genotypes. Post graduate society-Annual conference 26<sup>th</sup> June 2012.
- c. Fuller, M.P, **Al-Issawi, M.**, El-Sarkassy, N. Christopher, J. and Fredrick, T. wheat during flowering cannot resist frost, University of Zagazig, Egypt.

- d. Fuller, M.P, **Al-Issawi, M.**, El-Sarkassy, N. Christopher, J. and Fredrick, T. Frost tolerance of Wheat during ear emergence. Second Forum for Young Researchers Sunday 23 May 2010, Egypt.

### 3- Other conference attended

- a. Agrivision2020- The royal Showground, Wadebridge, Cornwall, sponsored by Clydesdale Bank and Mole Valley Farmers Ltd. 3<sup>rd</sup> April 2009.
- b. Rothamstad Research Institute Harpenden, Hertfordshire 15<sup>th</sup>-16<sup>th</sup> September 2009.

## Collaborative output

The following collaborative papers were produced during the period of registration of this PhD project

### - Publications

- 1- Rihan, Z., Hail, **AL-Issawi, Mohammed**, Burchett, Stephen. & Fuller, P., Michael. 2012. Artificial seed production from encapsulated microshoots of cauliflower (Brassica oleraceae var botrytis). Acta Hort. (ISHS), 961, 419-425.
- 2- Rihan, Z., Hail, **AL-Issawi, Mohammed**, Burchett, Stephen, & Fuller, Michael P. 2011. Encapsulation of cauliflower (Brassica oleracea var. botrytis) microshoots as artificial seeds and their conversion and growth in commercial substrates. Plant Cell, Tissue and Organ Culture, 107, 243-250.
- 3- Rihan, Z., Hail, **AL-Issawi, Mohammed**, AL-Swedi, Fadil. & Fuller, Michael P. 2012. The effect of using PPM (plant preservative mixture) on the development of cauliflower microshoots and the quality of artificial seed produced. Scientia Horticulturae, 141, 47-52.
- 4- Michael P., Fuller, Al-Juboori, H., Jalal, Rihan, Z., Hail. & **Al-Issawi, Mohammed**. 2012. Germination of primed seed under NaCl stress in wheat. ISRN Botany, , in press (published online).
- 5- Rihan, H.Z., **Al-Issawi, M.**, Al Shamari., Burchett, S and Fuller M.P. 2012. Cold tolerance and CBF/DREB1 up-regulation both cauliflower mature plants and microshoots. Centre for Agriculture and Rural Sustainability Postgraduate Symposium 2012. The New Continental Hotel, Plymouth, UK.

### - Presentations and posters

- 1- Rihan, H.Z., **Al-Issawi, M.**, Al Shamari., Burchett, S and Fuller M.P. 2012. Cold tolerance and CBF/DREB1 up-regulation both cauliflower mature plants and microshoots. Centre for Agriculture and Rural Sustainability Postgraduate Symposium 2012. The New Continental Hotel, Plymouth, UK. (Oral Presentation).
- 2- Rihan, H.Z., **Al-Issawi, M.**, Al Shamari., Burchett, S and Fuller M.P. Cold tolerance and CBF/DREB1 up-regulation in Brassica oleracea var botrytis. Plant and microbe adaptation to cold 2012. Hokkaido University, Japan, June 24-28, 2012. P-7, 60. (Poster presentation)
- 3- Rihan, H.Z, **Al-Issawi, M.**, Al-swedi, F, and Fuller, M. 2011. The improvement of cauliflower artificial seed cold tolerance. Plant

Environmental Physiology Group – First Annual Mini-Symposium 20th December, Darwin House, London (Oral presentation).

- 4- Al-swedi, F., Rihan, H.Z., **Al-Issawi, M.**, Al-Shamari, M and Fuller, M. 2011. Agrobacterium-mediated transformation of cauliflower (*Brassica oleracea* var. *botrytis*): optimization of protocol and development of transgenic cauliflower. Plant Environmental Physiology Group – First Annual Mini-Symposium 20th December, Darwin House, London (poster presentation).
- 5- Rihan, H.Z., **Al-Issawi, M.**, Al-swedi, F, and Fuller, M. 2011. An easy way to understand the mechanism of cold tolerance gene function in plants. University of Plymouth postgraduate conference 23th November, house stone theatre, Portland square building. (Poster presentation)
- 6- Rihan, H.Z., **Al-Issawi, M.**, Al-Shamari, M, and Fuller, M. 2011. Cauliflower artificial seeds cold storage. University of Plymouth postgraduate conference 29th June, house stone theatre, Portland square building. (Poster presentation)
- 7- Rihan, Z, H., **Al-Issawi, M.**, Fuller, P. M. and Burchett, S. 2011. The determination of the optimal cauliflower microshoot development stage suitable for capsulation as artificial seeds and the optimization of conversion using semi solid media and commercial substrates. Society of experimental biology annual main meeting, Glasgow, UK, P1.25, 187 (Poster presentation).
- 8- Rihan, Z, H., Fuller P, M., **Al-Issawi, M.**, and Burchett, S. 2011. Cloning of cauliflower (*Brassica oleraceae* var. *botrytis*) and the production of artificial seeds as a promising cost-effective alternative to conventional methods of seed production. University of Plymouth postgraduate conference. (Oral Presentation).

## List of abbreviation

ABA	Abscisic Acid
ANOVA	Analysis of variances
AO	Aldehyde oxidase
APS	Amonium Persulfate
BLAST	Basic Local Alignment Search Tool
BSA	Bovin Serum Albumin
bZIP	Basic Leucine Zipper Domain
CA	Cold Acclimation
CAT	Catalase
CBF	C-repeat Binding Factor
cDNA	Complementary deoxyribonucleic acid
COR	Cold Regulated
CRT	C-repeat
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleoside Triphosphates
DTA	Differential Thermal Analysis
DTT	Dithiothreitol
EC	Electrical Conductivity
EDTA	Ethylenediaminetetraacetic acid
ERD	Early Responsive to Dehydration
FT	Frost tolerance
GS	Growth stage
IAA	Indole acetic acid
IDPs	Intrinsically disordered proteins
INA	Ice Nucleation Active
IR	Infrared camera
IRVT	Infrared Video Thermography
K <sub>2</sub> HPO <sub>4</sub>	Dipotassium Hydrogen Phosphate

KDa	Kilo Dalton
KH <sub>2</sub> PO <sub>4</sub>	Potassium Dihydrogen Phosphate
KIN	Cold induced
LEA	Late Embryogenesis Abundant (LEA) proteins
LSD	Least Significant Difference
LT	Low temperature
LT <sub>50</sub>	Lethal temperature
LTI	Low temperature induced
Mo	Molybdenum
NA	Non acclimated
NADPH	Nicotinamide Adenine Dinucleotide Phosphate-oxidase
NCBI	National Centre for Biotechnology Information
NR	Nitrate Oxidase
PAGE	Polyacrylamide Gel Electrophoresis
PAR	Photosynthetically active radiation
PBS	Phosphate Buffer Saline
PBST	Phosphate Buffer Saline Tween
PCR	Polymerase Chain Reaction
Pn	Net photosynthesis
POX	Peroxidase
PPM	Part per million
PVDF	Polyvinylidene difluoride
QPCR	Quantitative Polymerase Chain Reaction
QTL	Quantitative Trait Loci
RD	Response to dehydration
REC	Relative Electrical Conductivity
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic acid
ROS	Reactive Oxygen Species
H <sub>2</sub> O <sub>2</sub>	Hydrogen peroxide

SDS	Sodium Dodecyl Sulfate
SO	Sulphate oxidase
SOD	Superoxide Dismutase
TaCBF	Triticum aestivum C-repeat Binding Factor
TEMED	Tetramethylethylenediamine
TTC	Triphenyltetrazolium Chloride
UV	Ultra Violet
VRN	Vernalisation
XDH	Xanthine dehydrogenase
ZCK	Zadoks scale



## **Chapter 1: General Introduction and Literature Review**

## **1.1 Introduction**

To be successful in regions of low temperature conditions and severe winter climates, plants have to be programmed to recognize and respond both to temperatures that are optimum for growth and environmental cues which indicate a threat of impending damage (Fowler and Limin, 2007). Wheat occupies a wide range of environments from sub-polar to sub-tropical and therefore it has to have the broadest adaptation possible (Reynold et al., 2001). This broad environmental range is largely because wheat has developed an extensive range of cold tolerance. Cold tolerance is the ability to withstand temperatures lower than minimum temperature for growth, i.e. below 4°C (Reynold et al., 2001). In fact wheat uses temperatures in the range of 0 to 4°C to stimulate an adaptation process which makes it more tolerant of increasingly sub-zero temperatures. The cold tolerance of wheat during its vegetative stage does not confer cold tolerance after spike emergence and it is most susceptible to frost damage in the period from pre-heading to flowering. Winter growth habit wheat varieties require long period of exposure to low temperature during winter months (vernalisation requirement) in order to prevent flowering until temperature becomes optimal for this important stage (Yan et al., 2003), while spring wheat does not have such requirements. It is thought that vernalisation when fulfilled, releases a gene product (VRN1) which initiates the transition from the vegetative to the double ridge stage of the apical meristems, and thereby down-regulates the cold resistance (CBF/COR) pathway (Galiba et al., 2009) leaving the plant in a vulnerable state for late frosts.

Spring radiative frost damage of winter wheat and other cereals is a significant problem in several regions of the world including Australia, the Mediterranean and South America (Shroyer et al., 1995). Single (1985) reported that frost stress during

the developmental stages of both male and female reproductive organs reduce wheat yield in parts of Australia by up to 40% in some years, while in China losses of yield of 30-50% have been reported (Feng et al., 1999). Also it has been reported that an estimated reduction of 7% of potential winter wheat in US is due to winter injury (Patterson et al., 1990). Furthermore, Tarik (1981) reported that total grain production had been reduced by 50% in northern Iraq in 1970 and 1980. Due to the sensitivity of reproductive meristems to frost damage compared with vegetative meristems, small differences in developmental stage can potentially affect floret survival and yield (Galiba et al., 2009). The understanding of the developmental regulation of Low Temperature (LT) tolerance has been improved by genetic analysis at the whole plant level, and has shown that vegetative/reproductive transition is a critical switch that initiates the down-regulation of expression of LT-tolerance genes. Accordingly, full expression of cold hardiness genes only occurs in the vegetative stage and the frost tolerance does not persist after spike emergence (Fredericks et al., 2004). Recently, Mahfoozi et al (2006) have stated that wheat genotypes can cold acclimate after reproductive transition and before heading evidencing that the vegetative/reproductive transition does not behave as an off switch for LT-tolerance genes. However, plants have only limited ability to cold acclimate during this period and they reach their maximum level of LT-tolerance very quickly once they are exposed to temperatures in the acclimation range but this response mechanism is operable up to the time of heading (Mahfoozi et al., 2001). The lack of field breeding progress in selecting for frost tolerance after spike emergence in Australia where this problem has vexed researchers for more than 50 years, suggests that the temperature to confer acclimation in wheat (0-5°C) are rarely experienced for

prolonged periods during flowering and so selection is not possible (Fuller et al., 2007).

Full expression of frost tolerance genes occurs in the vegetative stage and wheat plants in the vegetative stage can re-acclimate after exposure to warm temperature and de-acclimate. In contrast in the reproductive stage, wheat has a limited or no ability to re-acclimate once de-acclimated.

Some researchers maintain that the lack of frost resistance after spike emergence is due to an absence of sufficient acclimation temperatures at flowering time or insufficient environmental stimulus to up-regulate acclimation genes (Distelfeld et al., 2009, Fowler, 2007). Fuller et al (2007) confirmed these findings when they found that at spike emergence, wheat could only resist frost to its inherent or constitutive resistance level which appears to be  $-5^{\circ}\text{C}$ , but that even when given inductive environmental conditions acclimation was unable to be induced. They also found that the spikes of wheat had a high tendency to supercool in the absence of ice nucleators.

As a response to exposure to low temperatures a change in gene expression is induced in wheat plants. The product of this induction will mitigate the effect of subsequent low temperature stress and the acclimation process will be triggered (Beck et al., 2004). As a consequence, low temperature tolerance increases by accumulation of low molecular weight organic solutes, which are more commonly known as osmoprotectants such as sugar, polyols, amino acids (e.g. proline), their N-methyl derivatives (e.g. betaines) and poly amines (Naidu, 1998, Allard et al., 1998, Naidu et al., 1991). In addition, biochemical changes have also been associated with low temperature tolerance in plants including alteration in lipid composition and

increases in soluble protein content (Lin et al., 1990). Many of these proteins are COR proteins. Their function is still speculative but many have LEA type tertiary protective structures (Matthew, 2005, Hinch and Thalhammer, 2012). There is documented evidence that the polypeptide encoded by *COR15a* can help in stabilising membranes against freezing injury (Steponkus et al., 1998, Artus et al., 1996) and it can protect other proteins against freeze-thaw inactivation (Bravo et al., 2003). Acclimation is regulated by the CBF (C-repeat Binding Factor) regulon, and the induction of CBF is the first indicator of whether acclimation will be up-regulated by exposure to low temperature. It is hypothesised that the inability to acclimate during flowering is due to a failure of the up-regulation of CBF and therefore a failure to upregulate the entire regulon. If this hypothesis is upheld then one solution to lack of acclimation during flowering could be to constitutively express CBF through mutation or genetic modification. However it is also possible that CBF is up-regulated but the downstream regulon upregulation does not follow and acclimation does not occur. In order to determine if this is the case it will be necessary to examine the up-regulation of expression of CA (Cold Acclimation) genes for example *COR15a*.

Low temperature stress shares some signal transduction components with other abiotic stresses e.g. drought and salt; therefore there may also be an opportunity to manipulate its response utilising these networks. Recently it has been found that cold acclimation could be triggered through the application of Molybdenum (Mo) to wheat plants (Sun et al., 2009) evidencing that *CBF* gene can be up-regulated by the chemical cues and not just low temperature. Mo seems to be essential micro-element for all organisms and occurs in more than 40 enzymes catalysing many redox reactions (Mendel and Hänsch, 2002) including the regulation of abscisic acid (ABA) biosynthesis via Aldehyde oxidase (AO) and it may be via ABA that Mo

influences cold acclimation. The exogenous applications of chemicals such as Mo might open new horizons in the up-regulation of stress tolerance genes in plants.

The problem of frost damage to flowering structures of wheat becomes more complex because avoidance mechanisms like supercooling may also play a vital role when wheat plants are exposed to temperature just below freezing. Supercooling is the second mechanism that wheat has adapted to escape the damaging effects of freezing temperatures. In the laboratory pure water can supercool and remain unfrozen to its homogenous nucleation of approximately  $-40^{\circ}\text{C}$  (George et al., 1982). In the field supercooling may also occur although it is very difficult to monitor. The initiation of freezing following any degree of supercooling will be accompanied by an exotherm since the crystallisation of water is an exothermic process. Furthermore these exotherms can be detected by thermal analysis of plant tissues and infrared video thermography (IRVT) has been used for detecting the released heat of an exotherm from freezing tissues (Pearce, 2001). IRVT has been evaluated under both controlled and field conditions to observe and track ice formation directly (Fuller and Pearce, 2001). This technique has proved useful to determine the temperature and the location of ice nucleation and freezing pattern of plants and plant parts (Fuller and Le Grice, 1998, Fuller and Pearce, 2001, Fuller and Wisniewski, 1998).

According to this literature base, it can be concluded that wheat is unable to acclimate effectively during spike emergence, but it can supercool and escape freezing damage. With respect to supercooling however there are still issues which remain unclear such as: what causes ice nucleation in wheat spikes? is there any genetic variation in supercooling? and, can supercooling be controlled during exposure to freezing temperatures?

A number of research questions emerge which are addressed in more detail in the literature review and form the basis for the current experimental investigation:

- At what development stage are the CBF genes (cold acclimation) unable to be switched on any longer? Is it when vernalisation is complete or after a specific development stage as defined by for example by the apical growth stage?
- Is there any physiological evidence at all for acclimation at advance growth stages?
- What happens when a wheat plant at an advanced growth stage freezes - where does the ice form and what is the pattern of spread in the plant and how is damage manifested?
- If wheat at an advance growth stage cannot acclimate then is there any other way to avoid freezing and frost damage e.g. either by chemically inducing acclimation or encouraging supercooling?

This study reported in this thesis aimed to investigate and understand the characteristics of frost damage of wheat cultivars at spike emergence and later development stages. Different Middle Eastern wheat cultivars and European wheat (cv. Claire) were used to address these questions through:

- Frost hardiness screening of several wheat varieties.
- An investigation of frost damage in wheat during flowering.
- Tracking the formation of ice in spikes and using the IR thermography
- Investigating the molecular basis of frost tolerance and understanding the underlying regulatory network during flowering.

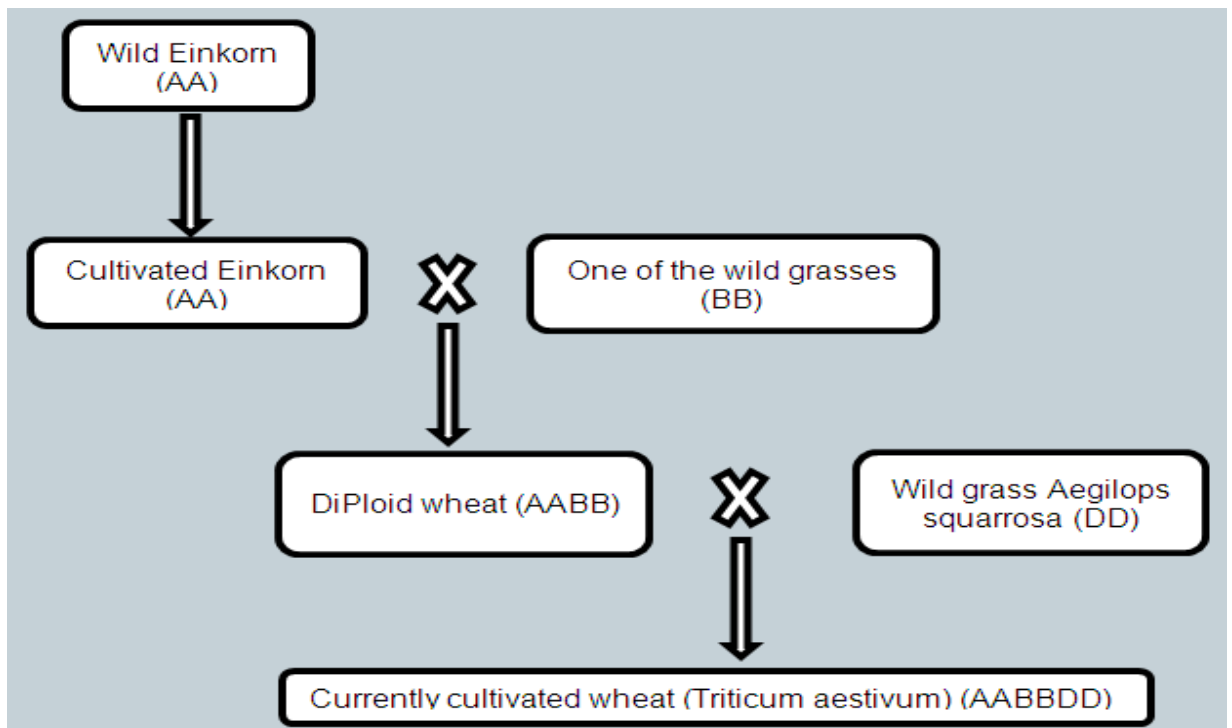
## 1.2 Wheat: history and types

Wheat (*Triticum aestivum*) is an agronomically important crop being a staple food for human nutrition. It is one of the most important source of carbohydrates on which a majority of countries depends (Tahir, 2008) and also its consumption has increased in many countries which originally were based on rice in their daily diet such as China and Japan and it can be partially used in many aspects of the food chain beside bread (Reynold et al., 2001). Wheat producers are facing increasing challenges in production as the population surges, climate change and emerging plant pest by trying to overcome difficulties that are facing wheat production. Cultivated wheat has exceeded in popularity over all other plant species that produce seeds, and it can be harvested in each month of the year somewhere throughout the world, occupying a wide range of environments from 65 °N to 45 °S. This wide adaptability has been favoured by its diverse growth habit (Lantican et al., 2005).

Archaeological and botanical evidence indicates that wheats originated in the upper area of the fertile crescent of the Near East (Tigris-Euphrates region) at about 7500 years BC (Zohary and Hopf, 1993). Two types of wheat have been found in Northern Iraq, the first type is very similar to one of the wild species which continues growing in the Near East. The other type called Einkorn is a currently cultivated wheat species. Historically, wild Einkorn (genome A) is regarded as very important, from which all cultivated wheat, except Emmer, has originated (Johnson, 1975). Recent genetic evidence indicates that Einkorn (*Triticum monococcum*) may have domesticated from wild Einkorn (*Triticum monococcum ssp. aegilopoids*) in the region of the Karacadag mountains in south east Turkey. The remains of cultivated Emmer (*Triticum turgidum ssp. dicoccum*) have been discovered at several archaeological sites in Syria dating to 7500 years BC (Zohary and Hopf, 1993). Recently, molecular evidences are



convincing that the B and G genomes of modern wheat were donated by (*Aegilops peltoides*). One diploid *Triticum* species and two species of the closely related taxon *Aegilops* (tetraploid) are involved in the phylogeny of polyploidy wheat (*Triticum aestivum*) (Figure 1) (Zohary and Hopf, 1993).



**Figure 1 .Phylogeny of polyploidy wheat.**

Sakamura (1918) from Japan and Sax (1918) from the United States have independently found three types of wheat according to chromosome number (diploid, tetraploid, hexaploid) species, which contain respectively 7, 14, 21 chromosomes. All species belonging to the *Triticum* genus have originated from these three types (Table 1).

**Table 1. Different types of wheat and their genome constitution. Adapted from Stallknech et al (1996).**

Species	Genome constitution	Grain	Common name
<b>Diploid (2n= 14)</b>			
<i>Triticum aegilopoids</i>	AA	Hulled	Wild Einkorn
<i>Triticum monococcum</i>	AA	Hulled	Cultivated Einkorn
<b>Tetraploid (2n= 28)</b>			
<i>Triticum dicoccoides</i>	AABB	Hulled	Wild Emmer
<i>Triticum dicoccum</i>	AABB	Hulled	Cultivated Emmer
<i>Triticum durum</i>	AABB	Free- threshing	Durum wheat
<i>Triticum turgidum</i>	AABB	Free- threshing	Rivet wheat
<i>Triticum polonicum</i>	AABB	Free- threshing	Polish wheat
<i>Triticum turanicum</i> <sup>2</sup>	AABB	Free- threshing	Kamut wheat
<b>Hexaploid (2n= 42)</b>			
<i>Triticum spelta</i>	AABBDD	Hulled	Spelt wheat
<i>Triticum aestivum</i>	AABBDD	Free-threshing	Common wheat
<i>Triticum macca</i>	AABBDD	Hulled	Macca wheat

Although, there are several types of wheat, all fall into one of two main categories: according to growth habit spring and winter based on flowering responses to cold temperature (Kiyooki et al., 1998). Winter-habit wheat is sown in autumn, and the growing plant must experience a period of cold temperature (vernalisation) before flowering can be initiated the following spring. In contrast, spring-habit wheat does not have to experience vernalizing temperature before flowering and can be planted in spring or in regions with mild winters in autumn (Liuling et al., 2004).

Generally, the difference between spring and winter wheat is primarily due to the length of the vegetative stage. Spring wheat will become reproductive soon after germination if the temperature and the day length are optimal. The transition from the vegetative stage to reproductive stage occurs when shoot apex becomes double

ridged (McMaster, 1997). The double-ridged formation occurs before internode elongation and eventually forms the spike. However, some spring plants are day length sensitive and when the day length is less than optimal, i.e., short day lengths, they will not enter the reproductive stage and will remain vegetative until the day length becomes longer. In regions with mild winters, some cold tolerant, day length sensitive spring wheat genotypes can be planted in the fall. Winter growth habit wheats will not enter the reproductive stage unless they are exposed to a period of low temperature less than 10 °C, and as a consequence have an increased vegetative stage in comparison to spring wheat. The vernalisation requirement is the need of the exposure to a period of low temperature (Fowler et al., 1999b) before the plant will switch from the vegetative stage to the reproductive stage and flower normally. Throughout the world spring wheat is the most common type grown but in the mild winter climates of western Europe winter wheat is used more extensively and with intensive inputs and high rainfall record wheat yields of 12 to 14 tha<sup>-1</sup> have been achieved compared to the world wheat average yield of 2 to 4 tha<sup>-1</sup>.

### **1.2.1 Vernalisation requirements:**

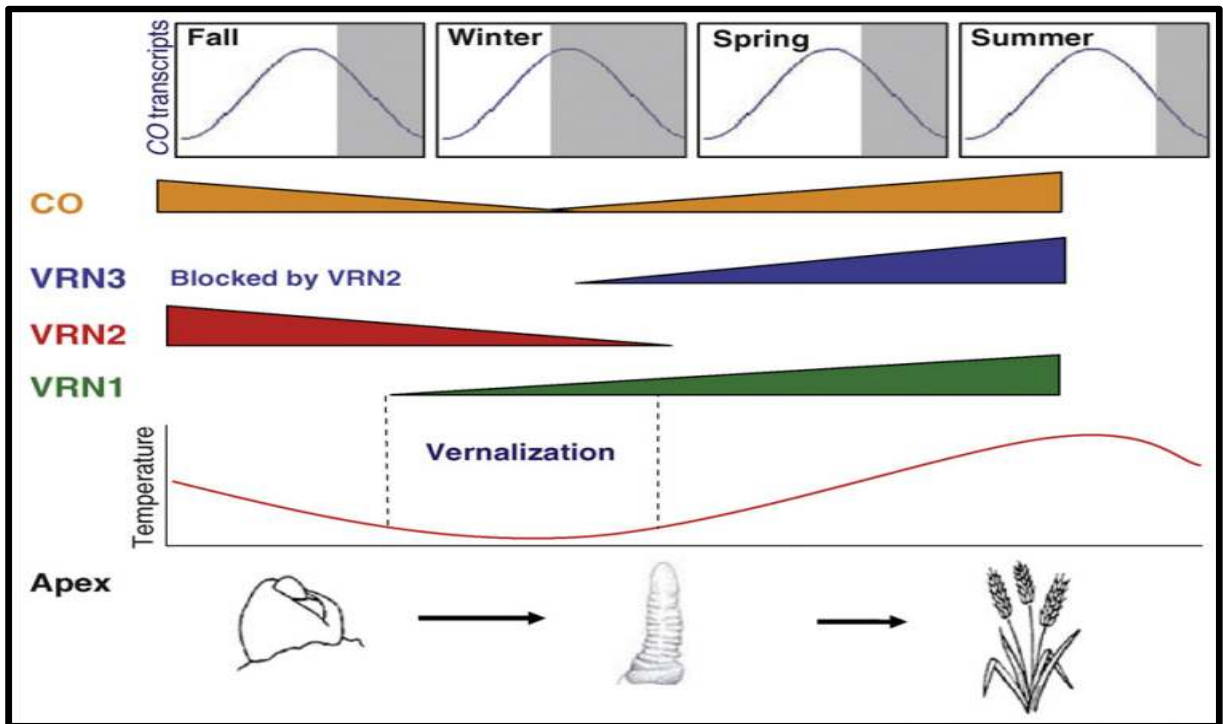
Vernalisation is the acquisition of the competence to flowering after exposure to periods of prolonged cold temperatures many plant species must go through periods of cold (4-8°C) before they can flower. This process ensures that reproductive development and seed production occur in optimal environmental conditions after the winter (Ilja et al., 2004). Law et al (1976) the vernalisation requirement prevents the onset of flower development during winter, thereby providing additional protection for environmentally sensitive floral organs. The different sensitivity of wheat varieties to vernalisation determines the growth habit. Low vernalisation requirement or its

absence is characteristic of spring growth habit, while a requirement for a long period of exposure to low temperature is a trait of winter wheat. If the requirement for vernalisation is not fulfilled, flowering of the plant is inhibited (Košner and Pánková, 2002), but long days can stimulate erratic flowering in winter wheats that are not fully vernalised.

### **1.2.2 Vernalisation genes:**

Genetic control of the vernalisation response has been investigated by several researchers. Christine and Law (1979) mentioned that Pugsley (1971) and (1972) found that spring and winter wheat are governed by at least four vernalisation genes, designated *Vrn-1*, *Vrn-2*, *Vrn-3*, and *Vrn-4*. *Vrn-1* and *Vrn-3* have subsequently been located on the long arms of chromosomes 5A and 5D respectively. More recently, Dubcovsky et al (1998) have reported that vernalisation in cultivated wheat is mainly controlled by three *Vrn* loci, *Vrn-A1*, *Vrn-B1*, and *Vrn-D1* located in the middle of the long arms of chromosomes 5A, 5B, and 5D respectively. However, *Vrn-A1* is a stronger spring/winter habit determining gene in *Triticum aestivum* than *Vrn-B1* and *Vrn-D1* (Dubcovsky et al., 1998, Loukoianov et al., 2005). The dominant *Vrn-A1* gene is present in spring habit genotypes (*Vrn-A1/Vrn-A1* and *Vrn-A1/vrn-A1*) and the gene is homozygous recessive (*vrn-A1/vrn-A1*) in winter habit genotypes with a vernalisation requirement. In contrast, the vernalisation gene *Vrn-2* is dominant for winter growth habit, and its transcription is down-regulated during vernalisation (Yan et al., 2003). *Vrn-2* encodes a protein that represses flowering. Prolonged cold exposure shuts down *Vrn-2* activity, after which a wheat plant can flower or is released for flowering. Galiba et al (2009) have recently reported that the vernalisation process is mainly regulated by differences in the regulatory regions of *Vrn-1* and *Vrn-3* genes or the coding region of *Vrn-2*. Studies have shown that *Vrn-3* is the integrator of both vernalisation and

photoperiod pathways in temperate cereals (Distelfeld et al., 2009). Wheat spike emergence during the fall is prevented by the *Vrn-2* down-regulation of the *Vrn-3* and low *Vrn-1* transcription. *Vrn-1* is induced by vernalisation, which is followed by the down-regulation of *Vrn-2*, thereby releasing *Vrn-3*. During the longer days in spring, photoperiod genes *PPD1* and *CO* are up-regulated *Vrn-3*, which induces *Vrn-1* above the threshold levels required for flower initiation (Figure 2), higher levels of the *CO* protein favour the activation of *Vrn-3* during long days only when the *Vrn-2* repressor is not present in the leaves (Turck et al., 2008). However, *Vrn-1* is slightly up-regulated during winter and then strongly induced by *Vrn-3* in the apices when day length increases. When *Vrn-1* reaches a threshold level, the vegetative apex stops producing leaves and starts producing spikelet primordial meristems (double-ridge state). When the day length gets longer and temperatures rise, most temperate cereals will complete the subsequent spike development and elongation phases of the stem and eventually the spike until emerge above the flag leaf (Distelfeld et al., 2009).



**Figure 2. Regulation of vernalisation and photoperiod genes by environmental changes during the growing season in a photoperiod-sensitive winter cereal. From Distelfeld et al (2009).**

### 1.3 Abiotic stress

Stress in physical terms is defined as mechanical force per unit area applied to an object and in response to applied force an object undergoes a change in the dimension (Mahajan and Tuteja, 2005). Biologically, the term stress has been adapted for any environmental factor, potentially unfavourable to living organisms and stress resistance for ability of the plant to survive that unfavourable factor. Levitt (1972) defined stress as any factor which disturbs the normal functioning of an organism.

Abiotic stresses, which include drought, temperature, salinity, heavy metals, ozone and other pollutant gases, can cause catastrophic yield reduction in most crops. Plants respond to stress stimuli in different and very complex ways. Unlike animals,

those stresses are much more intricate given the absence of an immune system in plants. However, plants have to exploit contiguous environment to upper limit. Plants adapt in order to deal with many stresses through physiological, biochemical, molecular or morphological changes. Plants have evolved different strategies to cope with different stresses, and ways to adapt to those stresses are numerous and interconnected. Some plants have morphological features; that allow them to avoid some stresses but, these are not universally applicable to other plants. Most plants only have the option to alter their physiologies, metabolic mechanisms, gene expression and various developmental activities to withstand the effect of certain stress and plants evolved complex mechanisms to tolerate abiotic stresses. Only plants that have these mechanisms (acclimation mechanisms) can survive unfavourable conditions. Thus gene products (e.g. proteins) make a fundamental function in the molecular mechanisms of stress tolerance in plants (Rao et al., 2006).

Each stress factor has its own specific effect on plants, but the most common target areas of most abiotic stress are the cellular membranes. Membrane systems under normal conditions perform many life maintenance processes such as signal perception, electron transport, ATP generation, Ion pumps and channels and carrier proteins and involve processes that can easily be affected by abiotic stresses. Abiotic stresses such as drought, salinity, low and high temperature, light stress and nutrients accelerate the production of Active Oxygen Species (AOS) in plants that cause membrane systems and other cellular process damage (Shanker and Venkateswarlu, 2011). Photosynthesis, respiration, nitrogen assimilation, protein synthesis and many other processes can be easily affected by abiotic stresses.

Plants have evolved some common mechanisms to withstand stresses. The degree of tolerance varies from one plant to another, but it starts with stress recognition followed by switching on genes that are involved in cellular protection and damage repair. The signal transduction pathways that detect stress have a crucial role in the induction of stress tolerance in plants (Smalle and Vierstra, 2004).

The perception of abiotic stress brings on signalling cascades that set off ion channels such as the production of Reactive Oxygen Species (ROS), Kinase cascades, accumulation of hormones such as Abscisic acid (ABA), Ethylene (ET), Jasmonic acid (JA) and Salicylic acid (SA). Ultimately, such signalling pathways activate the expression of sub-sets of defence genes that eventually lead to the assembly of genes products that effect stress tolerance (Sauter et al., 2001).

It has been shown that Calcium ( $\text{Ca}^{2+}$ ) and ROS constitute important and common signalling molecules in very early abiotic stress responses. Therefore,  $\text{Ca}^{2+}$  and ROS levels rapidly increase in cells of tissue when abiotic stress is exerted by environmental conditions. Calcium seems to be the most important cation as a main signal transducer in the signalling cascades activated in plant response to any kind of stress. Cytosolic  $\text{Ca}^{2+}$  levels increase in plant cells in response to various abiotic stresses and the  $\text{Ca}^{2+}$  cation is the important node at which cross talk between pathways occur. The signal activated by  $\text{Ca}^{2+}$ -influx is generally mediated through  $\text{Ca}^{2+}$ -binding proteins which act as  $\text{Ca}^{2+}$  sensors. The main  $\text{Ca}^{2+}$ -sensor in plants are Calmodulin (CaM),  $\text{Ca}^{2+}$ -dependent proteins kinases (CDPK) and Calcineurin B-like (CBL) proteins (Jenks and Wood, 2010). CaM is a highly conserved protein that has been considered to be the first sensor of changes that occur at the cytosolic level in all Eukaryotic cells (Rudd and Franklin-Tong, 2001, Ludwig et al., 2005). One of the key questions in biology is the understanding of sensing environmental triggers such



as low temperature and recently the key sensor molecules for the switching on and off the transcription factors are calmodulin genes controlling calcium fluxes. There is emerging evidence that physical changes in membrane fluidity caused by the stoichiometry of lipids could be the initial trigger for calcium influx into the cell (Marc et al., 2010).

Reactive oxygen species are also very important second messengers related with response to various abiotic stresses. In response to diverse abiotic stresses, an oxidative burst takes place (Rao et al., 2006). Low level of ROS lead to the increase of  $\text{Ca}^{2+}$  influx into the cytoplasm,  $\text{Ca}^{2+}$  in turn triggers NADPH oxidase to produce ROS by yielding  $\text{O}_2^-$  that is converted to  $\text{H}_2\text{O}_2$  by super oxidase dismutase (SOD), and the peroxide spread through the cell wall to the extracellular medium and then enters into cell (Rao et al., 2006). However, the production of ROS ( $\text{H}_2\text{O}_2$ ) is in a  $\text{Ca}^{2+}$  dependent manner and the  $\text{Ca}^{2+}$  concentration regulating in the cytoplasm by ROS through the activation of  $\text{Ca}^{2+}$  channels in the plasma membrane (Kwak et al., 2003). Thus, it has been recognized that crosstalk between the second messengers  $\text{Ca}^{2+}$  and ROS modulates the activity of specific proteins that act in the nucleus to control the expression of definitive defence genes. Recently, studies have identified and characterised several new genes such as Kinases and transcription factors which in turn are involved in the crosstalk between signalling cascades involved in responses against two or more kind of stresses (Rao et al., 2006). The plasma membrane is the main place of ROS production, but it has been reported that the mitochondria can also serve as the site of ROS production by abiotic stress exerted by copper in the marine alga *Ulva Compressa* (Gonzalez et al., 2010) and by cadmium in tobacco (Garnier et al., 2006). Likewise ABA can crosstalk with ROS; and  $\text{H}_2\text{O}_2$  generation is required by ABA regulation of stomatal closure (Rai and Takabe, 2006). The

importance of ABA in plant stress responses has long been reported (Bray, 2002, Ozturk et al., 2002). ABA biosynthesis is activated upon plant exposure to abiotic stresses (Xiong and Zhu, 2003). In response to water stress, ABA regulates ion channels and promotes stomata closure to minimize water loss (Schroeder et al., 2001). Also ABA can activate the expression of many stress responsive genes independently or together with other stresses. It has been reported that the importance of ABA as a regulator, is in the ABA responsive element (ABRE) which appears in the promoter of many defence genes (Thomashow, 1999).

Plants respond to abiotic stress in both ABA-dependent and ABA-independent pathways. When a plant is exposed to low temperature (LT), endogenous ABA activates basic leucine zipper (bZIP) transcription factor, and then triggers ABA dependent COR genes through responsive elements. Whereas, in ABA independent pathways, LT activates the expression of CBF, which activates downstream COR genes which in turn eventually confer LT tolerance in the plant (Thomashow, 1999b, Shinozaki and Yamaguchi-Shinozaki, 2000).

### **1.3.1 Wheat abiotic stress**

Wheat is a major cereal crop in many parts of the world and grown across very wide range of environments (El-Masry, 2010). Increasing the crop growth and productivity in regions which have been subjected to abiotic stress, which are variable in their timing and unpredictable in their intensity is a significant challenge to all plant scientists involved in crop yield production (Dolferus et al., 2011). Wheat growth and productivity throughout the world are limited by different abiotic stress notably drought, salinity and low temperature (Tester and Bacic, 2005).

Drought stress is one of the most important limiting factors of wheat yield in arid and semiarid areas; wheat performance can be severely reduced in these areas. When the soil water osmotic potential is decreased, osmotic regulation will take place by crops, where osmotic potential for water will be reduced by accumulation of soluble carbohydrates and proline (Martin et al., 1993, Keyvan, 2010). Salinity is not of less importance for drought; therefore it is a threat in intensive agriculture (Mer et al., 2000). It affects crops from germination (Mujeeb-ur-Rahman et al., 2000) and growth and productivity (Pandey and Thakrar, 1997). Consequently, plant growth will reduce through osmotic inhibition of water uptake by root or by specific ion effects. Photosynthesis and respiration are reduced because of the low water uptake caused by salinity. Likewise, physiological aspects, biochemical changes will take place in wheat plants as a result of salt stress. As wheat grows or is subjected to salinity there will be changes in the pattern of gene expression and both qualitative and quantitative in protein synthesis. Salinity also changes plant hormones levels such as ABA and cytokinin (Reynolds et al., 2001).

Low temperature (LT) represents one of the most significant abiotic stresses limiting the productivity, crop quality and the geographical distribution of plants.

## 1.4 Low temperature stress

Low temperature is a common abiotic stress condition that can adversely affect plant growth and crop production. Low temperature is one of the most important abiotic factors limiting the growth, development and distribution of agronomic species throughout the world and which causes significant losses in crop production (Pearce, 2001).

Stress below 0°C is also called frost stress, but technically, the word “frost” refers to the formation of ice crystals including a phase change from vapour or liquid to solid and it can occur on the surface of plants, either by freezing of dew (Hoar frost) or internally in a plants tissues. Ice crystallisation in plants is the process in which liquid water turns into solid when the air temperature falls below the freezing point. Crystallisation or ice formation consists of two major processes, nucleation and crystal growth. Nucleation is the step when water molecules start to gather into clusters, on the nanometre scale. These clusters need to reach a critical size and order to become a stable nucleus, at this stage of nucleation the atoms are arranged in a defined and periodic manner that defines the crystal structure of water. Subsequently, crystal growth is the subsequent growth of the nucleus or nuclei that succeed in achieving the critical cluster size (Sakai and Larcher, 1987).The plant and its parts will only be affected by frost stress when they cannot avoid nucleation and/or cannot prevent ice growth (Pearce, 2001).

Low temperature may impose stress on a plant in a two-fold manner: by the effect of low temperature alone and by dehydration of the cells and tissues when cellular water freezes. Many plants of the tropics and subtropics are readily affected by low

temperature above freezing and cannot acclimatize to cold. This kind of damage has been termed “chilling injury”, and results primarily from the loss of function of membranes connected with a decrease of their fluidity and loss of integrity or at least deceleration of their membrane-bound ion pumps. Also in some plants, the primary effect of the chilling injury has been found to be leaf stomata opening and closing, when the permeability of the roots to water is reduced (Muldrew and McGann, 1999). Therefore, the water will be lost faster than water uptake from the roots, and plants become dehydrated. In this case, cold hardening alters the stomata behaviour thus, they close under such conditions and the roots permeability will be increased. Whereas stomata in some plants seem to be acting properly at the chilling temperatures and the injury is thought to be metabolic. Some plants might undergo a decrease in respiration, photosynthesis and fatty acid synthesis (Muldrew and McGann, 1999). In contrast, freeze dehydration only follows ice nucleation and can only occur below zero degrees Celsius. The extent to which a plant is endangered by frost depends not only on the magnitude of the drop in temperature, but also on time of onset and duration of the negative temperature (Sakai and Larcher, 1987). Beck et al (2004) has explained that plants damaged by exposure to low temperature depends on many factors such as the developmental stage, the duration, severity of frost, the rate of cooling (and re-warming), and whether ice formation takes place intracellularly (within the symplast) or extracellularly in the intercellular spaces (within the apoplast). Intracellular freezing damages the protoplasmic structure and kills the cells once the ice crystals grow large enough to be detected microscopically (Lindow et al., 1982). In extracellular freezing, the protoplasm of the plant becomes dehydrated as cellular water is transferred to ice crystals forming in the intercellular spaces in response to a water-vapour deficit (Levitt, 1980).

Under natural conditions, damage to sensitive genotypes occurs between -2 and -5°C. At these temperatures, ice forms from supercooled cellular water and propagates throughout the plant (intra- and extracellularly), causing frost damage (Ashworth and Pearce, 2002).

Extracellular freezing is defined as ice formation on the surface of the cell or between the protoplast and cell wall (apoplastic freezing) (Sakai and Larcher, 1987). Usually, extracellular freezing occurs first, because of the large number of freezing nuclei, and because the extracellular solution has a larger bulk volume than the intracellular solution (Sakai and Larcher, 1987). During the extracellular freezing process, the solutes are gradually concentrated into a small quantity of unfrozen water, which has higher osmotic potential. Water will leave the cell in response to a negative water potential gradient created by the presence of ice in the apoplast (Forbes and Watson, 1992). The movement of water due to the chemical potential of water in the supercooled state and that at the extra plasmatic ice results in a net flow of water to the apoplast and consequently to cell dehydration (Wolf and Bryant, 1992). The remaining unfrozen water in the cell remains in a supercooled state with a high solute potential. The plant can avoid intracellular ice formation by deeper supercooling and by cellular processes which help protect important macromolecular structures from the dehydration process (Sakai and Larcher, 1987).

Generally, plants can be classified into three different classes according to their low-temperature tolerance (Stushnoff et al., 1984). The first group includes frost tender plants that are sensitive to chilling injury and can be killed by short periods of exposure to temperature just below freezing point. Beans (*Phaseolus lunatus*), corn (*Zea maize*) rice (*Oryza sativa*), and tomatoes (*Solanum lycopersicum*) are examples of plants in this category. Low-temperature acclimation of plants in the second group

allows them to tolerate the presence of extracellular ice in their tissues. Their frost resistance ranges from the broad-leafed summer annuals, which are killed at temperatures slightly below freezing point, to perennial grasses that can survive exposure to  $-40^{\circ}\text{C}$ . As temperatures decrease the outward migration of intracellular water to the growing extracellular ice crystal causes dehydration stress that will eventually result in irreversible damage to the plasma membrane. The final group is made up of very cold hardy plants that are predominantly temperate woody species. Like the plants in the previous group, their lower limits of cold tolerance are dependent on the stage of acclimation, the rate and the degree of the temperature decline, and the genetic capability of tissues to accommodate extracellular freezing and the accompanying dehydration stress. Deep supercooling allows certain tissues from plants in this group to survive low temperatures without formation of extracellular ice. However, plants have adapted two mechanisms to protect themselves from damage due to below freezing temperatures. First, supercooling is a low-temperature tolerance mechanism that is usually associated with acclimated xylem parenchyma cells of moderately hardy woody plants. The second and most common low-temperature response mechanism is acclimation. Acclimation is a gradual process during which there are changes in just about every measurable morphological, physiological, and biochemical characteristic of the plant. These changes are determined by genotype and environmental interactions that are quite complex (George et al., 1982).

### **1.4.1 Types of frost**

Freeze injury occurs when the plant tissue temperature falls below a critical value where irreversible physiological changes occur that are conducive to death or malfunction of the plant cells. Frost stress temperatures are caused by reductions in sensible heat content of air near the surface of the plants, mainly resulting from (1) a net energy loss through radiation from the surface to the sky (i.e. radiation frost); (2) wind blowing in subzero air to replace warmer air (i.e. advection frost); (3) some combination of the two process (Snyder et al., 1987, Kamla et al., 1992). Consequently, frosts are defined as falling into categories: Advective and radiative, according to nature of ice formation, the time of occurrence, and the damage it causes to the plant.

#### **1.4.1.1 Radiation frost:**

Radiation frost is a common occurrence in winter in many temperate zones following extensive radiative cooling characterised by a clear sky, calm or very little wind, temperature inversion, low dew-point temperature, and air temperatures that typically fall below 0°C during the night but are above 0°C during the day. Under a clear night-time sky with a long night period, more heat is radiated away from the surface than is received, so the temperature drops. There are two subcategories of radiation frost: a “hoar frost” occurs when water vapour deposits onto the surface and forms a white coating of ice crystals that is commonly called “frost”. A “black frost” occurs when the temperature falls below 0°C and no hoar frost ice forms on the surface. If the humidity is sufficiently low, then the leaf surface temperature might not reach the ice point temperature and no frost will form. On the other hand, when the humidity is high, ice is more likely to deposit and hoar frost can occur, because heat is released



during ice deposition process, hoar frost usually causes less damage than black frost (Perry, 1994). In temperate and maritime zones air humidities are usually high enough to mean that dew formation precedes freezing and as a consequence hoar frosts are typical during radiation freezing. In sub-tropical and continental zones low air humidity is more common and consequently black frosts are more commonplace than hoar frost.

#### **1.4.1.2 Advection frost**

An advection frost occurs when cold air blows into an area to replace warmer air that was present before the weather changed. It is commonly associated with moderate to strong winds, no temperature inversion, and often low humidity (Snyder et al., 1987). It is also associated with wind directions coming from continental cold zones or from the Polar Regions. Advection freezing can persist for days, weeks or even months depending on regional climatic factors. During a spell of advection freezing, overnight radiation frosts may also occur especially if the air mass is not below 0°C of plant height.

#### **1.4.2 Methods for testing frost resistance**

All methods which are used to investigate frost resistance in plants are based on the principles that determine the ice nucleation point and the place in which ice forms. As with all scientific measurements, the ultimate result must be an absolute rather than a relative measure of the quantity, the ultimate result is most simply met by determining the “frost-killing point”. Often the freezing temperature required to kill 50% of the plant or plant parts is determined. Occasionally the temperature resulting in 100% killing or “incipient frost killing point” or “ultimate frost killing point” is determined (Levitt, 1980).

The 50% killing point is the most readily measured and has now become standard. This point must be determined under standardised conditions so that experiments can be cross compared. All investigators have adopted the following five steps which have been proposed as basic requirements:

- 1) The plant or plant parts must be ice nucleated or inoculated usually with ice or another strong ice nucleation agents to ensure freezing as close to zero Celsius as possible.
- 2) Cooling must occur at a standard rate that is commensurate with natural freezing rates.
- 3) A single freezing point (or set point) must be used for a standard length of time to ensure equilibrium of the frost stress to be imposed.
- 4) Thawing must occur at a standard rate of warming commensurate with natural thawing rates.
- 5) Post thawing (recovery) conditions must be standardised (Levitt, 1980).

Excessive supercooling i.e. insufficient care with ice nucleation, may cause increased freezing injury when ice formation is eventually initiated (Levitt, 1980, Gusta and Fowler, 1977, Levitt, 1972). Freezing of detached samples can be ensured by placing small ice crystals into sample vessels, by touching a sample with a spatula cooled in liquid nitrogen, by wrapping sample in moist paper towels, or by placing them in moist sand. Large plants or plant parts are less likely to supercool extensively than small samples, because spontaneous ice nucleation is a chance event and stochastically is more likely to occur the bigger the sample (Burke et al., 1976). On the other hand, the mechanism of supercooling is less effective in excised stem section than in intact plant, because ice seeding may occur through cut surface where cell exudates exists (Fircks, 1993).

In terms of cooling rate, there are three types: direct, gradual, and simulation. In direct cooling, samples are brought to the test temperature quickly to try to avoid additional hardening during the freezing test using a cooling rate of 5 to 10°C h<sup>-1</sup>. In gradual cooling hardness levels can be increased and so this method enables the maximum hardness potential to be assessed by lowering the temperature stepwise and slowly enough to allow the samples to achieve their full hardening capacity (Lundheim, 2002). This involves cooling to zero for 1-2 days then to -1 to -2 for a further few days prior to freezing to sequential test temperatures. In simulation cooling, the temperature is lowered at a rate 1 to 2°C h<sup>-1</sup> to imitate natural cooling rates during frost events (Sakai and Larcher, 1987).

The low temperature exposure at test temperatures must be long enough for thermodynamic equilibrium to establish. Early reports of freezing tests generally reported that a longer exposure to a test temperature brings about increased damage (Rollins et al., 1962, Stoyanov, 1973). Larcher (1968) considered an exposure time 4 to 6 h as a minimum but this may be dependent on the heat exchange rates in the test equipment. As freezing occurs and subsequently as ice grows the latent heat of freezing is released and the testing environment needs to remove this heat. Ice is in fact a poor conductor of heat and so theoretically it can take a long time for the latent heat to be dissipated in frozen tissues.

Thawing is an integral part of the freezing test but is frequently overlooked by researchers looking at defining frost hardness. The ideal method of thawing is to warm the samples at a constant rate in the freezing unit itself. If a single freezing unit is available, this may only be applied to the lowest test temperature of a stepwise decreasing temperature regime. Therefore, a common approach is to transfer samples from the freezing device directly to a temperature environment of 0 to 5°C (

i.e. to a refrigerator) for a 12 to 24 h period of thawing (e.g. overnight) (McNamara and Pellett, 1998).

Among the numerous methods developed for the determination of tissue viability following freezing, visual observation and electrolyte leakage test are the most common (Lindén, 2002). Additional techniques used for deciduous woody plants include differential thermal analysis (DTA), observation on cellular fluorescence and cell plasmolysis/deplasmolysis, rate of hydrogen cyanide gas production, change in xylem pressure potential and measurement of electrical impedance (Calkins and Swanson, 1990).

Visual examination of thawed tissue samples or intact plants can detect many signs of freezing injury. Usually, freeze-injured tissues eventually develop a brown (Necrosis) or yellowish colour due to oxidation (chlorosis) of polyphenols but visual symptoms of freeze injury vary between plant species and cultivars as well as between different organs and tissues (Lindén, 2002). The visual observation of freeze injuries may be aided by using chemical compounds, such as neutral red or 2, 3, 5- triphenyltetrazolium chloride (TTC) which will penetrate living cells more deeply than non-living cells. This test was used for the test of tissue damage. However, formazan, the reduction product of TTC, stains living tissues with red whereas the non-living remains white (Pearce et al., 1998, Tanino and McKersie, 1985).

The damage to cell membranes is the principle on which the electrolyte leakage test is based. It is assumed that individual cells become progressively more leaky under increasing frost stress and within an organ or whole plant the electrical conductivity is used to measure the collective average of cell damage caused by freezing. Recording the amount of leakage will therefore provide an estimate of tissue damage. However, few researchers actually correlate the electrical

conductivity with visible frost damage and in some complex plant structures such as flowers or spikes economic damage may occur at low leakage levels. The electrolyte leakage test involves the measurement of the electrical conductivity of pure water in which detached tissue samples have been placed after a freeze thaw cycle (Lindén, 2002). Dexter et al (1930, Dexter et al., 1932) were the first researchers who applied the electrolyte leakage test in cold hardiness research. The validity of the relative leakage method on assessing frost damage in leafless shoots of *Quereus petraea* (Matt.) Lieb has been examined in detail and results indicated that samples should be incubated longer than for the usual 20 to 24 h to permit the initial leakage to approach asymptotic values. In addition, killing samples by autoclaving at 121°C for 15 minutes was found insufficient to release all diffusible electrolytes (Deans et al., 1995). Consequently, these workers recommended an initial incubation period of 5 to 7 days at +4°C to minimize microbial activity, followed by autoclaving for 90 to 120 minutes at 121°C and allowing 24 h before measurement of the final leakage value. For more herbaceous specimens however 12 to 24 h incubation and autoclaving at 121°C for 5 to 15 minutes or even just boiling for 15 minutes is sufficient for complete cellular release. Electrolyte leakage is commonly expressed as a percentage of total cell contents (i.e. of the autoclaved sample) in order to account for variability in sample size or tissue contents and the statistic Relative Electrical Conductivity (REC %) is computed for areas comparison.

## **1.5 Infrared thermal imaging for studying frost in plants**

Thermal infrared imaging techniques are very valuable as an aid in understanding the physiological effects of frost on plants and can be used both in the field and under artificial conditions. This technique is based on measuring the heat released when different fractions of water freeze within tissue (Ketchie and Kammereck, 1987, Quamme et al., 1972, Fuller and Wisniewski, 1998).

The thermal infrared camera produces an image in a way exactly analogous to forming images from visible light with familiar electronic imaging devices like video or digital cameras. Thermal cameras contain an array of detectors that convert infrared radiation (thermal energy) to electrical output signals whose amplitude is proportional to the amount of the energy detected. These output signals can be processed as a normal video image in grey-scale image of the scene. The grey-scale value in the images represents the amount of thermal energy detected from a given point in view and is proportional to the radiant temperature of that point. Hotter radiant temperatures are normally displayed as whiter tones in an image, and colder temperature as blacker tones (Colwell, 1983). More recently, greyscale images can be converted to colour enhanced images using computing software generating a range of colour palettes. Since freezing events are temporal it is necessary to record the images over time either as video images or digital video equivalents. The determination of the exact location of initial ice formation (point of ice nucleation) and how it progresses throughout the plant (ice spread) is very important information that needs to be known prior to devising methods to protect plants. Wisiniewski et al (1997) were amongst the first to use infrared thermal imaging technology to determine where ice first begins to form in a plant and at what temperature, based on

the principle of release of heat when ice is formed (exotherm which is the process that describe the chemical reaction when the energy is released usually in a form of heat). By using infrared video thermography, these researchers observed a plant freezing and followed the progress of ice formation throughout the plant. Subsequently, ice formation has been tracked by using infrared camera system in many species including apple (*Malus ssp*), peach (*Prunus persica*), and pear (*Pyrus ssp*) trees and bean (*Vicia faba L.*), potato (*Solanum tuberosum*), and strawberry (*Fragaria ssp*) plants (Fuller and Wisniewski, 1998, Wisniewski et al., 1997). Computer software has also been used to analyse recordings of infrared video tape to study all stages of ice formation and to quantify the magnitude of the exotherms observed and their duration. Furthermore, speed of ice front travel can also be determined and barriers to ice spread detected (Fuller and Pearce, 2001, Wisniewski et al., 1997). Of significance have been the observations that stem nodes hindered ice travel but ice forms quickly along the stem of the plant; but slows as it approaches the nodes, also, while shoots freeze quickly, ice formation progresses very slowly into the roots. In peach trees the stem was frozen before flowers and ice does not automatically form in the flowers, when shoots have frozen. Such differential freezing in various plant structures can begin to answer field observations of differential frost damage following natural freezing events.

Since the discovery that some bacteria (e.g. *Pseudomonas syringae*) can stimulate ice nucleation these have been used in freezing experiments to aid ice formation during infrared observation experiments (Fuller and Pearce, 2001, Fuller and Wisniewski, 1998, Pearce et al., 1998, Wisniewski et al., 1997). Typically, plants, which were treated with droplets of INA bacteria began to freeze first compared with samples or plants treated with water droplets only. It was observed that the drop of water on the

surface of a leaf froze independently, at least 2.5 minutes after the part of leaf containing the bacteria (Wisniewski et al., 1997). Fuller *et al* (2007) has reported that the ice nucleation temperature was uniform with *Pseudomonas syringae* but was lower in the presence of distilled water. The warmest temperatures that INA bacteria are active at is  $-2^{\circ}\text{C}$  whereas distilled water droplets can supercool significantly below this. Thus it would seem that in nature, plants will always supercool to some extent and it follows that eliminating INA bacterial cells from the plant tissue surfaces allows plants to protect themselves from freezing by enabling supercooling. This approach is being used in California to protect blossom in almond and peach orchards from spring radiation frosts (Lindow et al., 1982) but has not been used systematically elsewhere. Fuller et al (2007) have demonstrated *in-vitro* that the use of compounds that help shed surface water, decrease the incidence of dew and thereby increase the likelihood of supercooling and frost escape in potatoes, tomatoes and grapevine but these compounds have not been tested extensively in the field.

Infrared thermal imaging has been used by many researchers who have observed that whilst supercooling is a mechanism for avoiding frost damage, it does carry with it an inherent risk if the temperature drops too low and is followed by freezing. Ice crystals that develop after substantial supercooling have the potential to cause more injury than ice crystals that form at higher temperature (Cho et al., 1999). Slowly formed ice crystals are smaller than those formed more rapidly and in addition, when ice forms slowly, water has time to escape from plant cells so that ice grows in the intercellular spaces and intracellular ice formation, which is lethal, is less probable.



## **1.6 Low-temperature acclimation in winter cereals**

In the early autumn winter sown cereal plants will not survive subfreezing temperatures much better than spring cereal plants. However, winter cereals, which subsequently experience cool autumn temperatures, will cold acclimate or “harden off”. Cold acclimation of winter wheat plants begins once autumn temperatures drop below approximately 9°C. A translocatable substance that promotes cold acclimation is not produced when winter wheat plants are exposed to acclimating temperatures (Limin and Fowler, 1985). Consequently, the cold hardiness level of different plant parts, such as leaves, crowns and roots, is dependent upon the temperature to which each part has been exposed. Wheat is cultivated throughout the world (from South America and Southern Oceania to North America and the Northern parts of Europe and Asia, from sea level to about 3000 m), and its wide adaptability is based on complex developmental responses to environmental factors. As wheat has adapted to different regions, its development patterns have been modified through regional plant breeding and selection to suit particular environmental conditions. One key developmental issue is that anthesis (flowering) must occur when the risk of frost is small or non-existent. The important feature of wheat adaptability lies in its ability to sense the season so that development is accelerated or delayed depending on the environment (Reynold et al., 2001). In most regions of the world wheat is grown “in phase” with the seasons with flowering occurring in late spring/early summer but in a few regions e.g. east Australia and south Africa it is grown “out of phase” with flowering occurring in mid-winter when there is a chance of frosts occurring and subsequent frost damage. In continental climatic zones as the USA and Russia there is a chance and late frost during flowering of wheat.

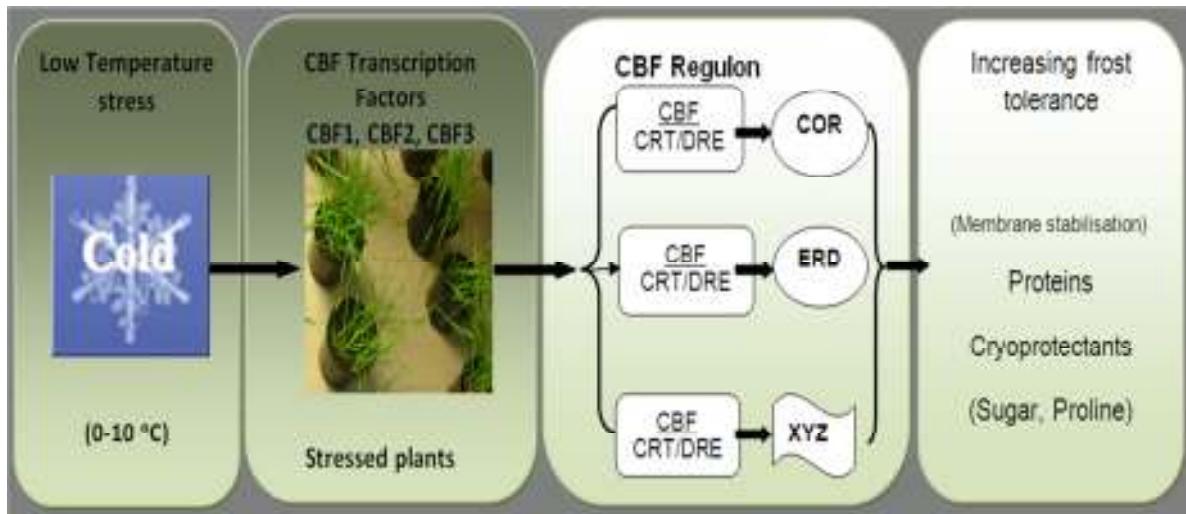
At the molecular level, cold acclimation requires recognition of low temperatures by cells through signalling processes to switch on the responsive genes to LT, and these eventually enable plant to have greater chance of survival of low temperatures. The increase in frost tolerance that takes place with cold acclimation is most likely to take in the action of several cold responsive genes that are activated upon exposure low temperature (Thomashow, 1999b). Many studies have identified that CBF cold response pathway to be a key regulatory pathway in cold acclimation (Smallwood and Bowles, 2002, Stitt and Hurrey, 2002, Xiong et al., 2002, Shinozaki et al., 2003, Sung et al., 2003).

### **1.6.1 The CBF pathway**

In general, during the low temperature exposure period, *LT-responsive* genes, *CBFs* (C- Repeat Binding Factor) and *COR* genes (Cold Regulated genes) are switched on and frost tolerance is maintained to survive winter conditions (Danyluk et al., 2003). In cereals vernalisation requirements and cold acclimation regulatory gene networks are interconnected, but regulated by different factors (Galiba et al., 2009). It has been reported that *VRN1* has been mapped on the long arm of chromosomes 5A, 5B and 5D (Dubcovsky et al., 1998, Laurie et al., 1995, Law et al., 1976) associated with Quantitative Trait Loci (QTL) for frost tolerance in both wheat and barley (Francia et al., 2004, Galiba et al., 1995, Laurie et al., 1995, Limin and Fowler, 2002, Sutka et al., 1999, Sutka and Snape, 1989, Hayes et al., 1993).

The CBF response pathway was first discovered for the first time through studies which were carried out on *Arabidopsis* to investigate the effect of low temperature (Thomashow, 1999). It was shown that *Arabidopsis* encodes a small family of Cold-responsive transcriptional activators which have been called CBF1, CBF2, and CBF3

(Stockinger et al., 1997, Gilmour et al., 1998) or DREB1b, DREB1c and DREB1a (Liu et al., 1998, Kasuga et al., 1999). These all are members of AP2/EREBP family of DNA-binding protein transcription factors (JL and EM, 1998). These recognize the cold and dehydration responsive DNA regulatory element which designated the CRT(C-repeat)/DRE (dehydration responsive elements) (Baker et al., 1994, Yamaguchi-Shinozaki and Shinozaki, 1994). Conserved 5-bp DNA-binding motif which is CCGAC for the CRT/DRE elements are present in the promoter regions of many cold and dehydration responsive gene of Arabidopsis including those designated as COR (Cold regulated genes) (Thomashow, 1999). Transcription factors (CBFs) are not expressed under normal conditions (~ 22°C). However transcripts of CBF1, 2, and 3 genes accumulate swiftly after 15 minutes from exposing to low temperature (normally +Ve temperatures). Accordingly after CBFs are activated, they induce the expression of genes containing CRT/DRE regulatory elements, i.e. "CBF regulon" (Gilmour et al., 1998). The expression of CBF regulons leads to an increase in frost tolerance over the following days provided low temperature is maintained (Jaglo-Ottosen et al., 1998b, Liu et al., 1998, Kasuga et al., 1999) (Figure 3). The maximum level of frost tolerance in plant will be reached after 1 to 2 weeks (Jaglo et al., 2001). But however, when the plant returned to the normal temperatures, the transcripts of the CBF transcription factors have been shown to quickly (within hours) return to the level found in plant grown in normal conditions (Jenks and Hasegawa, 2005).



**Figure 3. CBF pathway in plants. Low temperature leads to rapid induction of the CBF transcription factors, which in turn cause the expression of the CBF regulon of the CRT/DRE regulated genes such as COR (cold regulated genes, ERD (early responsive to dehydration) and presumably non discovered XYZ cold regulated genes, resulted in increasing cold tolerance. Adapted from (Thomashow, 1999).**

The function of CBF regulon is enhancing the freezing tolerance. Therefore, the transgenic plant become frost tolerant by induction of COR gene expression without exposure to low temperature (Gilmour et al., 2000, Jaglo-Ottosen et al., 1998b, Liu et al., 1998, Kasuga et al., 1999).

CBF proteins stimulate transcription of their target genes through recruiting to gene promoters' transcriptional adaptor complex that can modify chromatin structure (Jenks and Hasegawa, 2005). Among the genes which are the most significantly induced during cold acclimation COR genes alternatively KIN (cold induced), RD (response to dehydration), LTI (LT-Induced) or ERD (Early responsive to

dehydration) (Thomashow, 1999). All proteins encoded by these genes are hydrophilic. They are either new or members of dehydrin (Close, 1997) or LEA (Late embryogenesis abundant) (Wise and Tunnacliffe, 2004) protein families. Although, the activity of most of the COR/LEA/Dehydrin proteins is not clearly understood, however there is proof that the polypeptide encoded by COR15a imparts the stabilisation to the membranes against frost injury (Artus et al., 1996, Steponkus et al., 1998). The target place of 15 KDa polypeptide encoded by COR15a is the chloroplast. Lin and Thomashow (1992) have reported that chloroplast processes the 15 KDa polypeptide to 9.4 KDa mature polypeptide designated COR15m. It has been found that the constitutive expression of COR15a increased the freezing tolerance the chloroplast (Artus et al., 1996), indicating that the COR genes play an important role in protecting plant against frost damage. Therefore, COR15a can act directly as cryoprotective polypeptide (Steponkus et al., 1998). Some studies have found that COR15a proteins decrease the ability of membranes to form the hexagonall-II phase (deleterious non-bilayer structure that occurs due to cellular dehydration associated with freezing). Thus it suggested that COR, LEA and LEA-like polypeptides might contribute in membranes stabilisation (Thomashow, 1999b, Nakayama et al., 2007, Bravo et al., 2003b). In addition, they can protect other proteins against freeze-thaw inactivation *in vitro* (Bravo et al., 2003, Hara et al., 2003, Sanchez-Ballesta et al., 2004). More recently Thalhammer et al (2010) have reported that COR15A and COR15B proteins which belong the late embryogenesis abundant (LEA-group 3) are predicted to be intrinsically disordered proteins (IDPs) in the solution. Therefore COR proteins as well as LEA function as IDPs in fully hydrated state or alternatively they may acquire structure during partial or complete dehydration brought to plant by drought or freezing stress (Hinch and Thalhammer, 2012, Thalhammer et al., 2010).

COR15A and COR15B are predominantly unstructured under normal condition and become  $\alpha$ -helical under drying condition (e.g. freezing or drought) where they show 60-70%  $\alpha$ -helical in dry state. However the question of how could the structure of these proteins effect the stress tolerance still unanswered (Hincha and Thalhammer, 2012).

### **1.7 Frost resistance in wheat:**

Frost resistance is a complex trait, which involves different characteristics at the cell, tissue, and whole plant level, and so it is not surprising that it is under polygenic control. Earlier studies have shown that at least 10 chromosomes influence the plant defence response to low temperature (Law et al., 1970, Sutka, 1994, Sutka, 2001, Sutka et al., 1986). The key role of the homoeologous group 5 of chromosomes in the genetic control of this trait is well established. One of the most influential genes, *Fr1* is located in the long arm of chromosome 5A and plays a direct role in the plant response to low temperature (Ganeva et al., 2008). Molecular studies have shown a very close genetic linkage between the vernalisation (*Vrn*) and frost resistance (*Fr*) genes (Galiba et al., 1995b, Sutka, 2001). Developmental genes (vernalisation *Vrn*, photoperiod *Ppd*) which control the transition from the vegetative to the reproductive phase, also act to control genes affecting the expression of low temperature-induced genes associated with acquisition of frost tolerance. By using RFLP (Restriction Fragment Length Polymorphism) markers, the intra-chromosomal location of *Vrn-1* and *Fr1* loci were located closely linked on the distal of the long arm of chromosome 5A, but the recombination between them was found to be only 2 centimorgans. The other two homoeologous chromosomes were also shown to carry frost resistance

genes located close to vernalisation genes: *Fr-B1* and *Vrn-B1* on chromosome 5B and *Fr-D1* and *Vrn-D1* on chromosome 5D (Snape et al., 1997).

It can be concluded that vernalisation and frost resistance or cold acclimation are two important mechanisms that cereals have evolved to cope with low-temperature stress. Both are regulated through complex genotypic and environmental interaction inducing a large number of physical and biochemical changes in the plant. The gene for vernalisation requirement acts as a master switch regulating the duration of low temperature induced frost tolerance. In winter wheat, due to a longer vegetative phase, frost tolerance is maintained for a longer time and at a higher level than in spring wheat. After the saturation of vernalisation requirement, winter wheat (as in spring wheat) established only a low level of frost tolerance (Ilja et al., 2004).

Despite the fact that the effect of frost damage has dramatic effect on grain yield during reproductive development of cereals, it has received comparatively little attention. Plants have to develop a mechanism to avoid such losses. Cold acclimation can be activated by low temperature stimulus, but it seems that it is not the only stimulus to activate cold acclimation. It has been reported that COR genes are mediated by ABA dependent and independent pathways (Thomashow, 1999, Shinozaki and Yamaguchi-Shinozaki, 2000). It has also been found that Molybdenum (Mo) can regulate the biosynthesis of ABA via aldehyde oxidase (AO) which in turn mediates the expression of COR genes in wheat (Sun et al., 2009).

## **1.8 The role of Molybdenum in the frost tolerance in wheat**

Molybdenum (Mo) is one of the essential trace elements required by higher plants. Mo is a co-factor for more than 40 enzymes in all organisms and catalyses various redox reactions, but only four of these enzymes have been found in plants (Mendel and Hänsch, 2002). These four enzymes are: (1) nitrate reductase (NR) which is the key step of inorganic assimilation of nitrogen, (2) aldehyde oxidase (AO) which has been found to catalyse the final step of ABA biosynthesis, (3) Xanthine dehydrogenase (XDH) which has been found to be involved in purine catabolism and stress reactions, and (4) sulphate oxidase (SO) which is probably involved in detoxifying excess sulphate (Mendel and Hänsch, 2002, Sun et al., 2009, Mendel and Bittner, 2006). Mo has both structural and functional role in these enzymes (Vunkova-Radeva et al., 1988). Mo has to be in a complex in order to be biologically active and these are called Mo-co-factors (Mendel, 2007). Mo-co-factors are very important for the molybdenum-requiring enzymes (molybdoenzymes) that have been reported in most biological systems of plants and animals (Kaiser et al., 2005). Interestingly, Mo has been cited as involved in amelioration of frost damage in wheat (Li et al., 2001). Low temperature stress leads to the increase of Reactive Oxygen Species (ROS) in plants, and it has been reported that Mo increase the activity of anti-oxidant enzymes. These enzymes, including SOD, CAT and POX, increase the anti-oxidant defense in plants (Yu et al., 1999). ROS are produced once plants are exposed to low temperature and they have cellular effects in the plant tissues (Sattler et al., 2000). Mo addition has been found to increase the capacity of scavenging the ROS through catalyzing the enzymes, listed above, and thereby reduce membrane damage under low temperature stress (Sun et al., 2006). Mo also has a positive role in increasing frost tolerance in plants via catalyzing of the AO enzyme. AO has an



important role in the production of ABA, since AO has relatively broad substrate specificity for several aldehydes including abscisic aldehyde and indole-3-aldehyde. ABA triggers bZIP and the up-regulation of the ABA dependent COR gene expression pathways (Sun et al., 2009). Also Mo could enhance the nitrate assimilation via catalyzing NR enzyme. It has been reported that NR activity significantly increased in Mo-treated wheat and this was associated with protein accumulation (Yu et al., 1999, Hale et al., 2001, Hamdia et al., 2005).

## **Chapter 2: General Materials and Methods**

## 2.1 Evaluation of frost tolerance in wheat varieties

The thresholds of frost hardiness were determined as  $LT_{50}$  (lethal temperature 50,  $LT_{50}$ : The temperature, which kills 50% of the plants).  $LT_{50}$  indicates to the minimum temperature of the freeze-thaw cycle at which 50% of the plants were killed of both acclimated and non-acclimated plants. Half of the seedlings from each cultivar were acclimated and half un-acclimated. The plants were then sprayed with distilled water in order to facilitate the ice nucleation before treatment. Samples (n= 36) were taken after a 2 h hold at 0, -2, -4, -6, -8, and -10 °C. Plants were scored for survival (Table 2) and the lethal temperature for 50% kill ( $LT_{50}$ ) of the population determined by performing the logistic regression in differential equation for curve fitting using Minitab vs. 16 (Figure 4):

$$S\% = 100/(1 + e^{a-rt}).$$

*where: S is the survival percentage; a and r are constants; t is temperature*

*(Robert, 1974).*

**Table 2. Damage scores after 14 days recovery (after Fuller et al., 2007)**

Score	Description of plant damage
0	Un-damaged – plants same as unfrozen controls
1	Slight damage - Less than or equal 50% of plant damaged with plant showing regeneration
2	Severe damage - More than 50% of plant is damage with plant showing some regeneration
3	Complete damage - Plant is completely dead with no regeneration.

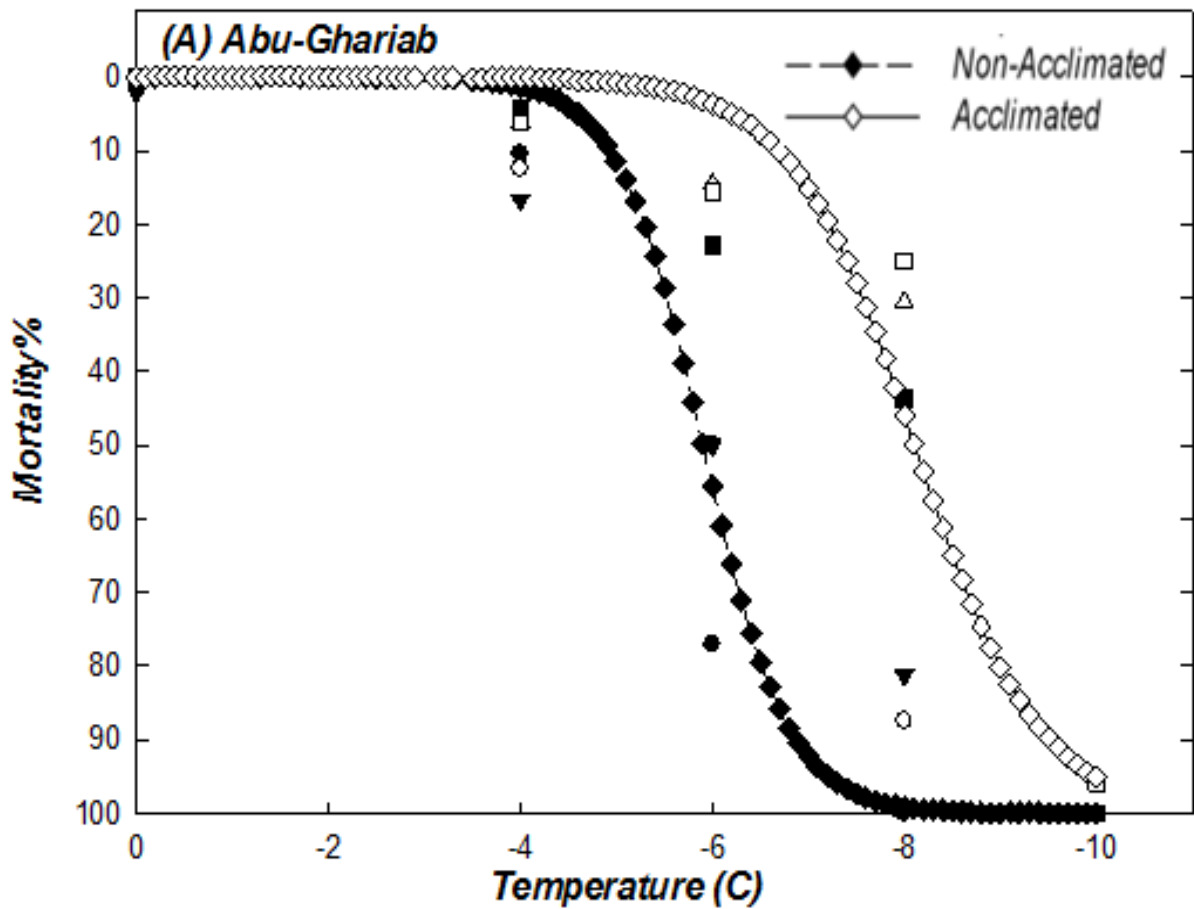


Figure 4. Example of the logistic curve used in determination of LT50 of cold acclimated (CA) and non-acclimated (NA) cultivars (each data point n = 36) (curves for other varieties and treatments are presented in Appendix).

## 2.2 Characterisation of the frost damage to wheat during spike emergence.

Wheat plants were sown in pots (30 × 30 × 25 cm) filled up with compost (John Innes: seed sowing compost, manufactured by Westland Ltd). The pots were sunk in a raised bed so that the compost was level with the soil surface. Plants were irrigated when necessary and nitrogen fertilizer (Sulphate of Ammonium 21%) was

added in two doses; first split dose 50 kg ha<sup>-1</sup> (2.4 g.pot<sup>-1</sup>) was applied when the plants reached Pseudostem erect (GS 30) and the second dose 100 kg ha<sup>-1</sup> (4.8 g.pot<sup>-1</sup>) at the early jointing stage (GS 32) according to Zadoks scale for cereals (Zadoks et al., 1974).

### **2.2.1 Cold acclimation before frost tolerance assessment**

Acclimation plants whether at the vegetative or reproductive stage, were acclimated by transfer of rooted plants into a cold store at 4°C with 8 h light (Photosynthetically active radiation (PAR) 177 µmolm<sup>-2</sup>s<sup>-1</sup>) for 14 days. During the acclimation, plants were irrigated as required. Samples for both frost damage and molecular analysis were collected after 0, 8, 24 hours and 14 days after transferring plants the cold store. Non-acclimated plants were also transferred to a chamber built specially for this purpose at 20°C and at the same level of light. Plants were irrigated as required and samples were collected at the same time as acclimated plants.

### **2.2.2 Relative Electrical Conductivity (REC%)**

Frost damage was assessed as Relative Electrical conductivity (REC%) as described in Fuller *et al* (2007). organs were cut and put in a boiling test tube and then exposed to this freezing regime 1°C for 1 h, 0°C for 2 h, -2°C for 2 h, -4 °C for h, -6°C for 2 h and then defrosted at 1°C for 1h inside the frost chamber according to the following steps:

The freezing regime was as follows:

- 1- After 2 h at 0°C small piece of ice was added to each tube and the 0°C sample one was removed. The ice was to facilitate ice nucleation in the plant material being tested
- 2- After 2 h sample 2 was removed.
- 3- After 2 h sample 3 was removed.
- 4- After 2 h sample 4 was removed.
- 5- All sample tubes were placed at 4°C overnight to facilitate slow defrosting.
- 6- Sufficient distilled water was then added to the tubes to cover the plant samples and a lid was placed on each tube. These samples then went through a standard
- 7- Electrical Conductivity (EC) testing.
- 8- The samples with distilled water were incubated at 20°C for 24 hrs.
- 9- The EC of distilled water in each tube was measured (EC1).
- 10-The samples were autoclaved at 121°C for 15 m.
- 11- The samples were incubated again at 20 °C for 24 hrs.
- 12-The EC of autoclaved samples was measured (EC2).
- 13-The Relative Electrical Conductivity (REC%) was calculated:

$$REC\% = \frac{EC1}{EC2} * 100$$

### **2.3 Infrared Video thermography**

The main aims of using the infrared video thermography (IRVT) are to visualise the first site of ice nucleation, its propagation and also to determine the temperature at which ice initiates (Wisniewski et al., 1997, Pearce, 2001, Pearce and Fuller, 2001).

Infrared video thermography was applied using a Digital infrared camera (ThemaCAMS65: FLIR system) in order to monitor the plants inside the frost chamber while the temperature was reduced. A SAYNO M533 Incubator was prepared specifically for this purpose using pre-programmed set temperature where the plant would be exposed to different temperatures ( $\leq 0^{\circ}\text{C}$ ) (Fuller and Le Grice, 1998, Pearce and Fuller, 2001). Frost chamber was set to run from temperature of 0 to  $-15^{\circ}\text{C}$  at  $2^{\circ}\text{C h}^{-1}$ , while the IR camera was able to detect the heat releases from plant within  $-3$  to  $-12^{\circ}\text{C}$  in some experiments. Plants in pots were grown in the field until they set spikes and then dug up to be taken to the Lab a single pot with several heading culms was used in each trial which was put in the frost chamber, during the frost test the temperature was recorded using datalogger (Gemini: TinyTag). Camera was set up in front of the frost chamber and then connected to a DVD recorder, which was in turn connected to a TV. In order to reduce the reflection interaction of the metal body of the frost chamber, a piece of cardboard was used as a background. In some trials half of plants were sprayed with water in order to facilitate the ice formations while the others were still without water (see chapter 5).

## **2.4 Molecular analysis**

Samples for molecular analysis (gene and protein expression) were collected accordingly after 0, 8, 24 hours and 14 days and then kept in  $-80^{\circ}\text{C}$  freezer. The total RNA was isolated from leaves and heads from acclimated and non-acclimated plant simultaneously. At each interval, three leaves were detached from each plant and placed immediately in liquid nitrogen then were kept in laddled plastic bags in  $-80^{\circ}\text{C}$  freezer. Leaves for each treatment were then frozen in liquid nitrogen and finally ground in liquid nitrogen in a pestle and mortar. After the liquid nitrogen

evaporated, 100 mg of frozen tissue powder for each sample was weighed in 1.5 mL RNase and Dnase free micro-centrifuge tubes and stored at -80°C to prevent RNA degradation before RNA isolation. Proteins in the wheat, the proteins were extracted from both non-acclimated and acclimated plants once after 14 days acclimation and then stored at -80°C until further analysis through sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE) and Western blotting techniques.

### **2.4.1 mRNA isolation from plant tissues**

Plant total RNA kit (STRNA50) (Sigma: Plant biotechnology) was used for isolating mRNA from plant tissues according to the method of (Ausubel, 2001, Farrell, 1998, Sambrook et al., 1989) method and as follow:

#### **Preparation instructions**

##### **1- Preparation Wash Solution 2**

Wash Solution 2 was supplied as a concentrate. Prior to first time use, 60 mL of 100% ethanol was added to the bottle of Wash Solution 2 concentrate, mixed briefly and then stored as diluted Wash Solution 2. Bottle was tightly capped to prevent the evaporation.

##### **2- Preparation plant tissue sample**

###### **2.1 Grinding plant tissue**

In order to make sure that all surfaces and equipment were used in the extraction process RNase ZAP (Sigma kit, Cat# R2020) spray was used for complete removal of any trace of RNase contamination. Plant tissues were harvested and submerged it in liquid nitrogen as soon as possible to prevent RNA degradation. Tissues were then ground into a fine powder in liquid nitrogen using a mortar and pestle. RNA yield is often dependent upon how fine the plant tissue has been ground, especially if the



tissue is difficult to grind. For best practice, the mortar was placed on dry ice and the plant materials were kept frozen at all times.

## **2.2 Weight tissue sample**

After liquid nitrogen has been evaporated from the frozen tissues powder, quickly weighs approximately 100 mg (90–110 mg and should not be exceeded 110 mg per tube) has been weighed of the tissue powder in a 2 mL microcentrifuge tube, pre-chilled on dry ice or in liquid nitrogen. The weighed samples were kept on dry ice or at  $-80^{\circ}\text{C}$  before lysis solution is added.

## **3- Prepare Lysis Solution/2-ME Mixture**

Lysis solution must be supplemented with 2-mercaptoethanol (2-ME) before use. Therefore 10  $\mu\text{L}$  2-ME were added to 1 mL of lysis solution in a clean conical tube, and then mixed briefly. Each RNA preparation requires 500  $\mu\text{L}$  of the mixture. For pipetting allowance when working with multiple samples some extra solution should be prepared. For best results, the mixture was prepared as close to the time of use as possible because the use of a lysis solution/2-ME mixture more than one day old after preparation may result in reduction in RNA yield.

## **4- Assemble Column and Collection Tube**

Filtration columns (blue retainer ring) were inserted into a 2-mL collection tube and closed with the lid for the use in step two under the procedure. Likewise, binding columns (red retainer ring) were also inserted into a 2-mL collection tube and closed with the lid for use in step three under the procedure.

## **Procedure**

### **1- Lyse tissue sample**

500  $\mu$ L of the Lysis solution/2-ME Mixture was pipetted to 100 mg of tissue powder and vortex immediately and vigorously for at least 30 seconds. Then the sample was incubated at 56°C (temperature can be changed according to the type of plant tissue) for 3–5 minutes.

### **2- Pellet Cellular Debris**

The samples were centrifuged at maximum speed (13000 rpm) for 3 minutes in order to pellet cellular debris.

### **3- Filter Lysate**

The supernatant lysate was pipette into a filtration column (blue retainer ring) seated in a 2-mL collection tube by positioning the pipette tip at the bottom of the tube but away from the pellet. Sometimes there is a layer of floating particulates, in such case the pipette tip below the floating layer positioned and away from the pellet before pipetting the supernatant. It is not matter if some of the floating particulates were carried over to the filtration column as long as pellets can be avoided. Then caps were closed and centrifuged at maximum speed for 1 minute in order to remove residual debris. The flow-through lysate was then saved.

### **4- Bind RNA to Column**

#### **-Add Binding Solution**

500  $\mu$ L of binding solution was pipette into the clarified lysate and mixed immediately and thoroughly by pipetting for at least 5 times and sometimes the vortex was briefly used.

Sometimes the RNA level is expected to be low, or more of the small-sized RNA needs to be recovered, the amount of the binding solution was increased to 750  $\mu$ L.

### **- Bind RNA**

700  $\mu\text{L}$  of the mixture were pipette into a binding column red retainer ring seated in a 2-mL Collection tube. Caps then closed and centrifuged at maximum speed for 1 minute in order to bind RNA. The flow-through liquid was decanted and the collection tube was tapped (upside down) briefly on clean absorbent paper in order to drain the residual liquid. Then columns were returned to the collection tube, the remaining mixture was pipette to the column, and the process was repeated.

### **5- First Column Wash**

500  $\mu\text{L}$  of wash solution 1 was pipette into the column. Then columns were closed with their caps and centrifuged at maximum speed for 1 minute. The flow-through liquid was decanted and the collection tubes were tapped (upside down) briefly on a clean absorbent paper in order to drain the residual liquid. Then columns were returned to the collection tube.

### **6- Second Column Wash**

500  $\mu\text{L}$  of the diluted Wash Solution 2 was pipette into the column. Then columns were closed with their caps and centrifuged at maximum speed for 30 seconds. The flow-through liquid was discarded and the collection tubes were tapped (upside down) briefly on a clean absorbent paper in order to drain the residual liquid. Then columns were returned into the collection tube.

### **7- Third Column Wash**

Another 500  $\mu\text{L}$  of the diluted Wash Solution 2 into the columns and step 6 was repeated.

### **8- Dry Column**

Columns were then just centrifuged without any addition at maximum speed for 1 minute to dry. Then columns-tubes assembly were carefully removed from the

centrifuge in order to avoid splashing the residue flow-through liquid on the dried columns.

### **9- First Elution**

Columns were transferred into a new; clean 2-mL collection tube (provided with the kit). A 50  $\mu$ L of elution solution was pipette directly onto the centre of the binding matrix inside the column. Then tubes were closed with their caps and let the tube sit for 1 minute. Finally columns-tubes were centrifuged at maximum speed for 1 minute to elute. The purified RNA is now in the flow-through eluate and ready for immediate use or storage at  $-20^{\circ}\text{C}$  (short term) or  $-80^{\circ}\text{C}$  (long term).

### **10- Second Elution (Optional)**

Sometimes the expected RNA yield is  $>20$  mg and an additional 10–30% of RNA yield could be recovered from the column with another elution. Therefore, columns were transferred into new, clean 2-mL collection tubes. A 30-50  $\mu$ L of elution solution was pipette directly onto the centre of the binding matrix inside the column. Then they were centrifuged at maximum speed for 1 minute to elute. The purified RNA was in the flow-through eluate and ready for immediate use or storage at  $-20^{\circ}\text{C}$  (short term) or  $-80^{\circ}\text{C}$  (long term).

Extracted RNA was quantified with a Nanodrop (NanoVuePluce) to estimate the concentration. In addition to measure the purity of RNA as the absorbance was measured using A260/A280 ratio procedure (Warburg and Christian, 1942). However, nucleic acids have a higher absorbance at 260nm than at 280 nm and therefore, the A260/A280 ratio was expected to be  $\geq 2$  for the pure samples.

## 2.4.2 cDNA library preparation and PCR (Polymerase Chain Reaction)

### **cDNA synthesis**

*cDNA* libraries were prepared for all treatment in both the vegetative and spike emergence investigations for the two varieties according to the standard operation procedure. Extracted total RNA was treated with DNase I (Sigma: AMP-D I) to purify it from any trace of DNA contamination. 1  $\mu$ L from DNase I and 1  $\mu$ L from its buffer were added to each sample in final volume of 10  $\mu$ L for each sample and then mixed gently. Samples were then incubated for 15 minutes in room temperature. In order to stop the reaction of DNA digestion, 1  $\mu$ L from stop solution (50mM EDTA) should be added to each sample to bind the calcium and magnesium ions and to inactivate the DNase I. Samples were then incubated at 70°C for 10 minutes to denature DNase I, then samples were placed either in ice for immediate use or into freezer for later use. The first strand cDNA was obtained by using M-MLV Reverse Transcriptase (Sigma: M1302) in 20  $\mu$ L volume on RNA isolated either from leaves or spikes of wheat. 1  $\mu$ g from mRNA from each sample was reverse transcribed to a first-strand cDNA as the following procedures.

### **Procedure**

The following Sigma-Aldrich reagents were used:

- 1- Deoxynucleotide Mix dNTPs, (product code D 7295, 10 mM dATP, 10 mM dCTP, 10 mM dGTP, 10mM dTTP).
- 2- Random nanomerase primer (Product code R7647).
- 3- M-MLV Reverse transcriptase (product code M 1427).
- 4- 10X M-MLV reverse transcriptase Buffer (Product code B8559).
- 5- Molecular grade water (product code W 1754).

cDNA was synthesized by combining 1 µg RNA and molecular grade water to 8 µl final volume. 1 µL from dNTPs and Random nanomerase primer respectively, were added to the sample and then incubated at 70°C for 10 min and samples immediately placed on ice. The master mix consisted of 1, 2, 7 µL from M-MLV reverse transcriptase, reverse transcriptase Buffer and molecular grade water respectively was added to each sample before incubating them at the following thermal profile for the PCR reaction: 21°C for 10 min, 37°C for 50 min, 94 °C for 2 min and holding at 4°C. The cDNA product was kept in freezer for the later use.

After cDNA synthesis, all samples were stored at -20 °C for later use in PCR. Ordinary PCR technique was used to investigate the expression of the *Cbf14* gene in both vegetative and flag leaves using the protocol presented in Chapter 2. The expression patterns of *Cbf14* were normalised with the endogenous control (18s rRNA) which is known to exhibit the most stable constitutive expression in plants grown under different environmental conditions (Jain et al., 2006). Primers for the genes were designed with gene sequences obtained from Blast software to give the following primer templates: forward 18s rRNA 5'-TGTGCCTAACCCGGGGGCAT-3' and reverse 18s rRNA 5'-GAGCGTGTTTTGGCGTGACGC-3'. All primers were obtained from Eurofins MWG.

### **PCR (Polymerase Chain Reaction)**

The forward and reverse primers were designed for the *Cbf14* gene primers for the gene were designed with wheat gene sequences from Blast software and CBF14-Int-F 5'-CCGTTTCAGCACCGCCAAGGC-3' and CBF14-Int-R 5'-CCATGCCGCCAAACCAGTGC-3' were obtained from Eurofins MWG. cDNA was used as a template for the PCR detection system. The master mix consisted of 1 µL

Red-Taq (sigma: product code D 4309), 2.5  $\mu$ L Red-Taq buffer (product code B5926), and 0.5  $\mu$ L forward and reverse primers, 0.5  $\mu$ L dNTPs, and 18  $\mu$ L H<sub>2</sub>O and was added to 2  $\mu$ L cDNA to final volume 25  $\mu$ L. The reaction mixture for each sample was run under the following thermal cycle: initial denaturation at 94 °C for 2 minutes once followed by 40 cycles of (denaturation at 94°C for 30 s, annealing at 60°C for 30 s, extension at 72°C for 30 s) and a final extension at 72°C for 5 minutes and then held at 4°C. A gel of 2% agarose was prepared in a 250 ml flask by melting 1.4 g of agarose (DNase and RNase free, Invitrogen cat# BPE 1356.100) in 70 mL of 10X TAE buffer (Tris-acetate + EDTA, Invitrogen cat# 15558-026) using a microwave to completely melt the agarose. The solution was allowed to cool for a couple of minutes to about 50°C and 7  $\mu$ L of SYBR safe dye (Fisher #VX33102) was added to the agarose solution and mixed before pouring the gel into the tray. The gel was cast and allowed to solidify for a minimum of 20 min at room temperature. 10X TAE buffer was added to submerge the gel. 10  $\mu$ L of the molecular weight markers of 1kb (Fisher #BPE2581-200) was loaded in a well as a reference ladder. The gel was run at 90 V until the bromophenol blue was about 3/4 through the gel (approx. 1 h). The tray with the agarose gel was carefully removed and taken to the UV trans illuminator and the gel was examined under UV light of Bio Rad universal Hood II (Gel-Doc XR: 170-8170). Band intensities proportions between the Cbf14 bands and 18s rRNA bands were analysed using Image J software.

In some experiments, Real-Time PCR (RT-PCR), from Applied Bio system (StepOne Plus) was used. The quantitative PCR protocol used was according to manufacturer's instructions (SYBR<sup>®</sup> Green Jump Start<sup>™</sup> Taq Ready Mix<sup>™</sup>, sigma:

cat# S4438). The cDNA library was obtained as mentioned in (2.4.2), 1  $\mu$ L of cDNA was mixed with a master mix consisting of (for each sample):

- 10  $\mu$ L Syber green master mix.
- 0.5  $\mu$ L forward primer
- 0.5  $\mu$ L reverse primer
- 0.25  $\mu$ L reference dye
- 3.75 Molecular grade water (Sigma cat, w1754)

The sample was prepared in 5  $\mu$ L Molecular water and then transferred to 96 well plates (Applied Bio system: MicrAmp cat #: 4360954). Then the plate was sealed with optical adhesive covers (Applies Bio system). The plate was put PCR machine under the following thermal cycle:

- 1- Starting stage at 94°C for 10 minutes.
- 2- 40 cycle of (95°C for 15 sec followed by 60°C for 1minute.

Melting stage, this consisted of 95°C for 15 sec followed by 60°C for 1 min followed by 95°C for 15 sec. Finally the results obtained were analysed to get the relative quantitation of the expression of the gene of interest (*Cbf14*) against the endogenous control (18s rRNA).

### **2.4.3 cDNA sequencing**

Leaves samples from plants that were expected to have positive *Cbf14* expression as well as plant must not have it were used in order to yield cDNA of this gene. cDNA sequences of *Cbf14* detected and purified using a cleaning kit protocol (Qiagen, Cat. no. 28004) described as follows:



- 500  $\mu$ L of binding buffer was added and mixed with 100  $\mu$ L of each PCR product samples obtained using *Cbf14* primers.
- The mixtures were applied to high pure filter tubes and centrifuged at the maximum speed (13.000 xg) for 60 sec.
- The flow through was discarded and 500  $\mu$ L of washing buffer was added to each of both filters. The filters were centrifuged at the maximum speed for 60 sec.
- The flow through was discarded and 200  $\mu$ L of washing buffer was added to the filters. The filters were centrifuged at the maximum speed for 60 sec.
- The flow through was discarded and the filters were transferred to new collection tubes. 75  $\mu$ L of molecular pure water was added to each filter centrifuged at the maximum speed for 60 sec to get the purified PCR product.

The purified DNA was subjected to sequencing by Eurofins MWG Operon (Germany). Multiple nucleotide sequence alignment of *Cbf14* comparison between the sequences obtained using *Cbf14* primers (in different treatments) and with other cold induced genes sequences were carried out using ClustalW 2. EMBL-EBI (Larkin et al., 2007) and BLAST (NCBI). Following this a phylogenic tree was constructed using sequence information.

## **2.5 Protein extraction**

### **Extraction buffer preparation:**

The extraction buffer consisting of 100 mM potassium phosphate buffer (200 ml of 2 M KPO<sub>4</sub> stock solution was prepared by dissolving K<sub>2</sub>HPO<sub>4</sub> 63.2 g and KH<sub>2</sub>PO<sub>4</sub> 5.0 g in distilled water), pH 7.8, 5% β-Mercaptoethanol, 1 mM EDTA, 1% Triton X-100, 10% glycerol modified from (Ni et al., 1996).

### **Procedure**

Plant materials from the -80°C freezer were thawed on ice and placed in a mortar and pestle. 2 ml of extraction buffer (Ni et al., 1996) was added to 1 gram tissues and ground. 1 ml of the liquid grindate was transferred to a microfuge tube (1.5 ml) and placed on ice and the mortar and pestle were washed in preparation for the next sample. Centrifugation was carried at 14000 xg for 15 minutes at 4°C in refrigerated centrifuge (Heraeus: FresCo 21). The supernatant was pipetted into a new microfuge tube (1.5 ml) and centrifuged again for 10 minutes. Then the clear supernatant was distributed in aliquots and stored at -80°C until used.

#### **2.5.1 Total protein estimation of homogenate tissue**

The total protein content was evaluated using a BCA (bicinchoninic acid) assay kit (Pierce, product NO. 2161297A). Following the manufacturer's micro plate protocol; 200 µl of the working reagent (prepared by mixing BCA reagent A with B at of 50:1 part respectively) was added to 25 µl of samples. The plates were incubated at 37°C for 30 min then plates were allowed to cool to room temperature. Samples of protein concentrations were determined by calibration against standard curve where 2 mg/ml

bovine serum albumin (BSA) stock was used to prepare serial dilution of 0.025-2 mg/ml. All samples and standards were used in triplicate in 96 well plates and the absorbance was measured at 562 nm using a plate reader (Spectra Max, Molecular Devices, Sunnyvale CA.)

### **2.5.2 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS- PAGE) and Western blotting.**

Protein samples were separated according to their electrophoretic mobility (a function of the length of a polypeptide chain and its charge) using the SDS-PAGE technique. The concentration of 12 µg/µL of each sample was loaded in the gel wells. Therefore, samples were prepared in 100 µL, the concentrated samples were diluted with the extraction buffer in 50 µL, and the volume was completed to 100 µL using the loading buffer. The samples were heated up to 95°C for 5 minutes before loading them into the gel wells.

The main stacking gel of 14% acrylamide was prepared from the following reagents:

- 1- 4.67 ml from solution A (30% Acrylamide/bis-acrylamide).
- 2- 2.5 ml solution B (1.5 M Tris-CL, 0.4% SDS, PH8).
- 3- 2.83 ml distilled water.
- 4- 50 µL APS (Amonium Pre-Sulphate, 10%).
- 5- 5 µL TEMED (Tetramethylethylenediamine)

All these materials were mixed except the TEMED which was left to the end. Due to its quick solidification, it was added before pouring the gel into the vertical glass plates that fit in the acrylamide gel electrophoresis apparatus (Small mighty II, USA).

Then the stacking gel also was prepared using solution C (0.5 M Tris-CL, 0.4% SDS, PH6.8) instead of solution B as follow:

1. 0.67 ml solution A
2. 1 mL solution C
3. 2.3 ml distilled water
4. 30  $\mu$ L APS 10%
5. 5  $\mu$ L TEMED.

All these materials were mixed and poured on the top of the main gel and then comb was inserted directly in the stacking gel and left to solidify.

The gel was then run in the running buffer consisting of Trizma base (12 gm), Glycine (57.6 gm) and SDS (4 gm) in Bio-Rad system.

Some of the gels obtained from above were visualised by using coomassie blue staining solution. The dye was prepared by mixing 0.2% coomassie Brilliant blue R-250 with 30% Methanol and 10% Acetic acid. The dye was poured on the gel page in a suitable box and left on the shaker overnight. The page then was washed with destain solution consists of 40% (V/V) methanol and 10% Acetic acid for 2 or 3 times until it became clear, finally page was pictured.

Unstained gels were used for Western blotting and the proteins were transferred to a nitrocellulose membrane by electro blotting.

### **2.5.3 Western blotting (Immunoblot Analysis) for detection of COR15**

Before conducting western blotting, a transfer buffer was prepared and chilled at 4 °C for at least two hours. The transfer buffer contained 14.4 g/L glycine, 3.03 g/L Trizm base and 200 ml Methanol and the volume then made up to 1 Litre with d. H<sub>2</sub>O. The Bio-Rad procedure was followed for western blotting. The nitrocellulose membrane was soaked membrane in methanol for 15 seconds then H<sub>2</sub>O for 2

minutes then transfer buffer for at least 5 minutes. The apparatus of the western blotting was in the order of (- electrode sponge/filter paper/GEL page/membrane/filter paper/sponge + electrode). All were tightly clamped after making sure that there were no bubbles could be formed between the membrane and the gel. The sandwich was then submerged in the transfer buffer so that the gel was close to the negative electrode. The electrical field was 90 volt for 30-35 minutes. The membrane was then incubated in the blocking buffer consisting of PBST (Phosphate Buffer Saline+0.05% Tween) + 0.05 milk for one hour at room temperature. After the blocking process, the membrane was washed two times with PBST for 5 minutes each. The membrane was then hybridized with specific antibody for COR15a (Diluted at 1:1000) obtained from Michael Thomashow's lab (Michigan University, USA) in PBST solution and then put in the cold room (4°C) overnight. The membrane was then washed three times with PBST 10 minutes each. The membrane was incubated with the secondary antibody (abcam) that was diluted at 1:20,000 for 2 hours and then washed three times with PBST 10 minutes each. In order to visualise the bands of interest in the membrane, the chromogenic reaction was performed using a Bio-Rad kit. The reaction for each membrane consists of 1 mL from HRP-Colour buffer, 9 ml ddH<sub>2</sub>O, 60 µL reagent B and 2 ml Reagent A. Finally, the membrane was incubated with the developing solution for 5-10 minutes and the digital images were taken of the membrane.

## **2.6 Statistical Analysis**

Results are presented as means + standard error (s.e.). The statistical software Minitab v.16 was used for the analyses of data. Balanced analysis of variances (ANOVA) was performed and the means were compared using least significant difference (LSD) test. The probability table (Fisher & Frank, 1948) was used for determination of the significance level of the F test. Sigmaplot v. 12 was used in plotting all graphs. Correlation was also calculated between the field observation and the fertility ratio (chapter 4) using Minitab v. 16 software.

## **Chapter 3: Frost Hardiness of vegetative seedlings of wheat cultivars**

### **3.1 Introduction**

Frost hardiness level in winter cereals is especially temperature dependent (Fowler and Carles, 1979) and the difference in cold hardiness potentials among cultivars are apparent from the early stages of acclimation. Skinner and Garland-Campbell (2008) claimed that the ability of wheat plants to tolerate the frozen state for extended periods of time is crucial to staying alive during the hard winter months in Canada and the northern US. The ultimate measure of winter-hardiness of a cultivar is its survival (Skinner and Garland Campbell, 2008). However, field survival is not fit as a tool in selection because it is unreliable because of the variable winter severity leading to differential winter kill over years (Gusta et al., 2001, Skinner and Garland Campbell, 2008). Microgeographic environmental variation can also result in the plants throughout a field being exposed to a wide variation in temperature stress within a relatively small location and if the field temperatures are close the the thresholds for survival then patchy observations will be made in the field and whilst experimental design can help it cannot eliminate false positives being picked up (Fowler and Carles, 1979). Despite this caveat winter survival has been the most employed screening test for the genetic variation in cold hardiness of wheat and other cereal crops, but it has been suggested that laboratory determined frost hardiness is more consistent than just survival scoring in the field for cereals (Pomeroy and Fowler, 1973). This study aimed at determining for the first time the genetic level of frost resistance of Middle Eastern varieties brought to the UK from Iraq in comparison with a European winter wheat cultivar (cv. Claire) of known frost resistance. It is intended that this information will provide basic information for the subsequent studies on these cultivars.



### 3.2 Materials and Methods

Six varieties were used, five Iraqi wheats:

- 1- Abu-Ghariab.....winter wheat
- 2- IPA 99.....winter wheat
- 3- IPA 95.....winter wheat
- 4- Sham 6.....spring wheat
- 5- Fatah.....spring wheat

and one European wheat

- 6- Claire.....winter wheat

The experiment was carried out at Plymouth University during July 2009. The wheat cultivars were established in plastic trays (37 x 23 x 5 cm). Each tray contained three rows (12 plant each row) with two trays representing one replicate. The plants were raised outside during July and August when light levels were high and mean temperatures were optimal for growth. The acclimated group was sown two weeks before the non-acclimated one to ensure that all plants should be placed into frost chamber at the same growth stage (decimal growth stage GS Z<sub>1.4</sub>& Z<sub>2.2</sub>) when plants had established 3-4 leaves. A Complete Randomised design was used with three replicates. The number of plants was duplicated in order to attain two groups for each cultivar, one for acclimation (two weeks at 4 °C in cold store), and the other one for non-acclimation. The Target temperature of the cold store was not achieved but was found to be 1.5°C with an oscillation of +/- 1.0°C.

The total treatment design was as follows:

6 varieties x 2 acclimation treatments x 6 freezing temperatures x 3 replication (2 trays each)

A SANYO M533 freezing chamber was programmed to the following regime: 0, -2, -4, -6, -8, -10°C with a two hour hold at each test temperature. The plants were removed at the end of the two hours hold at each temperature and immediately placed at 4°C to defrost overnight. So -10°C treatment had experienced -2, -4, -6, -8°C and finally all trays were transferred to the field to recover for two weeks prior to damage score being collected table (2).

### **3.3 Results:**

Seedling wheat varieties were increasingly damaged as the temperature was lowered to  $-10^{\circ}\text{C}$  (Table 3 & 4). Analysis of variance indicated that there was a significant difference in the damage between varieties. All of the Middle Eastern varieties were damaged approximately to the same degree except Abu-Ghariab which was similar to the European cultivar Claire. All varieties demonstrated a tendency to become cold acclimated when they were incubated at  $4^{\circ}\text{C}$  with less damage after acclimation. Significantly, the non-acclimated plants were damaged at temperatures higher than the acclimated plants. The analysis showed a significant effect of the interaction between the genotypes and acclimation treatments indicating that varieties responded differently to acclimation. However, acclimation and temperature were correlated in their effect on seedling damage. The acclimated plants tolerated temperatures much lower than non-acclimated plants. There was no significant three-way interaction between factors.

**Table 3. The average of score damage to wheat cultivars in acclimation (CA) and non-acclimation (NA) state after exposure to negative temperatures.**

Varieties Temp. °C	Abu-Ghariab		Fatah		IPA 95		IPA 99		Sham 6		Claire		Mean
	NA	CA	NA	CA	NA	CA	NA	CA	NA	CA	NA	CA	
0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
-2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
-4	0.5	0.2	0.6	0.4	0.5	0.3	0.6	0.3	0.6	0.3	0.3	0.2	0.4
-6	2.0	0.7	1.8	1.0	1.6	1.1	1.6	0.9	1.6	1.1	1.2	1.1	1.3
-8	2.6	1.2	2.6	1.4	2.9	1.7	3.0	1.5	2.9	1.5	2.5	1.3	2.1
-10	3.0	2.9	3.0	2.9	3.0	3.0	3.0	2.9	3.0	2.9	3.0	2.9	3.0
Mean	1.3	0.8	1.3	0.9	1.3	1.0	1.4	0.9	1.3	1.0	1.2	0.9	R <sup>2</sup> = 97.66 %
Mean	1.1		1.1		1.2		1.2		1.1		1.0		

**Table 4. L.S.D values of study factors**

Variables	P value	L.S.D (0.05)
Varieties	0.027*	0.101
Temperature	0.000***	0.101
Acclimation	0.000***	0.058
Varieties × Temperature	0.787	N.S
Varieties × Acclimation	0.067*	0.143
Temperature × Acclimation	0.000***	0.143
Varieties × Temperature × Acclimation	0.422	N.S

\*-significant. \*\*-Very significant. \*\*\* -Highly significant. N.S-non-significant.

LT<sub>50</sub> (50% killing point) was determined from the logistic differential curves of the seedling mortality (Table 5). It was very clear that all varieties in the non-acclimated state had a LT<sub>50</sub> approximately at the same temperature but the acclimated plants showed different responses. Genotypes were different in their LT<sub>50</sub> with Abu-Ghariab and Claire cultivars were similar and showed significantly lower LT<sub>50</sub> temperatures than the other cultivars.

**Table 5. The LT<sub>50</sub>s for the varieties in acclimated and non-acclimated state (n=36).**

<b>cultivars</b>	<b>Type and origin</b>	<b>LT<sub>50</sub> Non-acclimated</b>	<b>LT<sub>50</sub> Acclimated</b>
Claire	WW European	-5.926 °C	-8.065 °C
Abu-Ghariab	WW Middle Eastern	-5.803 °C	-8.005 °C
Fatah	SW Middle Eastern	-5.774 °C	-7.266 °C
Sham 6	SW Middle Eastern	-5.640 °C	-7.1268 °C
IPA 95	SW Middle Eastern	-4.608 °C	-7.199 °C
IPA 99	SW Middle Eastern	-5.713 °C	-7.386 °C

### 3.4 Discussion

Differences in cold hardiness among the wheat cultivars were apparent from this experiment. For the non-acclimated plants, all cultivars, even the European cultivar (Claire), behaved similarly with small differences in their  $LT_{50}$  varying from only -4.6 to -5.9. This actually demonstrates that even in an un-acclimated state, wheat does demonstrate constitutive frost resistance down to about -5 to -6°C. This is consistent with previous reports (Fuller et al., 2007) when the cultivars were acclimated, all showed increased frost resistance but response to cold acclimation (CA) differed between the cultivars. The Iraqi cultivars with the exception of Abu-Ghariab typically only showed a 1 to 2°C improvement in  $LT_{50}$  and there were only small cultivar to cultivar differences which were not significant. Abu-Ghariab however responded to cold acclimation better than the rest of the cultivars and was more similar to the European winter wheat cultivar Claire. Both of these cultivars are classified as winter wheats and they are therefore expected to have a genetically greater ability to acclimate and this was upheld in this experiment. As shown in table (5) the  $LT_{50}$  for Claire in the non-acclimated state was -5.93°C which was almost identical to that previously quoted by Fuller et al (2007) (-5.95°C), but it differed by 1.45°C difference in  $LT_{50}$  when acclimated. The reasons for this difference are unclear but may reflect slightly different acclimation treatments.

Acclimation improved the  $LT_{50}$  by 0.493°C in Sham 6 while the improvement was 2.591°C in IPA 99 and the other wheat cultivars ranged between these two. Generally, there is no information in the literature regarding the frost hardiness levels of Iraqi wheat cultivars so this is new information. It was clear that the classified Iraqi spring wheats were less able to acclimate than the Iraqi winter wheat, demonstrating the restricted frost hardiness associated with the spring habit as described by

Christine and Law (1979). This is attributed to an associated negative selection when selecting for low vernalisation characteristics of the spring wheats. As has been shown (Sutka et al., 1999) frost resistance QTL's are closely linked to the VRN genes on chromosome 5 and this linkage is difficult to break so any selection for spring habit is likely to inadvertently counter select for less of frost resistance .

It is clear from the results that all cultivars had an  $LT_{50}$  in the non-acclimated state around  $-5^{\circ}\text{C}$  and considered as inherent constitutive moderate frost resistance (Fuller et al., 2007). Matthew (2005) mentioned that the herbaceous plants from temperate regions can tolerate temperature ranging from  $-5^{\circ}\text{C}$  to  $-30^{\circ}\text{C}$  depending on the species. One of the most extreme examples is rye plant which when grown at non-acclimating temperatures ( $\sim 22^{\circ}\text{C}$ ) is killed by  $-5^{\circ}\text{C}$  but when it has been acclimated is able to withstand temperature down to  $-30^{\circ}\text{C}$ . Within the wheats there is great variation in frost resistance with  $LT_{50}$ 's from  $-5$  to  $-25^{\circ}\text{C}$  with the more resistant varieties selected for severe winter climate zones in continental America and continental Europe. Maritime wheat varieties require less frost resistance and typically, UK wheats only show moderate frost resistance as demonstrated here by the cultivar Claire. It is interesting to note that the Iraqi winter wheat tested (Abu-Ghariab) showed the same moderate frost resistance as Claire despite being selected under a totally different set of environmental conditions.

Frost resistant cultivars reportedly harden faster and deharden more slowly than the susceptible genotypes because the acclimation processes have different threshold induction temperatures differing them in cold tolerance (Gusta et al., 1979). It was not possible to test this in the current experiment but it is a useful characteristic for wheat to possess. There is also potentially a difference among cultivars regarding the threshold temperatures (above  $0^{\circ}\text{C}$ ) at which acclimation is initiated and

threshold induction temperatures for cold acclimation in wheat, barley and rye have been reported to be positively correlated with frost tolerance between and within species (Fowler, 2008). The genotypes under study here might have varied in their frost tolerance according to differences in threshold induction temperatures for acclimation but it is anticipated that the temperature used, +1.5°C, should have up-regulated acclimation in all cultivars. Acclimation has also been reported to proceed in two stages in cereals, depending on the sequential action of chilling (> 0°C) and sub-lethal freezing (-1 to -5°C) temperatures. Cold acclimation requires energy to be maintained and with seedlings this energy is supplied by seed reserves or by photosynthesis (Fowler and Limin, 2007, Limin and Fowler, 1985). The development and maintenance of available carbohydrates supply with retarded vegetative growth is essential for cold temperature acclimation of wheat plants to occur efficiently. This observation has led to the suggestion that acclimated and non-acclimated cultivars with high growth activity at low temperature might be reflecting an inability to acclimate in autumn. Under controlled experimental conditions, it is necessary that sufficient light for photosynthesis is provided during acclimation (Gabriella et al., 2009) and the levels used in this experiment were within the range that can stimulate photosynthesis during acclimation.

It can be concluded from the findings of this study, that all Middle Eastern varieties tested showed very similar  $LT_{50}$ 's in the non-acclimated state and this demonstrated that they have an innate ability to survive freezing temperatures down to approximately -4 to -5°C similar to European and North American wheats. Following acclimation, all varieties showed significant improvement in their response to freezing but the degree of response varied among the cultivars. Abu-Ghariab and Claire both showed the best response to acclimation commensurate with their winter wheat



status whilst the remaining cultivars were much more like spring wheats. Both of Claire and Abu-Ghariab cultivars were used in the next experiments and the others were excluded.

## **Chapter 4: Characterisation of the frost resistance of wheat during spike emergence**

## 4.1 Introduction

In Australia particularly and other regions in the world such as subtropical zones and the Mediterranean, wheat can be exposed to frost during spike emergence and can be damaged (Fuller et al., 2007). During late developmental stages both male and female reproductive organs are sensitive to frost and this frost damage reduces wheat yield production in parts of Australia by up to 40% in some years (Single, 1985). The economic losses come directly from sporadic frosting weather after spike emergence in wheat and barley (Frederiks et al., 2012), because of the rapid progression from relatively resistant vegetative stages to the susceptible reproductive stages (Single, 1984). In Iraq, during the crop season 1979-1980, frosty weather prevailed in the northern parts (Nineveh governorate, which grows 50% and 25% of the country's wheat and barley area respectively) when cereals were in the late flowering to early dough stage. The damage recorded was of the head frosting type and whole or parts of spikes turned white colour and failed to develop further. During this season an average visual estimate of the damage was placed at 50% grain loss (Tarik, 1981). Zhong et al (2008) reported that the probability of frost damage to wheat in Iraq was nearly 40% in the 1970s, rising about 50% in 1980s, and 78% in 1990s. Damage occurs when low temperature coincides with sensitive plant growth stages, due to the greater vulnerability of the male and female organs and also to the more exposed position of the growing point (Single, 1985). Crop damage at this time could be caused by decreased photosynthesis (Zhong et al., 2008), leaf death or stem damage (Shroyer et al., 1995). Also spike death or malformed spike formation has been reported (Zhong et al., 2008). Single (1985) reported that although many studies have been held by numerous investigators for more than a century, there is still no clear picture of wheat frost resistance during flowering and little genetic

progress has been gained. In the 25 years since this statement there has still been remarkably little progress, either genetically or in the physiological understanding of the problem. The freezing damage in plant tissue is probably a physical process which can be moderated by factors such as plant developmental stage (Single, 1964), availability of ice nucleators and temperature (Lindow et al., 1982). Furthermore, the physical processes occurring during frost damage in wheat during late developmental stages are still not completely characterised. This problem is occurring in areas where the drought and the summer heat restrict the main growing season of wheat to the late winter and spring. In such areas, the daytime temperature is ideal for growth but the temperature at night might fall to damaging levels. Therefore, the aim of the current study was to focus on frost resistance in wheat during reproductive stages. It was a trial to examine and characterise the frost damage and ice nucleation in spikes in two wheat varieties. Due to the unpredictable nature of frost events in terms of timing and severity, controlled environments were used to achieve the aims of this investigation.

## 4.2 Materials and Methods

### 4.2.1 The effect of frost damage to fertility of wheat

36 pots were used in this study each pot containing 16 plants (213 plants. m<sup>-2</sup>) of wheat cv. Claire. Three spike growth stages (A: Early spike emergence, B: Mid spike emergence, and C: Late spike emergence) (Table 6) were chosen to represent the full development stages of wheat spike production (12 pots each). In these 12 pots, coloured wires were used to mark each growth stage, because not all culms on one pot at the same growth stage. At treatment time pots were dug out of the bed and then transferred either to a cold store or frost chamber.

On May 21<sup>st</sup> 2009, the first growth stage was identified and the allocated 12 pots were randomly assigned to the 4 treatment groups, each group comprising 3 pots.

**Table 6. Zadoks scale for cereals (Zadoks et al., 1974).**

<b>Growth Stage</b>	<b>Zadoks scale (Zxx)</b>	<b>Description</b>
A	Z51-Z60	From: 10% of spike visible (spike peep) to: whole spike visible
B	Z61-Z69	From: early 20% spikes with anthers to: late 90% of spikes with anthers
C	Z70.2-Z79	From: Kernels middle spike extended 20% to: very late milk, half solid in milk

Four treatments were applied to each of the three growth stages pots:

**T1:** 3 pots stayed in field (control – non-acclimated and non-frozen).

**T2:** 3 pots directly transferred to the frost chamber (non-acclimated) and exposed to freezing regime.

**T3:** 3 pots transferred to the cold store at 4°C for 14 days and then returned back to the field (acclimated but not frozen).

**T4:** 3 pots transferred to cold store at 4°C for 14 days and then to the frost chamber (acclimated and frozen) using the same freezing regime which was applied to T2.

On June 4<sup>th</sup> 2009 the same treatments were repeated on the second growth stage plants.

Two further weeks on the third growth stage, visual observations of the plants were taken 14 days after each growth stage being treated. Plants were then grown on to maturity; the spikes were collected from the field and stored for later analysis.

At treatment for each individual spike, the spikelets per spike were counted, the spike threshed, and the grain number counted. The mean number of grains per spikelet was then calculated (fertility ratio):

$$Fertility\ ratio = \frac{Grains}{Spikelets} \text{ (Scott and Langer, 1977)}$$

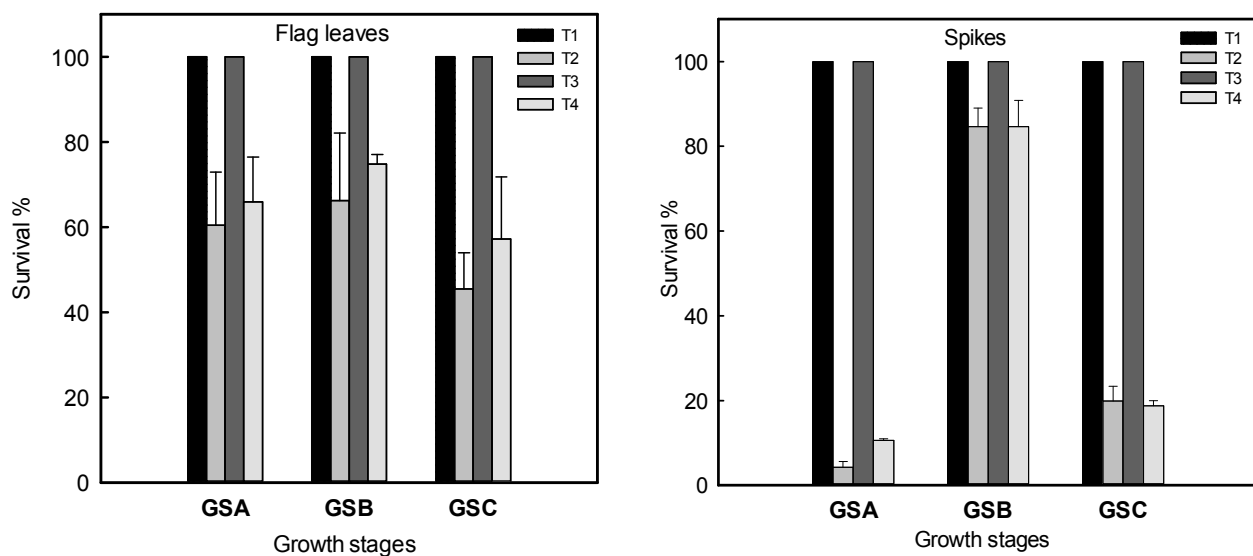
#### **4.2.2 Frost damage (REC %) with isolated flag leaves and spikes of European and Iraqi wheat after spike emergence.**

Two cultivars were used: a European winter wheat (cv. Claire) and Iraqi winter wheat (cv. Abu-Ghariab). The genotypes were sown each in its season in pots (30 × 30 × 25 cm) filled up with compost (John Innes: seed sowing compost, manufactured by Westland Ltd). The pots were buried in a raised bed outside at Portland square. Flag Leaves and flag spikes were cut and placed in test tubes, and then they underwent the freezing regime (chapter 2, 2.2.2.).

### **4.3 Results:**

#### **4.3.1 Fertility ratio**

Visual observations after 14 days recovery from treatments were taken as proportional damage (%) to the whole tissue. Data in the figure (5) showed that there were no difference between plants grown in the field (T1) and plants that were acclimated (T3), while there were considerable difference between acclimated plant (T3) and acclimated-frozen plants (T4). This was the case for both flag leaves and spikes, and in all growth stages under study. Frost damage was not significant between growth stages for leaves regardless of the treatments, whereas the spikes showed significant damage specially GSA & C where the survival percent was significantly lower than GSB (Figure 5).



**Figure 5. The visual observation of plant organs (flag leaves and spikes) damage after 14-day recovery at three growth stages (GSA, GSB and GSC) for European wheat (Claire)**

Plants were left to grow and set seeds until maturity and at harvest time spikes were collected from each treatment and the fertility ratio was as calculated (Table 7). There was considerable variation in fertility ratio because of the freezing regime. The non-acclimated/unfrozen control (T1) showed a fertility ratio of around 2.5 grains per spikelet equating to approximately 50 grains per spike, which is typical for European winter wheat (Scott and Langer 1977) (Table 7). Exposure to acclimation (4°C and 8 h light) for 14 days during flowering (T3) had a slight negative effect on fertility ratio, which was most noticeable at GSB when the plants were closest to anthesis, and the mean ambient temperature in the field prior to acclimation was around 13°C



according to Plymouth university weather station. When the plants were exposed to sub-zero treatment (T2 and T4), then fertility was drastically reduced indicating a big effect of freezing. However, fertility was not reduced to zero and observations during threshing showed that spikes contained mostly spikelets with zero grains but with a few fully fertile spikelets (Plate 1).

**Table 7. The effect of treatments on the fertility ratio (grain number/spikelet) maturation of wheat cv. Claire.**

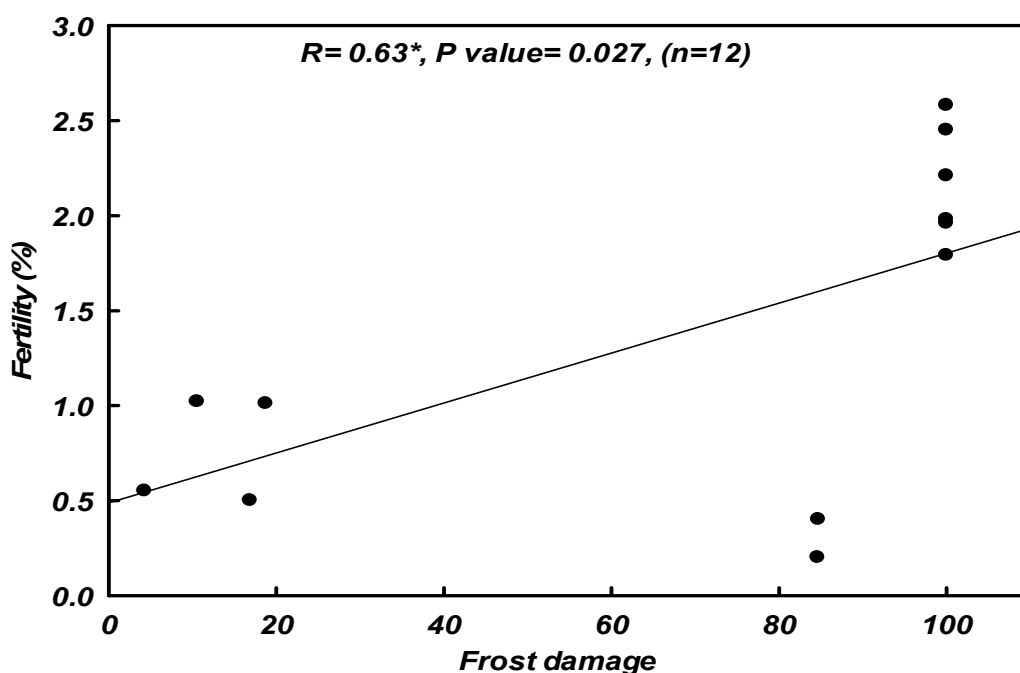
Treatments		No. Of spikes	Grains no/spikelet no.	SE
T1	GSA	64	2.41	0.03
	GSB	38	2.42	0.05
	GSC	44	2.58	0.04
T2	GSA	63	0.46	0.03
	GSB	75	0.16	0.03
	GSC	88	0.51	0.05
T3	GSA	55	2.25	0.02
	GSB	54	2.20	0.05
	GSC	29	1.96	0.10
T4	GSA	69	1.03	0.06
	GSB	82	0.40	0.01
	GSC	69	1.01	0.10

These observations suggest that the frost damage was not distributed evenly across the spike and that some spikelets appeared to escape frost damage completely (Plate 1). The results showed that the acclimation process only had a small positive effect within each growth stage with the acclimated and frozen treatment (T4) showing slightly higher fertility ratio than their non-acclimated and frozen equivalents (T2).



**Plate 1. Photographs of spikes to show the degree of damage caused by frost; completely infertile spikes (GSA-T3 and GSB-T2) and partially infertile spikes. (Growth stage = GSA/B; T = Treatment 2/3/4 (see text for detail).**

However, sometime the damage may not be at the level that can be visualized, and it is hard to be taken as a measurement to frost damage in the field. In this experiment as we used artificial freezing, therefore the field observation was an indicator for the frost damage. However, the field observation in this experiment was generally correlated with the fertility ratio at harvest ( $R= 0.63^*$ ,  $P\ value= 0.027$ ) for all treatments (Figure 6). But non-significant association between the field observation and fertility ratio might be found in each treatment individually, since the survival rate was very high in spikes GSB at (T2 and T4) (Figure 5 Spike) while the fertility ratio was significantly lower comparing with other growth stages and the rest of treatments (Figure 6).



**Figure 6.** The correlation between the frost damage as observed in the field after two weeks recovery and the fertility ratio at harvest for wheat cv. Claire (\*Correlation is significant at 0.05 level and 2-tailed).

#### **4.3.2. Frost damage (REC %) of flag leaves and spikes of winter and spring wheat genotypes after spike emergence.**

Wheat in a vegetative stage as well as in reproductive stage can inherently tolerate low temperatures down to about  $-5\text{ }^{\circ}\text{C}$  (Fuller et al., 2007). Data from the logistic curve presented in table 8 showed that wheat from both spring and winter wheat is able to tolerate low temperatures whether it is grown in the field or in a growth cabinet ( $20^{\circ}\text{C}$ ) at late developmental stage from  $-4$  to  $-5^{\circ}\text{C}$ . However, as consequences of exposing the plant to acclimating temperatures the ability of wheat to respond to low temperatures becomes much lower than vegetative plants. The  $\text{LT}_{50}$  in acclimated winter wheat has been decreased  $1.86^{\circ}\text{C}$  (Table 8) after spike emergence directly (e.g. GSA); while in vegetative winter wheat the acclimation enhanced the  $\text{LT}_{50}$  for  $2.13\text{ }^{\circ}\text{C}$  (Table 5 chapter 3). The enhancement was trivial in late developmental stage e.g. GSB and GSC, where the  $\text{LT}_{50}$  was changed by  $1.40$  and  $0.89^{\circ}\text{C}$  respectively.

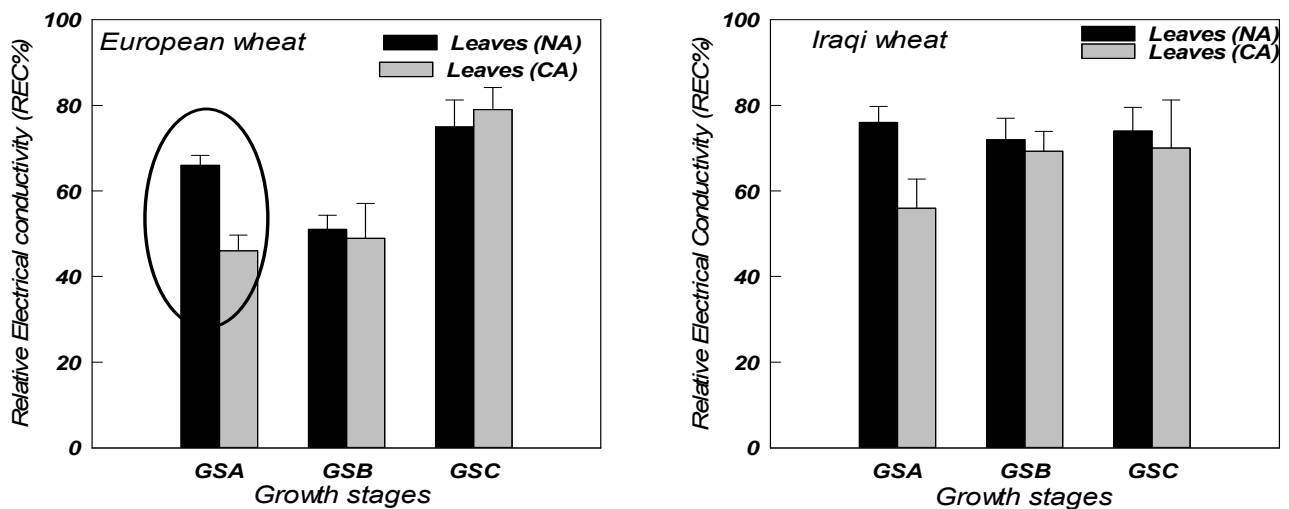
**Table 8. LT50 for wheat genotypes flag leaves from field, growth cabinet condition and acclimated plant (cold store)**

Wheat genotype growth stages		Untreated	Non acclimated (14-days)	Acclimated (14-days)	$\Delta$ LT50 due to acclimation
Claire	GSA	-5.08	-5.61	-7.47	1.86
	GSB	-4.86	-5.41	-6.81	1.40
	GSC	-4.41	-4.71	-5.60	0.89
Abu-Ghariab	GSA	-4.99	-4.03	-6.14	2.11
	GSB	-5.97	-4.03	-5.74	1.71
	GSC	-5.04	-4.47	-4.40	0.07

It is clear from the above data that Abu-Ghariab behaved the same as Claire in responding to the acclimation process. However, the  $LT_{50}$  had increased by 2.11°C in acclimating plant in GSA compared with 1.86°C (Table 8) in winter wheat at the same stage. Nevertheless, wheat was more tolerant to low temperatures under normal conditions (field and growth cabinet condition), since the  $LT_{50s}$  of spring wheat were -4.41 and -4.03°C whereas there were -5.08 and -5.61 in winter wheat for the field and growth cabinet respectively (Figure 7 and Table 9). The  $LT_{50}$  in the rest of growth stages (e.g. GSB and GSC) spring wheat likewise winter wheat has not been very well enhanced. Statistically this result was confirmed in table (9) and figure (7) where the acclimation significantly reduced the frost damage in GSA in the case of winter wheat (46.04%), nonetheless the reduction was not significant in the case of spring wheat (56.27%). Whereas wheat in late developmental stages, e.g. GSB and GSC, do not show any response to the acclimating temperature that have been imposed to plants from both genotypes.

**Table 9. ANOVA analysis of the interaction between growth stages and acclimation treatments in Claire and Abu-Ghariab wheat. (Means with different letters are significantly different, \* significant at 0.05 ANOVA)**

Genotypes	Treatments	GSS	REC%	SE	P-value (GSS×Treatment)
Claire	NA	GSA	66.23 <sup>a</sup>	2.29	0.003* (n=12)
		GSB	51.47 <sup>a</sup>	3.365	
		GSC	75.14 <sup>a</sup>	6.24	
	CA	GSA	46.04 <sup>b</sup>	3.69	
		GSB	49.07 <sup>a</sup>	8.09	
		GSC	79.17 <sup>a</sup>	5.20	
Abu-ghariab	NA	GSA	76.87 <sup>a</sup>	3.75	0.269 (n=12)
		GSB	72.85 <sup>a</sup>	5.01	
		GSC	74.13 <sup>a</sup>	5.53	
	CA	GSA	56.27 <sup>a</sup>	6.79	
		GSB	69.35 <sup>a</sup>	4.66	
		GSC	70.01 <sup>a</sup>	11.23	



**Figure 7. Frost damage (REC%) to leaves of European and Iraqi wheat for both acclimated (CA) and non-acclimated (NA) plants for three growth stages (GSA, GSB, GSB) (n=3, means + se).**

Unlike the response of the leaves in wheat, the spikes showed frost damage considerably less than leaves. However, the  $LT_{50s}$  presented in table (10) are very low compared with the  $LT_{50s}$  in leaves (Table 8). It is clear from this data that all growth stages in both genotypes and under the two treatments (NA & CA) are mostly close to each other.

**Table 10.  $LT_{50}$  for wheat genotypes spikes from field, growth cabinet condition and acclimated plant (cold store)**

Wheat genotypes growth stages		Untreated	Non acclimated (14-days)	Acclimated (14-days)
Claire	GSA	-8.78	-8.47	-9.09
	GSB	-9.05	-10.06	-12.55
	GSC	-9.36	-6.88	-4.20
Abu Gharaiab	GSA	-8.81	-5.53	-9.04
	GSB	-8.62	-9.49	-9.27
	GSC	-8.24	-9.41	-8.44

It seems that there is no response to the low temperature in the spikes, since all growth stages were damaged at the same range of freezing temperatures. Furthermore, from the data presented above there was no correlation between the frost damage in leaves and spikes. It might not be a good idea to rely on the REC% measurements in the spikes since there is no clear response to low temperatures. Both genotypes in general, have the same trend towards frost damage during spike emergence either in flag leaves or in spikes. Although there is no overall difference in acclimation treatments over the genotypes, there was a huge difference between flag leaves and spikes, where the frost damage was much higher in leaves.

#### **4.4 Discussion.**

Based on the relative electrical conductivity (REC %) method, wheat flag leaves showed similar level of hardness ( $LT_{50}$ ) to vegetative seedlings in both Iraqi and European wheat varieties. This suggests that the REC % method is suitable for testing frost damage in wheat leaves and producing a survival rating ( $LT_{50}$ ). This is in contrast with REC % in spikes which indicated high frost hardness (i.e. low REC %) but damage was still evident. It was concluded that the REC % method is not a suitable method to investigate frost damage in spikes.

As wheat has spring and winter growth habits, it has to have a wide range of frost tolerance. Wheat genotypes are most susceptible to frost damage at the reproductive stage. This susceptibility, in specific, is from spike emergence (e.g. GSA) until grain filling stages of development (e.g. GSB & C). The temperature that causes damage to spikes is unpredictable and its duration length is unknown, but it might correlate to the potential damage symptoms seen. Normally, symptoms of frost damage are developed 7-14 days after damaging events (Frederiks et al., 2012). Frost damage during spike emergence (e.g. anthesis) in wheat is the main reason for the reduction in grain set and eventually the yield.

One of the observations in the field was that some spikes were kept inside their boots and they could not normally emerge due to the growth slowdown caused by low temperature. Another observation was discolouration where the spikes' colour turned to yellowish instead of green. Such spikes were then aborted. The most important stage at which the low temperature can cause catastrophic economic losses is the anthesis stage (GSB). It has been reported that male and female organs are the most sensitive parts to low temperature (Single, 1984, Single, 1985, Zhong et al., 2008). Therefore; if the low temperature coincides at this stage the economic losses



will be very high, due to the sensitivity of reproductive organs. Moreover, wheat spikes and its parts will be more vulnerable to the nature (Zhong et al., 2008). When freezing temperatures are imposed on wheat during anthesis the male organ of the flowers will be killed and will reduce the fertility (Shroyer et al., 1995). It is thought that the male parts of the flowers are the first organs that can be affected because wheat is self-pollinated (Shroyer et al., 1995). Therefore, sterility caused by freezing temperatures marks in poor kernel set and eventually reduces the grain yield.

Flowering in wheat starts from the centre of spikes and progresses for both top and bottom and lasts quickly within 5 days (Herbek and Lee, 2009). Therefore, whenever low temperature is imposed, full or partial sterility occurs (Plate 1) either in the centre or one of the two terminals of the spike. The grain will be set however because the flowering had not started or was already developed in those frozen spikes.

As shown in plate 1 there some spikes that were not damaged even in freezing treatments (e.g. T2 & 4). Therefore some florets can escape frost events even when plants are subjected to sub-zero temperatures ( $-6^{\circ}\text{C}$ ). However, it has been reported that plants have the ability to be supercooled during artificial frost (Fuller et al., 2009), thus some frozen spikes might not be damaged. For that reason, no frost damage will be experienced (see chapter 5)

At the molecular genetics level, many wheat genotypes at the maximum level of frost hardiness are still very sensitive to low temperatures at the late developmental stages. Therefore, the implementations of the genetic approach to enhance frost tolerance in wheat are very complex and difficult (Rizaldi et al., 1993) (see chapter 6).

It seems that winter and spring wheat became less hardy in early and late winter and in early spring, which increases frost damage. During the flowering stage and the

start of grain growth in wheat, frost damage reduces the number of grains per spike. Therefore the visual observation results are correlated with the future yield reduction ( $R=0.63$ ,  $P$  value  $=0.027$   $n=12$ , figure 6) as the fertility ratio was reduced due to the low temperature effect. The chilling effect was clear between T1 and T3 but in the field, it was difficult to recognise the difference between acclimated (T3) and acclimated-frozen plants (T4). However, wheat might respond to the effect of low temperature at the molecular level, as will be explained in chapter 6.

Frost chambers, sometimes offer an efficient method to investigate frost damage in plants, because of the unpredictability of the frost in nature in terms of the occurring of frost especially during flowering and the intensity. In addition, this offers precise control of it and allows for study of frost events at any time of the year (Fuller and Le Grice, 1998, Frederiks et al., 2012).

Based on that, the response of wheat is different between vegetative and reproductive stages (Fuller et al., 2007), and frost damage was investigated in both flag leaves and spikes of winter and spring wheat. The field growing and the non-acclimated winter and spring wheat were tolerant to low temperatures down to  $-4$  to  $-5$  °C, as proved in Fuller et al (2007) where it was stated that all wheat genotypes inherently can tolerate temperature of  $-5$ °C but not in temperate crops which can only tolerate low temperatures in the range of  $-1$  to  $-2$ °C as inherent frost tolerance in non-acclimation plants (Fuller, 2002).

The results in table 8 showed that both wheat genotypes still have the same constitutive tolerance to low temperature stress even after spike emergence and in all spike growth stages. However, not all spike growth stages responded to the following acclimation ( $4$ °C). The acclimation enhanced wheat tolerance to low temperature in early spike growth stages (GSA) in both genotypes since the  $LT_{50}$  was

increased by 1.86 and 2.11°C in winter and spring wheat respectively. The enhancement was gradually decreased in GSB & C in both varieties confirming that wheat has limited ability to cold acclimate after heading (Galiba et al., 2009b). In addition, there were some evidences that wheat genotypes can be acclimated after the transition from vegetative to reproductive stage but before heading proving that this transition is not the off switch of cold tolerance genes (Fowler, 2007, Al-Issawi et al., 2012).

Statistically it has been found that winter wheat directly after spike emergence (GSA) can be significantly ( $P$  value= 0.003) cold acclimated since the frost damage was less compared with late spike growth stages (Table 9 and figure 7). The acclimation trend in winter and spring wheat was at the same direction but not significant as in winter wheat especially at GSA. Fuller et al (2007) stated that the acclimation enhanced the frost tolerance in cv. Clair but not in spring wheat (cv. Hartog). Despite the fact that Ohno et al (2001) and Kume et al (2005) have reported that long term of acclimation has increased the transcripts level of cold tolerance genes in winter wheat compared with the spring type, therefore this expression profile was then correlated with the frost tolerance at physiological level.

Frost damage to wheat genotypes during spike emergence typically happened and was easily identified (Figure 5, table 7 and plate 1). There were some missing grains from spikes because of the effect of low temperature on the fertility of spikes flowers. As wheat spikes progress in growth, low temperature may cause death of formed grains or even reduce the grain weight (Cromey et al., 1998, Single, 1984). Despite the presence of the severe impact of the low temperature to wheat during flowering in terms of spikes, the opposite has been found when the method of the Relative of Electrical Conductivity was used. It is clear from the result presented in table 11 that

there was no response to acclimation in terms of wheat formed seeds. However, the  $LT_{50}$  for the plant growing either in the field or in the laboratory were mostly higher comparing with their counterparts in leaves. However, despite all this survival of low temperature according to the EC, there was a huge loss of the yield due to the freezing damage. It can be concluded that the method of EC is not suitable to study frost damage in wheat grains and these economic losses belong to the sensitivity of reproductive organs in the spikes. In addition, the effect of freezing damage to spikes might be useful to study the effect of low temperature on the seed structure and its quality, which is not the interest of this work.

As a whole, wheat genotypes at the flowering stage are different in their response to acclimated temperature from the vegetative stage. However, it has been reported that during flowering wheat can only tolerate low temperatures to inherent level, which has also been reported to be  $-5^{\circ}\text{C}$  (Mahfoozi et al., 2001, Prasil, 2004, Fuller et al., 2007, Galiba et al., 2009), proposing that the vernalisation genes act as an off switch for the frost tolerance genes. Ilija et al (2004) stated that when the vernalisation requirements were fulfilled, winter wheat (as in spring wheat) established only a low level of frost tolerance. Nevertheless, Galiba et al (1995) and Sutka (2001) have stated that there was strong linkage between vernalisation and frost tolerance in wheat. In an attempt to separate between frost tolerance development and vernalisation requirements, Båga et al (2007) have confirmed that there is linkage between the two traits, but also found that only proportion of the frost acclimation is affected by vernalisation. Fowler (2007) also has stated that either spring or winter wheat can be acclimated after reproductive transition but before heading, thus it been demonstrated that this transition does not act as an off switch for the frost tolerance genes.

**Chapter 5: Observations of ice nucleation and spread in spikes  
using infrared video thermography**

## 5.1 introduction

Freezing can be detected on the basis of the physical changes in opacity, latent heat release, expansion in volume or the changes in the relative electrical conductivity that go along with the phase change of water from liquid to ice (Lee et al., 1995). The signal of these physical changes is approximately proportional to the amount of ice formed in a certain period and can also be measured by the latent heat release. Infrared thermal imaging, which is based on detecting and visualising the infrared heat released from objects can be valuable in determining the ice nucleation point in the freezing process and can monitor how ice progresses throughout the tissue of plants (Wisniewski et al., 2003). The technique of Infrared video Thermography (IRVT) has advantages over other methods in that it can be used in ice nucleation studies with plants as a non-invasive and non-perturbing technique. IRVT offers live direct visualisation of the freezing process *in-planta* either in the field or in the laboratory. By using this method no thermocouples need to be attached to the plant and therefore there are no influences of these attachments on the freezing process (Pearce and Fuller, 2001, Pearce, 2001, Wisniewski et al., 2003), as it has been shown that thermocouples could themselves induce ice formation in plants (Fuller and Wisniewski, 1998). IRVT can be used in controlled environments where it has been used to evaluate ice nucleation and propagation directly on and in plants (Wisniewski et al., 1997). IRVT can be used in woody plants e.g. apple, peach, pear trees (Wisniewski et al., 1997) and gymnosperms (Hacker and Neuner, 2007). Even though it has been used in a very wide range of woody plant to investigate how ice propagates in the xylem elements it still needs deep explanation because of the lack of direct measurements (Pearce and Fuller, 2001). In addition it can be used to monitor field crops e.g. bean, cranberry, tomato and potato (Wisniewski et al., 2003,

Fuller and Wisniewski, 1998, Chaerle et al., 2003, Wisniewski et al., 2002, Wisniewski et al., 2002) where it has been possible to monitor whole plants either inside a frost chamber or in the field. Moreover, and relevant to the current study it has been shown to be useful as a technique in cereal crops including barley (Pearce and Fuller, 2001) and wheat (Al-Issawi et al., 2012, Fuller et al., 2009, Fuller et al., 2007). IRVT has the advantage that it can give an idea about the first location at which ice is formed and how it initially spreads (Pearce, 2001) and it has been used extensively to understand frost damage in plants including where and what particular tissue the initiation of the ice nucleation begins and how it spreads through that tissue (Wisniewski et al., 1997, Hacker and Neuner, 2007, Fuller et al., 2007, Fuller and Wisniewski, 1998, Pearce and Fuller, 2001). This has led to speculation how possible damage may be characterised.

Some plants or their parts remain unfrozen even when the temperature goes down below 0 °C and this can depend on various factors that induce ice formation and its spread. This state, referred to as supercooling, is a mechanism by which plants or plant parts can avoid ice nucleation even when they are exposed to freezing temperatures. However, when ice eventually forms in deeply supercooled cells severe injury is usually experienced compared to when ice crystals are formed at warmer temperatures and allowed to grow slowly. When ice forms more slowly at warmer sub-zero temperatures it gives the plant cells a chance to equilibrate their water potential and avoid dramatic changes. In addition, the crystals will be smaller when formed slowly. With a low rate of ice formation, water will have the chance to move slowly out from cells and the ice will be formed between cells instead of inside them. In contrast, sudden ice formation will cause a large water potential gradient which can rupture the plasmalemma and cause physical damage to cells. A

knowledge of how ice is being formed and how it progresses inside plants, tissues or cells will help in developing an insight into how the ice could be avoided either genetically or mechanically (Wisniewski et al., 2002, Wisniewski et al., 2002). Plants do not normally freeze until a few degrees below 0°C and the extent of supercooling is dependent on the presence of ice nucleators e.g. surface proteins on *Pseudomonas syringae*, that can be associated epiphytically with plants and catalyses crystallisation. Ice nucleator activity occurs at similar temperatures that cause damage to sensitive plants at about -2 to -5°C (Lindow et al., 1982, Ashworth, 2010). As ice nucleators can be manipulated they might significantly be used in limiting freezing damage in plants or in parts of the plants (Wisniewski et al., 2002). Ice nucleators can also be used to facilitate ice formation in frost damage studies (Wisniewski et al., 1997, Fuller et al., 2007, Fuller et al., 2009, Ashworth, 2010, Pearce and Fuller, 2001). The IRVT technique has been used to monitor ice formation in wheat spikes in both the field and in controlled environment (Fuller et al., 2009, Fuller et al., 2007, Al-Issawi et al., 2012). Ice formation is a very complex process in wheat spikes compared to leaves. The complexity comes from the complex morphology of a spike and the presence of nodes along the spike, which can restrict ice travel. In the current study IRVT was used to study the frost damage in spikes and the aims of this study were to determine the start point of the ice nucleation, how it progresses throughout the spikes and the time it takes to be completely frozen. The aims of this study were achieved by monitoring the ice nucleation live on plants inside a frost chamber by the use of an IR camera. The time and the temperature of the ice nucleation as well as the pattern of ice progression are the main interest of these investigations.



## 5.2 Materials and Methods

The infrared video thermography technique was applied using a digital infrared camera (ThermaCAMS65: FLIR system) in order to monitor plants inside a frost chamber while the temperature was gradually lowered below zero. The thermal sensitivity of this camera was 0.08°C and had a 76000-pixel display, which provides extremely high resolution measurements in real time. In addition, it has video imaging rate of 60 Hz, which allows fast inspection of moving objects without image smear and therefore, it could be used to detect and monitor rapidly moving temperature changes and could even be used in the field in windy conditions.

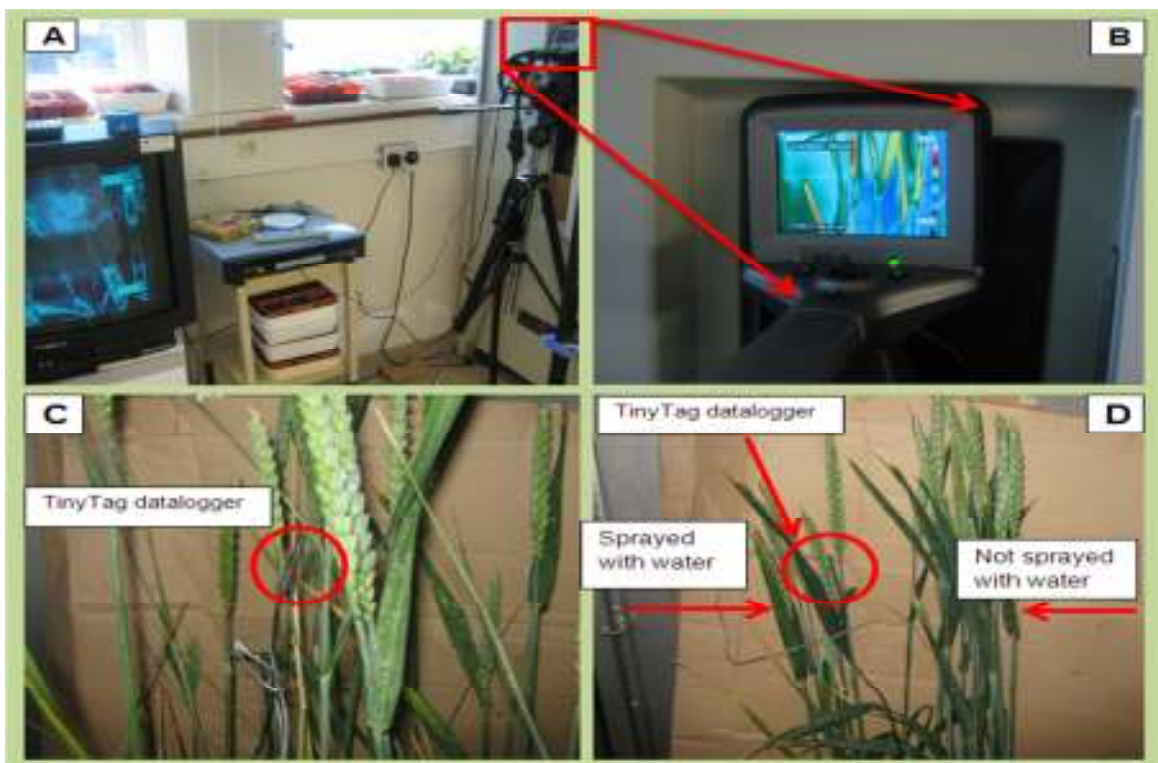
Convective freezing was used in a frost chamber (SAYNO M533 Incubator) prepared specifically for this purpose using pre-programmed set temperature where the plant would be exposed to different levels of temperatures ( $\leq 0^{\circ}\text{C}$ ) (Fuller and Le Grice, 1998, Pearce and Fuller, 2001).

Wheat plants were grown outside in pots buried in a raised bed prepared specifically for this purpose (see chapter 2). There were 16 plants in each pot and these were left to grow in the field until spikes emergence when some plants were moved the acclimation chamber at 4°C for two weeks and then used in this study together with plants which had been left growing in the field.

A single pot was used in each run in the frost chamber and 13 runs were undertaken. The temperature was recorded inside the frost chamber using a datalogger (Gemini: TinyTag) (Plate 2 C & D). The camera was set up in front of the frost chamber on a tripod with the lens positioned to peer through a hole cut in the plexiglass front of the chamber. It was connected to a DVD recorder and to a video monitor (Plate 2). In order to reduce the reflection interaction of the polished metal interior of the frost chamber, a piece of cardboard was used as a neutral background to the plants with an emissivity close to 1.0 (Plate 2). In some runs half of the plants

from acclimated (CA) and non-acclimated (NA) treatments were sprayed with water in order to facilitate ice formation.

Data was collected by watching each DVD individually and recording the observations. Data included the total number of spikes in each run and the number of frozen spikes in order to get the per cent of freezing in each run. The temperature at which ice started to be nucleated also was recorded according the colour bar in the right side of each picture. The time taken from ice nucleation to the complete freezing of the spike was also recorded. Still pictures were taken directly from the recorded DVD for illustrative purposes.



**Plate 2. Photographs to show the infrared video thermography study set-up. A & B: the camera set up in front of the chamber and its connection with the DVD recorder and TV. C & D: pots with plants inside the frost chamber, datalogger was fixed close to the spikes.**

### 5.3 Results

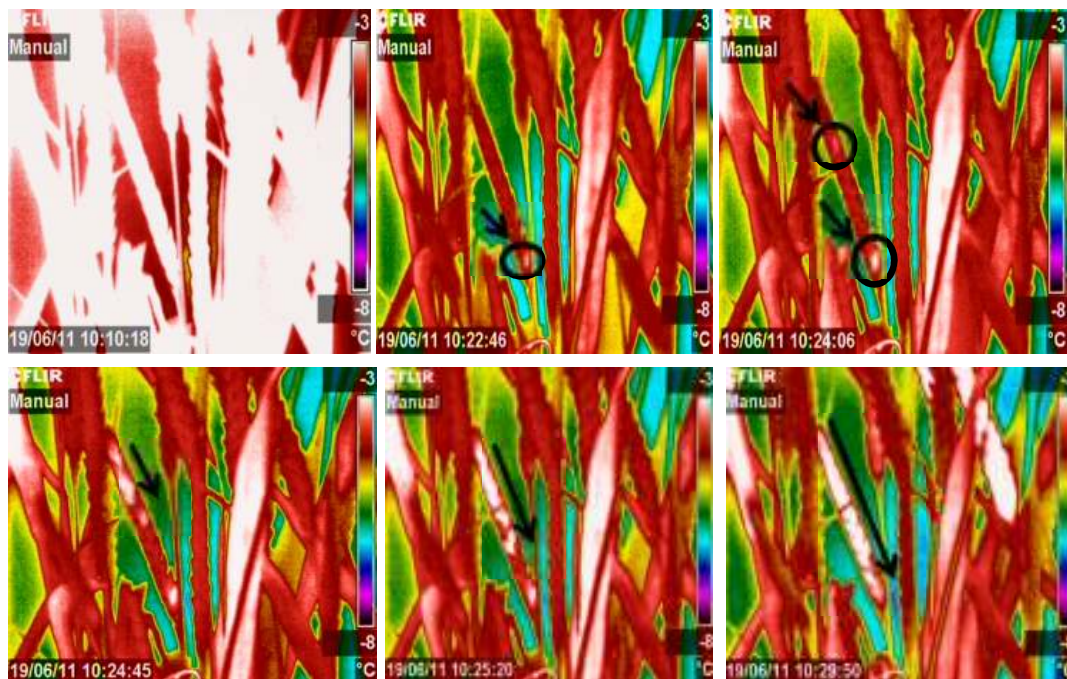
It was noticed that not all spikes under study were frozen, and when ice nucleation was experienced, not all spikes froze at the same time or at the same temperature, or with the same speed.

Many ice nucleation events were observed in most of the frost test runs, but not all these events developed to the ice progression stage. The results presented in Table (11) show the total number of the spikes in each run (pot), and the number of completely frozen spikes.

**Table 11. The total number of spikes in each run and the per cent of frozen spikes in sprayed and un-sprayed plant of both treatments (NA & CA).**

Treatments		Pots No.	Spikes/pots	Frozen spikes	(%) Frozen
Un-sprayed plants	NA	1	17	4	23.53
		2	15	7	46.67
		3	4	1	25.00
		4	4	0	0.00
	CA	5	15	0	0.00
		6	5	1	20.00
Sprayed plants	NA	7	18	5	27.78
		8	5	0	0.00
		9	5	1	20.00
		10	7	0	0.00
	CA	11	7	3	42.86
		12	4	1	25.00
Sprayed/unsprayed plants in the same pot		NA	13	8	37.50

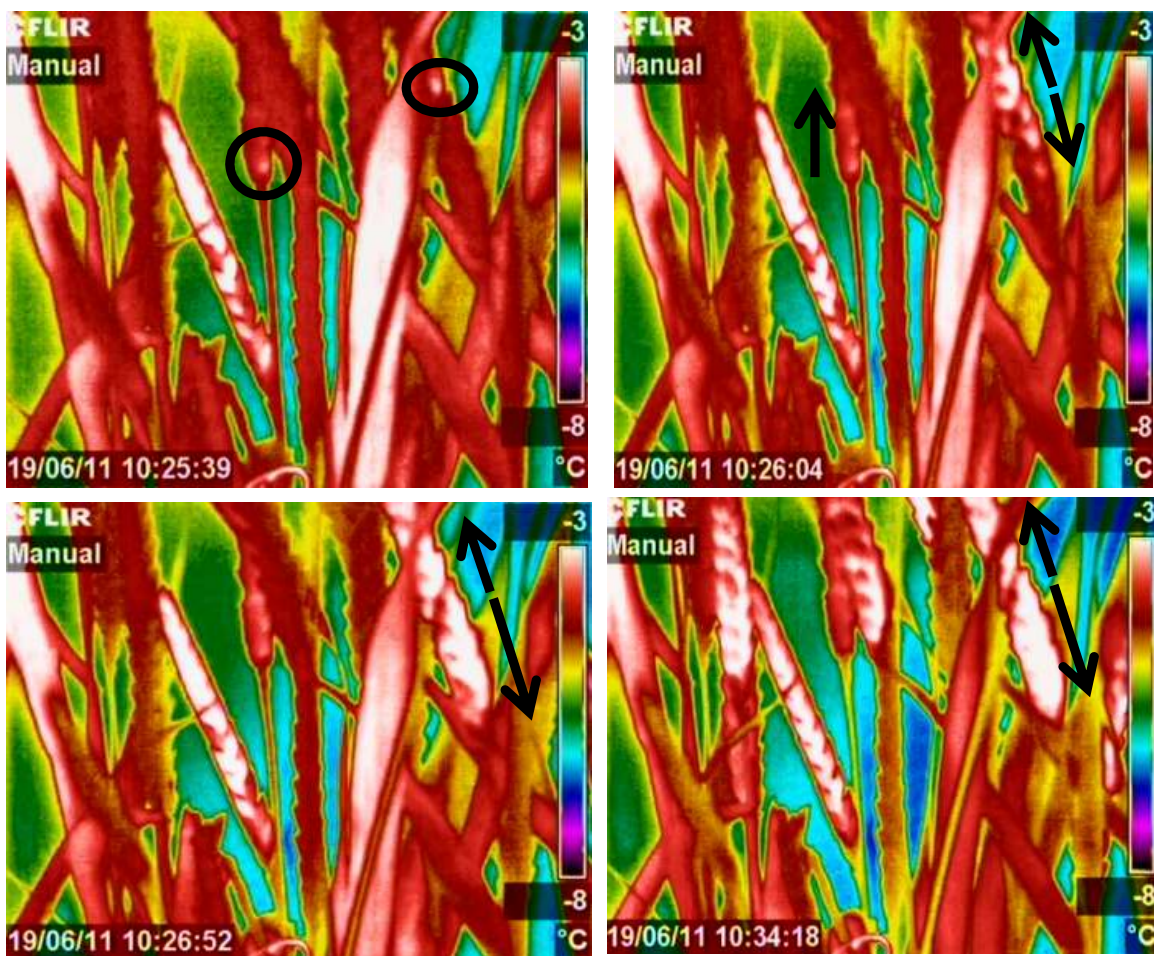
The total number of frozen spikes across the 13 runs was 26 out of 114 spikes observed (22.81%). The 26 spikes which froze showed different patterns of ice nucleation and progression. Several pictures were taken to explain these patterns and also to determine at which temperature the ice started to be nucleated and how long each spike took to be completely frozen. For instance, when un-sprayed and non-acclimated wheat plants were observed, a spike (circled) nucleated first in the bottom spikelet and then the ice formed in the upper third of this spike at a temperature of -4 to -5°C. The ice spread was from the upper site down to the bottom not in the opposite way (Plate 3A). The spike took around 7 minutes until it was completely frozen.



**Plate 3A. Ice nucleation event of a spike which ice-nucleated in two sites (circled) in range -4 to -5 °C of and the ice progression (arrowed) of un-sprayed and non-acclimated wheat (NA).**

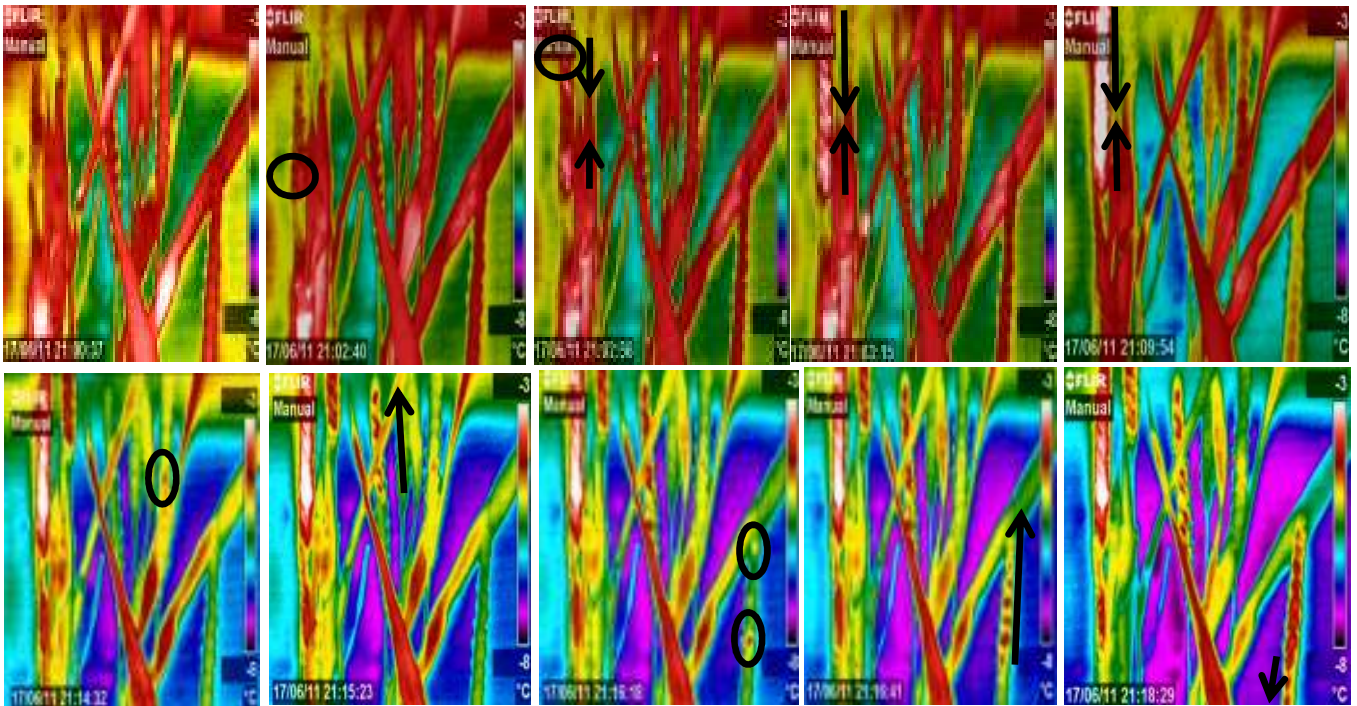


In the same pot there were two other spikes that experienced an ice nucleation event, approximately 3 minutes after the first ice nucleation event. The ice formed in the bottom spikelet for the middle spike (circled) and then progressed towards the top and this spike also froze at a temperature of  $-4$  to  $-5^{\circ}\text{C}$  and took 10 minutes to be completely frozen. While the other spike (on the right), the ice formed in the middle of the spike and progressed both up and down but travelled firstly to the top and then to the bottom (Plate 3B) and took around 10 minutes to be formed in the whole spike.



**Plate 3B.** Ice nucleation events on the same plants in plate 3 A on two spikes showed different pattern of ice nucleation and progression in the range of  $-3$  to  $-8^{\circ}\text{C}$  according to the temperature scale.

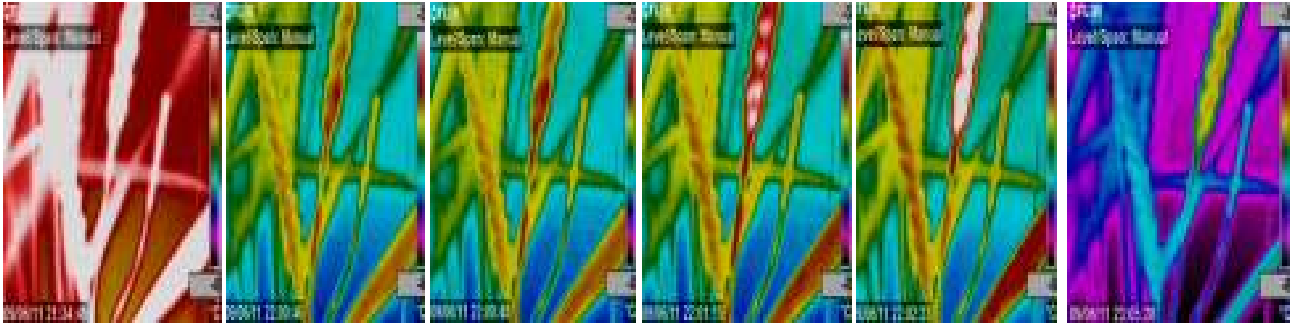
The same pattern was observed when ice formation was recorded in one spike of another group of plants at temperature of  $-4$  to  $-5^{\circ}\text{C}$ . The first site of ice nucleation was in the second spikelet from the bottom and then formed on the second spikelet from the top. When the ice nucleated in the middle of the spike it travelled faster towards the top compared towards the bottom. This spike took around 10 minutes to be completely frozen at the temperature about  $-4^{\circ}\text{C}$  (Plate 4). When the temperature was gradually reduced to approximately  $-5^{\circ}\text{C}$  another two spikes also experienced an ice nucleation event. In the first one the ice nucleated in the lower third of the spike at  $-5$  to  $-6^{\circ}\text{C}$  and then progressed to the top. While the spike that was located on the left of picture frames in plate (plate 4) there were two sites of the ice nucleation. One was on the top of the spike and another at the bottom. The ice progression was from the upper point first down and then ice progressed from the bottom point also downwards until the complete spike was frozen. These two spikes were froze faster and only took round 5 minutes to be completely frozen.



**Plate 4. Two patterns of ice nucleation (circled and arrowed) in unsprayed plants (NA) in range of -4 to -6 °C, these two spikes took around 5 minutes to be completely frozen.**

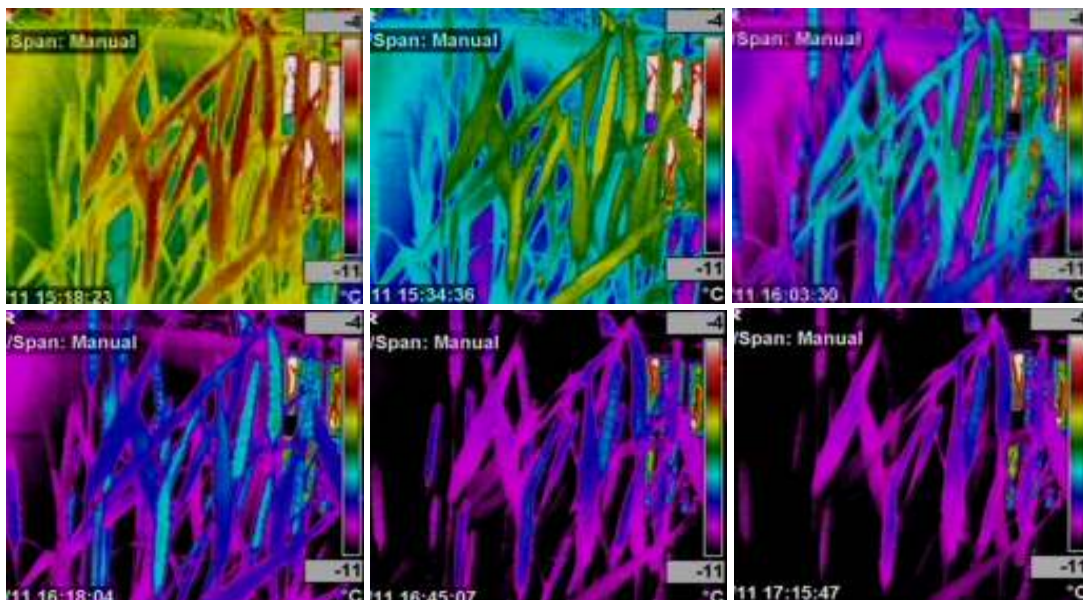
In all the DVDs that were observed there were some spikes still not frozen. This was clear in plate (5) when there were two spikes; one was frozen in the first two spikelets from the bottom and then in other spikelets individually on the spike at approximately -6°C, while the other were still not frozen.





**Plate 5. Different ice nucleation sites on one spike and the other spike were supercooled even when the temperature went down to  $-8^{\circ}\text{C}$  in non-acclimated plants.**

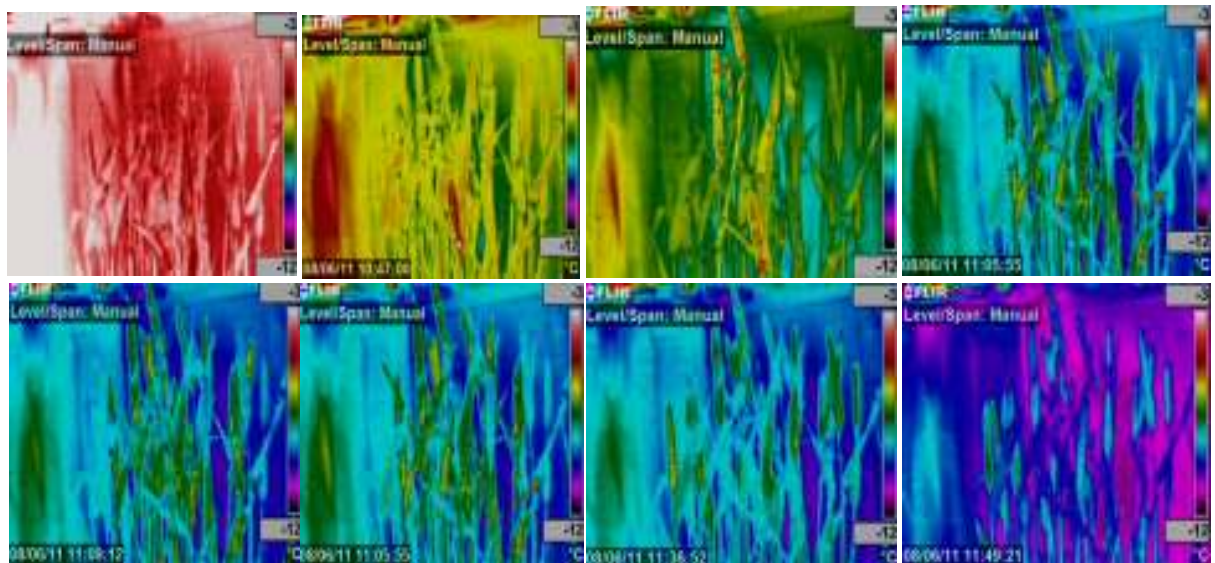
It is worth mentioning that there was no ice nucleation events experienced in plant as shown in plate (6) even when the temperature was gradually declined to  $-11^{\circ}\text{C}$ . It also should be mentioned that those plants were not acclimated and not sprayed with water. However, there were some leaves that were frozen at various temperatures when the DVD was observed but the ice nucleation in leaves was not the interest of this study.



**Plate 6. Examples of supercooling of spikes of non-acclimated and un-sprayed wheat frozen to  $-11^{\circ}\text{C}$ .**

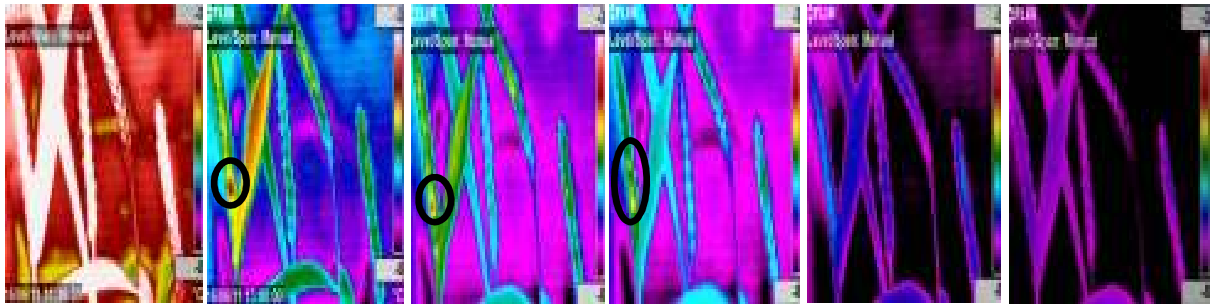


In the current study, IRVT technique has been used to detect the ice nucleation point and its progression in some plants after spraying them with distilled water to facilitate the ice nucleation before placing them into the frost chamber. There were however also many freezing patterns, which were not significantly different from the unsprayed plants. There were often several ice nucleation sites due to spraying plants with distilled water whether they were on the leaves or spikes. However, there were also some spikes that escaped from freezing even in the presence of sprayed water freezing on its surface and frozen to very low temperatures ( $\sim -10^{\circ}\text{C}$ ). It appears therefore that the surface freezing can penetrate the tissue and make ice nucleation inside spikes or leaves (Plate 7) but also plants can resist this ice penetration.



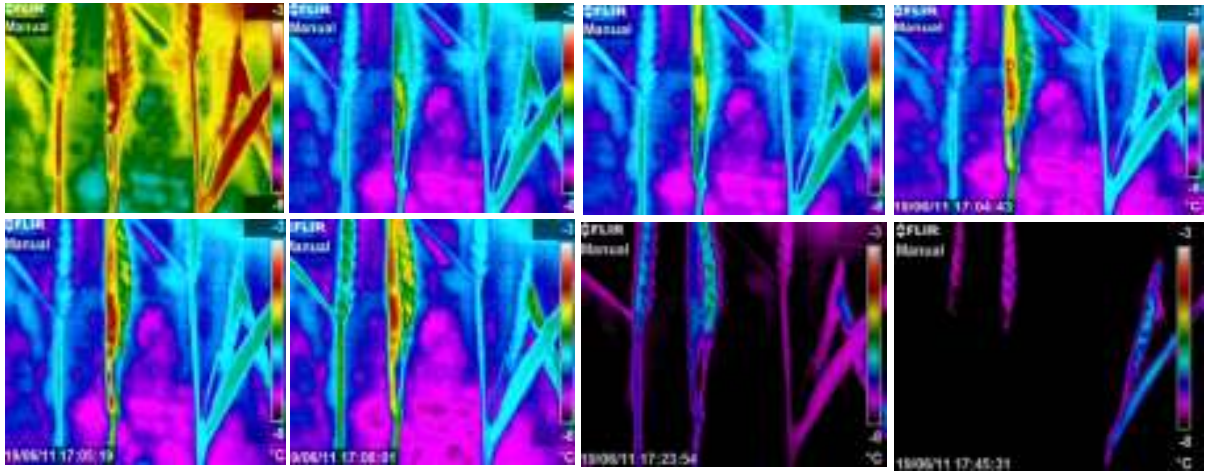
**Plate 7. Many ice nucleation events on sprayed and non-acclimated plants but there was no ice progression within range of  $-3$  to  $-12^{\circ}\text{C}$ .**

It was observed that there sometimes can be no plant ice nucleation events in spite of the presence of water droplets on the surface. It is observed in plate (8) that ice was formed on the tip of one of the spikes but it did not progress into the spike in spite of declining the temperature down to  $-8^{\circ}\text{C}$  suggesting that ice may not develop to an *in-planta* ice nucleation event .



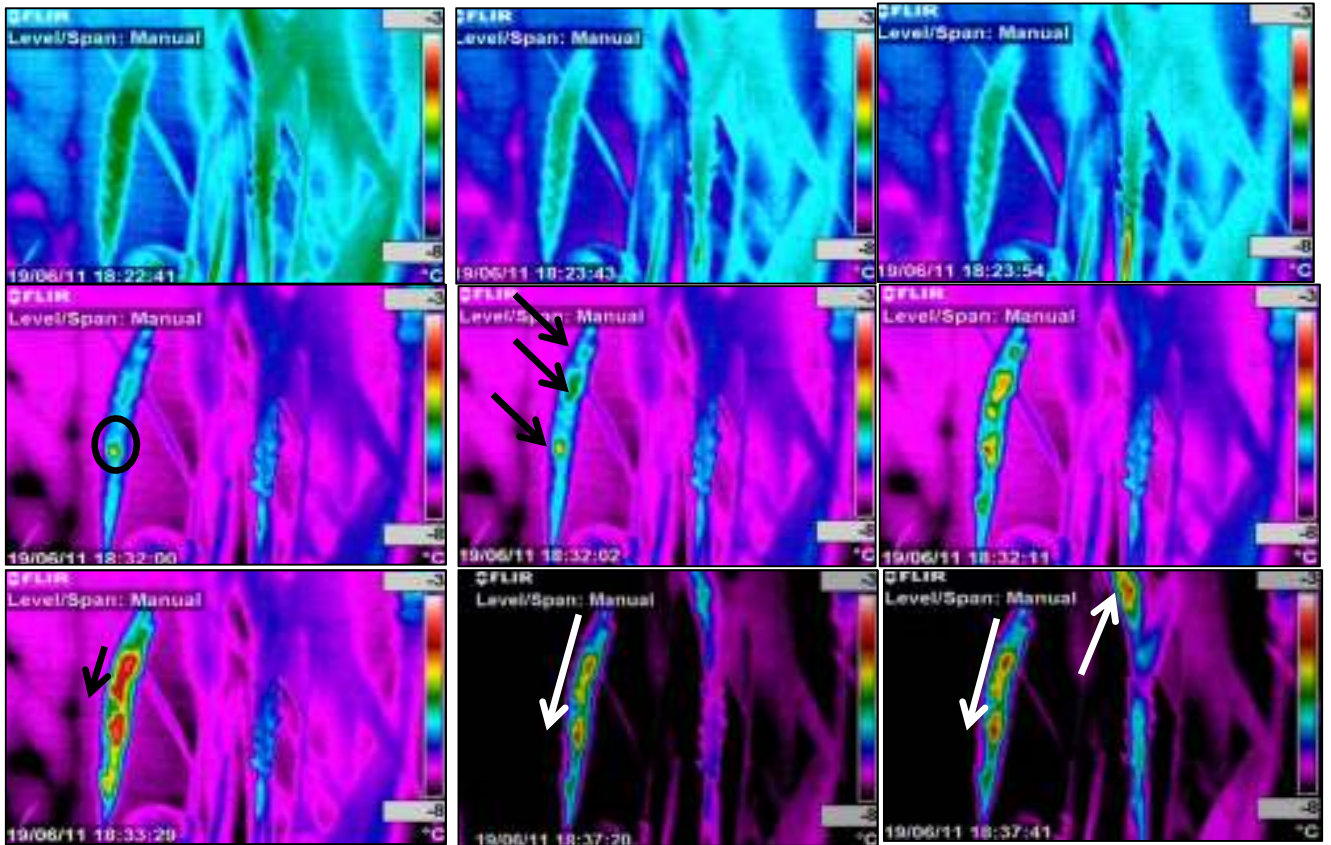
**Plate 8. Water-sprayed and non-acclimated wheat plants shown that there is no progression of the ice nucleation in the range of  $-3$  to  $-8^{\circ}\text{C}$ .**

It is also observed that water droplets generally froze quickly on the tissue surfaces. However, this freezing event did not mean that freezing in tissue would follow. The flag leaf in the plate (9) was firstly frozen very quickly due to the presence of the ice nucleators on its surface. Moreover, the spike in the middle also started to be frozen at approximately  $-7^{\circ}\text{C}$ . The ice on this spike progressed first to the top and then to the bottom until it completely froze within  $\sim 4$  minutes. There were some spikes still not frozen within the range of  $-3$  to  $-8^{\circ}\text{C}$ .



**Plate 9. Water-sprayed and non-acclimated wheat plants, some leaves and one spike experienced ice nucleation.**

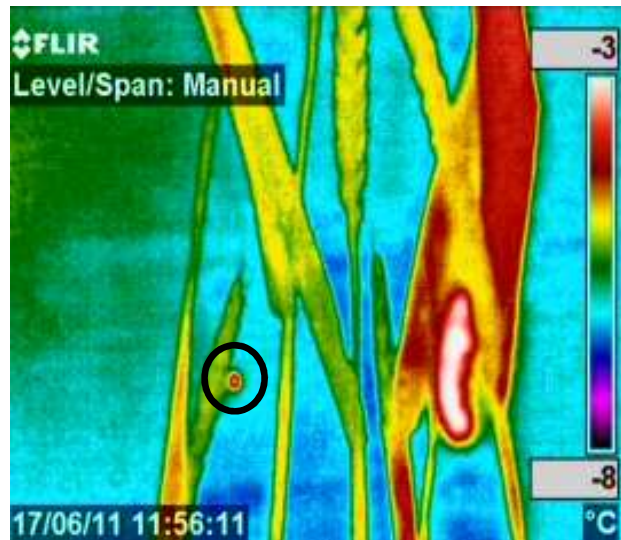
Through the observation of the freezing process in acclimated wheat plant after spike emergence, there were several themes of the ice nucleation as found for non-acclimated plants. For instance when 14 day-acclimated wheat was subjected to freezing down to  $-8^{\circ}\text{C}$ , ice nucleation started at approximately  $-7^{\circ}\text{C}$  (spike circled). Then the ice also then formed in the upper third of the spike in two places before travelling to the rest of the spike. When the temperature declined down to  $-8^{\circ}\text{C}$ , another spike was ice nucleated from its middle within approximately 2 minutes while the rest of spikes in this pot supercooled (plate 10).



**Plate 10. Ice nucleation events in two sites in un-sprayed and acclimated wheat plants. It seems that the ice in this case was formed inside the spike rather than its surface since its expand was transversly.**

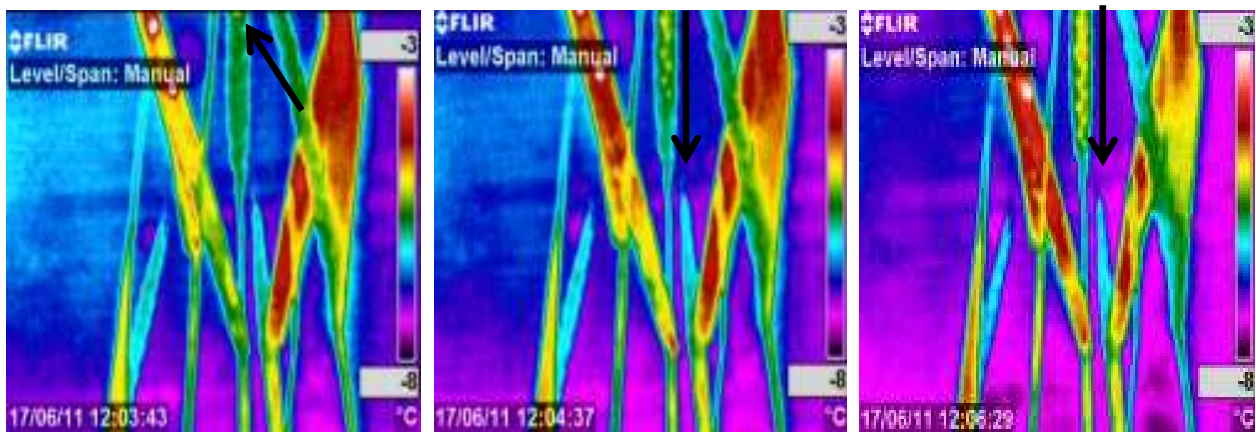
Also when acclimated plants were sprayed with distilled water the same pattern of ice nucleation events was found as with unsprayed plants. Thus, it was observed that the ice was formed on the tip of the spike in plate (11) at around  $-5^{\circ}\text{C}$ , but this ice did not progress into the spike.





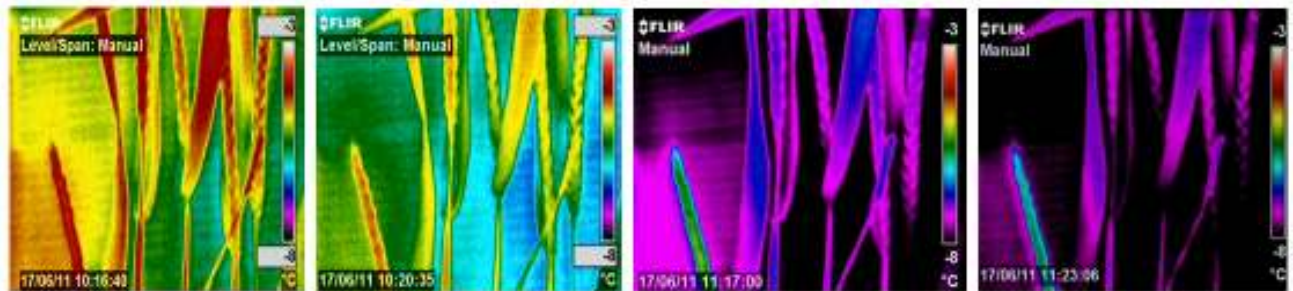
**Plate 11. Ice nucleation of Water sprayed on acclimated wheat plants; the ice did not travel into the spike.**

On the same group of plants, the ice nucleation events also were independently observed on the top of one of the spikes in plate (12) and then progressed down to the bottom through the rachis of the spikes. It took around 4 minutes to be completely frozen. Ice nucleation events were also observed in some leaves.



**Plate 12. Ice nucleation event on spike (arrowed) and its progression through a spike in acclimated and sprayed plant.**

Also there were some spikes which avoided ice nucleation events in the full range of temperatures used (-3 to -8°C). It was also observed that even some leaves were not frozen in this pot at the limit of the temperature range (Plate 13).



**Plate 13. Supercooling of spike frozen to -8 °C in acclimated plants.**

From the above descriptions, the ice nucleation events can be summarised as taking one of the following themes:

- 1- Ice can form in two places, at the top and at the bottom of the spikes. The progress in such cases took three subsequent patterns:
  - Ice may not spread at all.
  - Ice spread from the upper point only to the whole spike.
  - Ice may travel from either direction however; ice travel will always be faster from the top down.
- 2- Ice can start forming in the middle of spike and can take one of the following subsequent patterns:
  - Ice may not spread at all.
  - Ice may spread up and down, but travel up faster than down.
- 3- The ice may form in the lower third of the spike and then take the following pattern

- Ice may not spread at all.
- Ice may spread up to the whole spike
- 4- The ice might form in more than two places, which may not spread at all.

The ice nucleation also can be categorised into two other categories other than the previous four, which are:

- A- The ice may form on the Rachis of the spike and in this case, the ice progression was uniform along the rachis.
- B- The ice may form on the surfaces and in this case could fall into subcategories:
  - Ice may not travel into the tissue. This case happened when plants were sprayed with water.
  - Ice can form on the surfaces of the tissue and then penetrate it, causing the freeze damage.

In all the observations that were made, it was found that many spikes escaped from freezing events and supercooled.

## 5.4 Discussion

Ice nucleation can occur spontaneously when ice nucleators are absent and this is referred to as homogeneous nucleation when the temperature is dropped below very far below 0°C. Alternatively, it can be formed when ice nucleators are present and this is referred to as heterogeneous nucleation and can occur at only a few degrees below 0°C (Lee et al., 1995). Generally, plants have a greater ability to supercool when ice nucleators are absent. This case in this study may not agree with this, where the supercooled spikes were not different from the frozen one. Fuller et al (2007) also confirmed this and they also reported that ice nucleation was not necessary to reduce the frost damage in spikes. However, according to these findings, supercooling may become an effective way to reduce frost damage to wheat at spike emergence if it can be controlled in the field. Therefore, the challenge for researchers could be how to control supercooling in the field and it is different between wheat genotypes.

The ice nucleation seems to be formed in different places in different tissues depending on many factors that can help in predicting the pattern of ice progression (Hacker and Neuner, 2007, Ashworth et al., 1985). However, Pearce (2001) has reported that the exact location of ice in plant tissues still needs to be investigated. Findings presented here showed that ice can be formed in different places either in leaves or in spikes, whereas its spread took many patterns. Ice nucleation in leaves was more consistent due to the absence of barriers (nodes) which hinder ice progression (Pearce and Fuller, 2001, Marcellos and Single, 1984). Ice can spread in either two ways wherever it is nucleated. In the case of heterogeneous nucleation, the ice nucleation site will mostly depend on the location of the ice nucleators. However, frost damage was also consistent in leaves and it



was in line with the REC results (Chapter 4). Ice nucleation in spikes takes place in different locations and shows different patterns in its progression. The nodal regions in the spike might assist the distribution system of ice or it might stop it or slow it from spreading (Marcellos and Single, 1984). However, as seen with the observations presented here, ice nucleation is a very complex process in spikes, and since they cannot be uniformly frozen at the same temperatures and subsequent observations of damage must take this into account. Thus, it was found that for the same group of plants, which were subjected to same rate of freezing temperatures both frozen and non-frozen spikes can be observed. Moreover, even on the same spike it was found that some florets escaped from freezing events while others were frozen (Plate 1, chapter 4).

In order to be able to establish effective methods to protect plants from freezing, the ice nucleation site must be firstly determined and then how ice progresses through the plant tissue (Pearce and Fuller, 2001, Wisniewski et al., 1997). In the current experiment, the first site of ice nucleation was found in various places and the ice progression pattern varied according to the first place of nucleation. The results here showed different patterns of ice progression through spikes from initial ice nucleation. When the initial ice nucleation was on the rachis then the movement of the ice was most consistent and a drastic freezing pattern was observed. However, sometimes the ice can be initiated on the tips of the spikelets or in the floret; in this case the ice does not necessarily spread back to whole spike. Hacker and Neuner (2007) also stated that external ice may not propagate into tissues whereas, Wisniewski et al (2002) have stated that ice may travel into tissue through stomata, broken cuticles or other types of lesion when it physically grows enough.

Moreover, ice can be independently formed in different places (Al-Issawi et al., 2012b), and some spikes will be partially damaged while other spikelets will be supercooled and escape freezing damage. Generally, ice in water-sprayed plant was nucleated according to the availability of the water droplets on the surfaces of the spike, but it seems that this ice was hindered from penetrating the spike more than its counterpart nucleation in the rachis was. The result of using IRVT here showed that not all spikes that were subjected to low sub-zero temperatures freeze at the same time and the subsequent ice propagation did not necessarily must follow the ice nucleation. However, these results were agreed with what have been found previously (Al-Issawi et al., 2012, Fuller et al., 2009, Fuller et al., 2007). It is therefore no wonder that it has been found that some spikes show partial damage (Plate 1) after freezing, and the REC results were inconsistent as it was in flag leaves (Chapter 4).

The reason for this inconsistency in ice nucleation and progression in spikes is not determined yet, but it might be because of the availability of the ice nucleators e.g. water droplets in current study (dew in the field or INA bacteria). These water droplets could provide an explanation about the first site of ice nucleation in case of extrinsic ice nucleation. In addition, the ice may start form firstly when the spike spikelets guttate during the night and then ice will be formed and might spread back into the spike. Conversely, if the atmosphere has a very low relative humidity, the external ice may not form and supercooling is likely to happen. It has been mentioned by Al-Issawi et al (2012) that in the field in Australia where frost damage is severe due to the low probability incidence of dew formation during spike emergence since the low RH% in the atmosphere. The spikes in such situations have a tendency to supercool rather than freeze. In case of the absence of ice nucleators, the first site

of the ice nucleation is still not known. It has been thought that some large polysaccharide may help in initiating the ice nucleation in *Prunus sp* which exhibit very high specific activity at low concentration (Brush et al., 1994). However, these authors also reported that such ice nucleators do not fluctuate from one season to another, they cannot be extracted by sonication and they are not proteinaceous (reviewed by Brush et al, 1994). Plants can avoid ice formation when they are supercooled or they can be tolerant to it when they are acclimated. This usually makes plants more tolerant to Low temperature through different pathways. However, solutes including sugars, proline, betaines and polyols can be accumulated during acclimation as well as the highly hydrophilic proteins can also be accumulated including late embryogenesis abundant (LEA) and dehydrin proteins (Close, 1996). It has been reported that these solutes and proteins help in protecting cells against freezing damage by stabilising cell membranes either directly or indirectly (Xin and Browse, 2000). Steponkus et al (1993) confirmed that in the freezing, the availability of those compounds could be different from acclimated and non-acclimated plants. Likewise Wisniewski et al (2003) have also reported that the acclimation and presence of some materials like anti-freeze proteins that could affect the response of plant to freezing temperatures. Therefore, they stated that acclimated plants could supercool for longer compared with non-acclimated plants. However, Pearce and Willison (1985) have a different point of view where they thought that the mechanism of the freezing damage was the same in acclimated and non-acclimated plants. The case in this study supports Pearce and Willison, because it seems that acclimation after spike emergence in wheat did not change the mechanism of ice nucleation in spikes.

The ice nucleation experienced same nucleation pattern in acclimated and non-acclimated spikes. This confirms that wheat after spike emergence has limited ability to be cold acclimated (Mahfoozi et al., 2001, Fuller et al., 2009, Fuller et al., 2007, Al-Issawi et al., 2012). (Details are in chapter 6).

**Chapter 6: Molecular analysis of the expression of the CBF transcription factor and COR15a in Iraqi and European wheats.**

## **6.1 Introduction**

Low temperature is one of the most important environmental factors that has a major impact on plant survival (Levitt, 1980) and wheat is subjected to low temperature during much of its growth period. Low temperature injury during the reproductive stage can be particularly destructive. Winter wheat goes through a complex process of cold acclimation during vegetative growth in the autumn that increases its tolerance to cold during winter months. This tolerance can be lost quickly once growth resumes during the spring. The period from pre-heading to flowering is the most susceptible to frost damage in wheat. After vegetative/reproductive transition the sensitivity of wheat to frost changes noticeably. Usually this susceptibility comes from the warmer weather which promotes rapid shoot growth and these new tissues only have low frost tolerance, therefore young spike tissue is much less tolerant to frost than vegetative growth (Quinlan, 2000).

In Australia particularly and in other regions of the world such as subtropical zones, the Mediterranean and continental climatic zones, wheat and other temperate grain crops can be exposed to freezing during or after spike emergence and huge loss of yield can occur (Fuller et al., 2009, Fuller et al., 2007, Chen et al., 2009, Thakur et al., 2010). During these late developmental stages, both male and female reproductive organs appear to be frost sensitive and frost damage reduces grain set and thereby yields (Thakur et al., 2010). In Iraq, during the crop season 1979/1980, frosty weather prevailed in the northern parts (Nineveh governorate, which accounts for 50% and 25% of the country's wheat and barley area, respectively) when cereal crops were in the late flowering to early dough stage, and average frost damage recorded was 50%. Grain yield was lost owing to damage to whole or parts of spikes when they failed to develop further and took on a bleached appearance (Tarik, 1981).

In southern Queensland, Australia, frost damage losses to wheat crops during flowering are estimated to cost the Australian grain industry A\$100 million per year (Tshewang et al., 2010). In China, Zhong et al. (2008) reported that the probability of frost damage to wheat was nearly 40 % in the 1970s, rising about 50 % in 1980s and 78 % in 1990s and damage occurred when low temperatures coincided with sensitive late plant growth stages. Whilst crop damage at this time can be caused by decreased photosynthesis, leaf death or stem damage, it is apparent that spike or spikelet death is responsible for most of the recorded losses (Zhong et al., 2008). Wheat in the vegetative stage is tolerant of freezing and this tolerance is determined by a complex interaction between physical and biochemical factors that are dependent on both genotype and environmental stimuli leading to acclimation induced by low non-freezing temperatures (Thomashow et al., 2001). Plant growth and differentiation are continuously adjusted to any of the impending environmental factors and with the variations in environmental factors and stimuli each process has therefore a potential for perpetual variation. During low temperature stimuli, molecular and biochemical processes are very complex depending not only the degree of low temperature but also on the developmental stage and morphological/anatomical parameters of the plants (Bartels and Souer, 2004, Rizhsky et al., 2002). After exposing plants to low temperature, different signalling pathways are activated in order to convert the environmental signal to a biochemical response (De Leonardis et al., 2007). In general, plants response to low temperature in three steps. Firstly is the perception of the downward shift in temperature which might be accomplished by a two-component system (histidine kinase and a response regulator). This system has been recognised to play a role in osmo-sensing processes as well as to act as an ethylene and cytokinin receptor in plants (Urao et

al., 2000). Suzuki et al (2001) have reported that histidine kinase and a response regulator have been identified as a component of the pathway for perception and transduction of low temperature signals in the photosynthetic bacterium, *Synechocystis sp.*. Also they stated that the inactivation of this system depressed several cold-induced genes. Secondly, generation and transmission of cascades of signals follow the perception of low temperature stress. In higher plants, the putative cold sensor might be calcium ( $\text{Ca}^{2+}$ ) channels.  $\text{Ca}^{2+}$  channels tend to be opened under low temperature when there is a decrease in membrane fluidity and the  $\text{Ca}^{2+}$  ions that enter cells activate a signal transduction pathway (Monroy and Dhindsa, 1995). Calcium dependent protein kinases are up-regulated when the level of cellular  $\text{Ca}^{2+}$  increased Thirdly, are the subsequent changes in the downstream biochemical processes (Hughes and Dunn, 1996, Thomashow, 1998, Thomashow, 1999, Shinozaki and Yamaguchi-Shinozaki, 2000). Activation of the signal transduction cascade increases the expression of cold acclimation genes (Sangwan et al., 2002). In many plants and in wheat particularly cold acclimation is regulated by the CBF (C-repeat Binding Factor) regulon, which was discovered through the investigations of cold-regulated genes in Arabidopsis (Fowler et al., 2007). CBF up-regulation is considered to be the first indicator of whether acclimation is being induced following exposure to low temperature (Zarka et al., 2003, Thomashow, 2010). CBF genes are generally found to be induced rapidly in plants upon exposure to low temperature (Badawi et al., 2007, Jenks and Hasegawa, 2005). Many studies have demonstrated that changes in gene expression occur in response to low temperature following CBF induction. Different designations have been given to these genes including *COR* (cold regulated), *LTI* (low temperature-induced), *KIN* (cold-induced) and *RD* (responsive to dehydration). The expression of these genes is found to be increased



dramatically within 2 to 4 hours upon transferring plant to low temperature (Thomashow, 1999). Studies have shown that the promoter regions of these genes included a DNA regulatory element (CRT/DER: C-repeat/dehydration responsive element) that imparts responsiveness to both low temperature and drought stress (Yamaguchi-Shinozaki and Shinozaki, 1994). CBF1, CBF2 and CBF3 DNA-binding proteins were the first three CBFs designated (Stockinger et al., 1997, Gilmour et al., 1998, Medina et al., 1999) however, in wheat it has subsequently been shown that *Cbf14* shows the highest transcripts levels (Vágújfalvi et al., 2005). In vegetative wheat it seems that the induction of CBF genes starts within 15 minutes of exposure to low temperatures around 4°C and triggers a cascade of up-regulation of target cold genes (e.g. *Cor15*) after about 2 to 3 h (Thomashow, 2010). Full expression of frost tolerance genes occurs in the vegetative stage and wheat plants in the vegetative stage can re-acclimate after exposure to warm temperature and de-acclimation (Paulsen and Heyne, 1983, Cromeý et al., 1998). In contrast in the reproductive stage, wheat has been reported to have limited or no ability to re-acclimate (Mahfoozi et al., 2001) and *Cbf* and *Cor* gene expression is assumed to be reduced after vegetative/reproductive transition (Fowler et al., 1999, Mahfoozi et al., 2001, Limin and Fowler, 2006). However, there is very little detailed information in the literature on the frost characteristics of wheat during spike emergence but supercooling ability and thereby frost avoidance has been observed both in frost tests and in the field (Fuller et al., 2007, Fuller et al., 2009). It can be hypothesised that the frost damage that occurs during late development stages in wheat is owing to the failure of the up-regulation of *Cbf* following exposure to normally inductive low temperature but such studies have not been reported to date. This study aimed to investigate the possibility of the up-regulation of CBF genes (*Cbf14*) and the

subsequent up-regulation of their regulon (using COR15a as an indicator of this) in wheat of both Iraqi and European origin.

## **6.2 Materials and Methods**

### **6.2.1 Plant materials**

#### **Vegetative seedlings test materials.**

For vegetative frost tolerance assessment, seeds of 2 varieties were tested an Iraqi (cv. Abu-Ghariab) and a European wheat (cv. Claire). Seeds were sown in plastic seed trays (37 x 23 x 5 cm) filled with compost (John Innes No 2: seed sowing compost, manufactured by Westland Ltd). Plants were raised to the 4–5 leaf stage (ZGS14 –15). Trays were randomly divided into two groups for pre-freezing temperature treatment: un-acclimated at 20°C and acclimated at 4°C. Trays were transferred to the respective controlled environment rooms for 14 days under 8 h light (PAR: 177  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ). Vegetative samples (leaves and pseudostem) were then collected after 0, 8, and 24 h and 14 days for later assessment of *Cbfl4* expression and after 0, 14 day for later assessment of COR15a protein expression. All samples were immediately transferred to -80°C in order to prevent nucleic acid degradation for subsequent mRNA and protein extraction.

#### **Plant materials during reproductive stage assessment.**

For the reproductive stage frost tolerance assessment, the experimental set-up described in chapter 4 (4.2.2) that was used in frost damage investigations were also used for the molecular analysis. Samples for frost damage testing and molecular analysis were taken simultaneously. Because of the sheer volume of work involved in the molecular analysis only two of the spike growth stages were selected rather than

all three growth stages used in the frost damage test. Flag leaf samples for molecular analysis were collected from growth stages GSA and GSB exposed to either 20°C (un-acclimated) or 4 °C (acclimated). Pots at each of the spike emergence growth stages were lifted from the raised bed and transferred to the respective controlled environments. Samples were then collected after 0, 8, and 24 h and 14 days for assessment of *Cbf14* expression and after 0, 14 days for assessment of COR15a expression. Samples then were immediately transferred to -80°C and stored prior to subsequent mRNA and protein extraction.

### **6.2.2 Molecular analysis**

#### **mRNA isolation from vegetative seedlings and flag leaves**

Total mRNA was isolated from plant samples according to the manufacturer procedure using plant total RNA kit (STRNA50) (Sigma: Plant biotechnology). cDNA libraries and cDNA sequences also were prepared and those method are fully explained in Chapter 2.

#### **Protein extraction, estimation and separation in sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE).**

Protein was extracted from vegetative seedlings and flag leaves according to the standard operation procedure presented in chapter 2. Because COR genes require 10 to 14 days for full expression under acclimation conditions, only samples at 0 and 14 days were extracted in order to investigate COR15a expression.

#### **Detection of COR15a protein using western blotting**

The Western blotting technique has been explained in general in the materials and methods (Chapter 2) but some modifications needed to be carried out in order to get

reactive bands at the expected molecular weight of 15 kDa. These modifications included changing the protein concentration in each well, gel concentration and type and the detection reagents and are explained in the results. Beside all these empirical evaluations the way the samples were prepared was also changed and the samples had to be prepared according to an immunoprecipitation protocol (IP).

### **Immunoprecipitation protocol (IP)**

Immunoprecipitation is a widely used method to purify specific proteins from complex samples such as cell lysates or extracts. Conventional immunoprecipitation protocols use Protein A/G-coupled to an insoluble resin, such (e.g. agarose beads) to capture an antigen: antibody complex in solution. The antibody/antigen complex will then be pulled out of the sample using the protein A/G-coupled agarose beads by centrifugation. This physically isolates the protein of interest from the rest of the sample. The sample can then be separated by SDS-PAGE for Western blotting analysis.

Samples were prepared according to the manufacturer procedure (Santa Cruz Biotechnology, Inc: Protein A/G PLUS-Agarose, immunoprecipitation Reagent: SC-2003). After estimating the concentration of each protein sample, 500 µg from each sample was transferred to 1.5 mL micro centrifuge tube. Two sets of these tubes were prepared for each sample. Then 10 µL of the primary antibody and also 10 µL of pre-serum were added to each set of tubes respectively. Then all tubes (500 µg+10 µL COR15a primary antibody or pre-serum) were incubated for 1 hour at 4°C. Samples were then incubated with 20 µL of the re-suspended volume of Protein A/G PLUS-Agarose (SC-2003). Tubes were then closed and incubated at 4°C on a rotator (Grant bio PS-M3D multi-function 3D rotator from Grant Instruments Ltd (Cambridge UK) overnight. The immunoprecipitates were then collected by centrifugation at

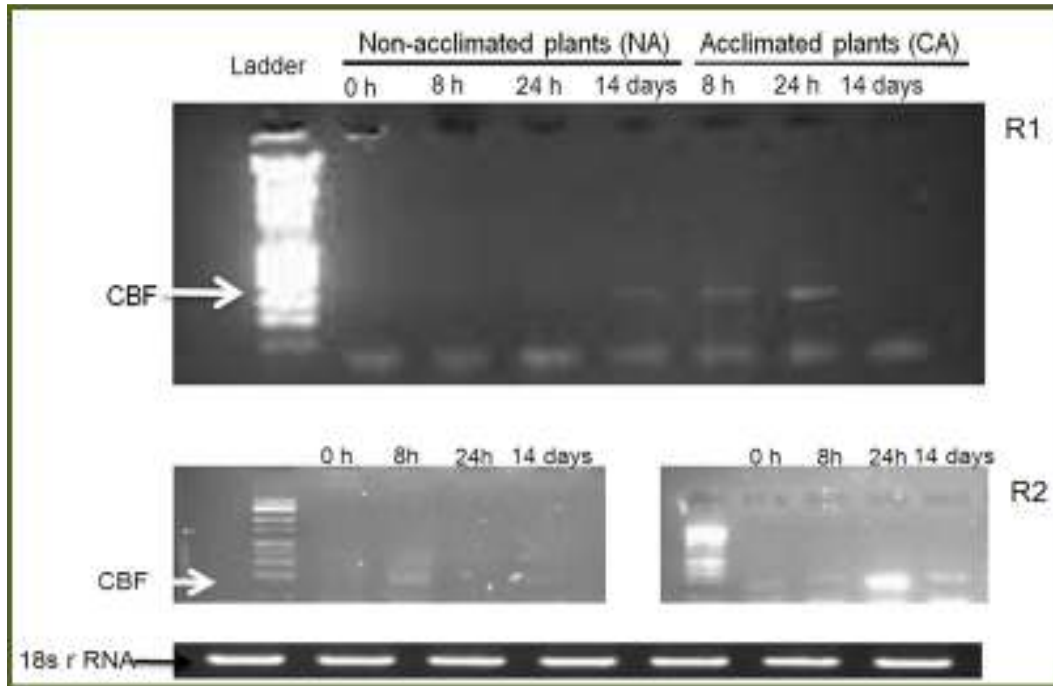
2500 rpm (approximately 100x G) for 5 minutes at 4°C. The supernatant was then discarded carefully and the pellets were kept in the tubes. The pellets were then washed four times with 1 mL of PBS buffer and the centrifuging repeated after each wash. After the final wash the pellets were re-suspended in 50 µL of sample buffer. Samples were then ready for SDS-PAGE and then western blotting as described previously (Chapter 2).

## **6.3 Results**

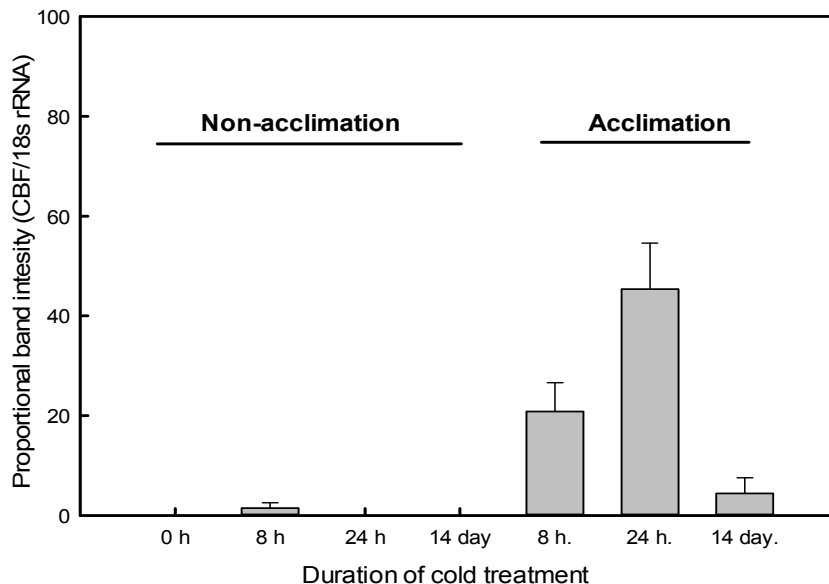
### **6.3.1 Cold acclimation (CBF expression):**

#### **6.3.1.1 CBF expression in vegetative wheat.**

The results of ordinary PCR showed that the *Cbf14* was typically expressed in Iraqi wheat. Cold acclimation increased the level of *Cbf14* transcripts up to 24 h cold acclimation (4°C) and then expression decreased up to 14 days. The expression level was higher in vegetative Iraqi wheat after 24 h exposure to low temperature in both replicates while there was no expression in non-acclimated plants (Plate 14 and figure 8).

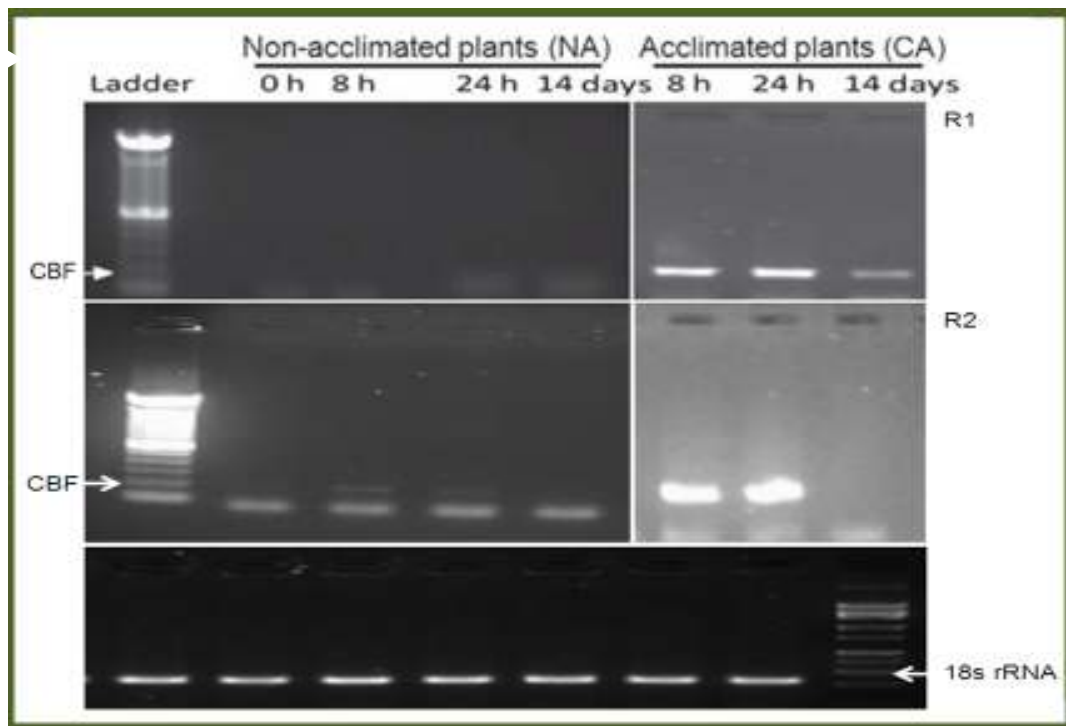


**Plate 14. Cbf14 transcripts level in two replicates (R1 and R2) of Iraqi wheat (Abu-Ghariab) after 0, 8, 24 hr and 14 days from cold treatment of acclimated and non-acclimated plants.**

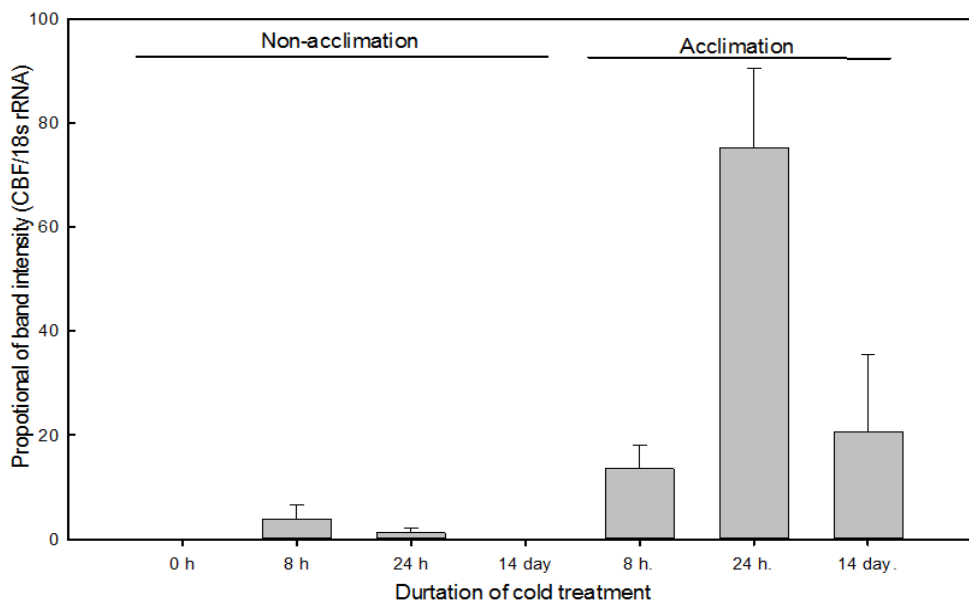


**Figure 8. Proportional band intensity of Cbf14 to endogenous control (18s rRNA) in acclimated and non-acclimated plant of Iraqi wheat (Abu-ghariab) after 0, 8, 24 hr and 14 days from cold treatment (n=3, means+se).**

The *Cbf14* gene also attained maximum expression levels after 8 and 24 hr of cold treatment in the European wheat and similarly returned to basal levels after 14 days of treatment. In some of replicates *Cbf14* products were still detectable after 14 days but the bands were very weak indicating that the transcription possibly declines until it disappear (Plate 15 and figure 9). In non-acclimated plant the PCR results showed that there was no *Cbf14* expression.



**Plate 15. *Cbf14* transcripts level in European wheat (Claire) after 0, 8, 24 hr and 14 days from cold treatment of acclimated and non-acclimated plants.**

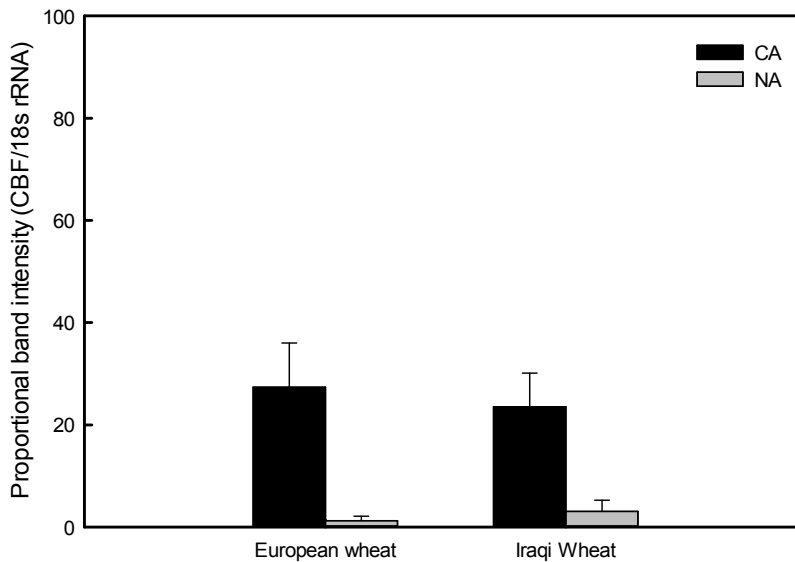


**Figure 9. Proportional band intensity of Cbf14 to endogenous control (18s rRNA) in acclimated and non-acclimated plant of European wheat after 0, 8, 24 hr and 14 days from cold treatment (n=3, means + se).**

The results in figure (10) demonstrated that there were no differences between European and Iraqi wheat in terms of *Cbf14* expression pattern and proportional band intensity. Although Iraqi wheat was sown in winters with mild temperatures for a long time, it has been found the genetic history was not affected by the environment. Therefore, the *Cbf14* expression was associated with the winter cultivar's superior frost tolerance (FT) development capacity. However, there were no differences in the quantitative accumulation of certain *CBFs*. In non-acclimated plants, it has been found that there was some expression of *Cbf14* especially in Iraqi wheat but the expression was relatively very low and still far to do physiological response comparing to the expression in acclimated plant. These results were in line with frost hardiness level of these two varieties (see chapter 3) where the  $LT_{50s}$  of European and Iraqi wheat were close to each other and both were different from other varieties



under study. However there was slight superiority to European wheat over the Iraqi wheat in the level of *Cbf14* transcripts level.

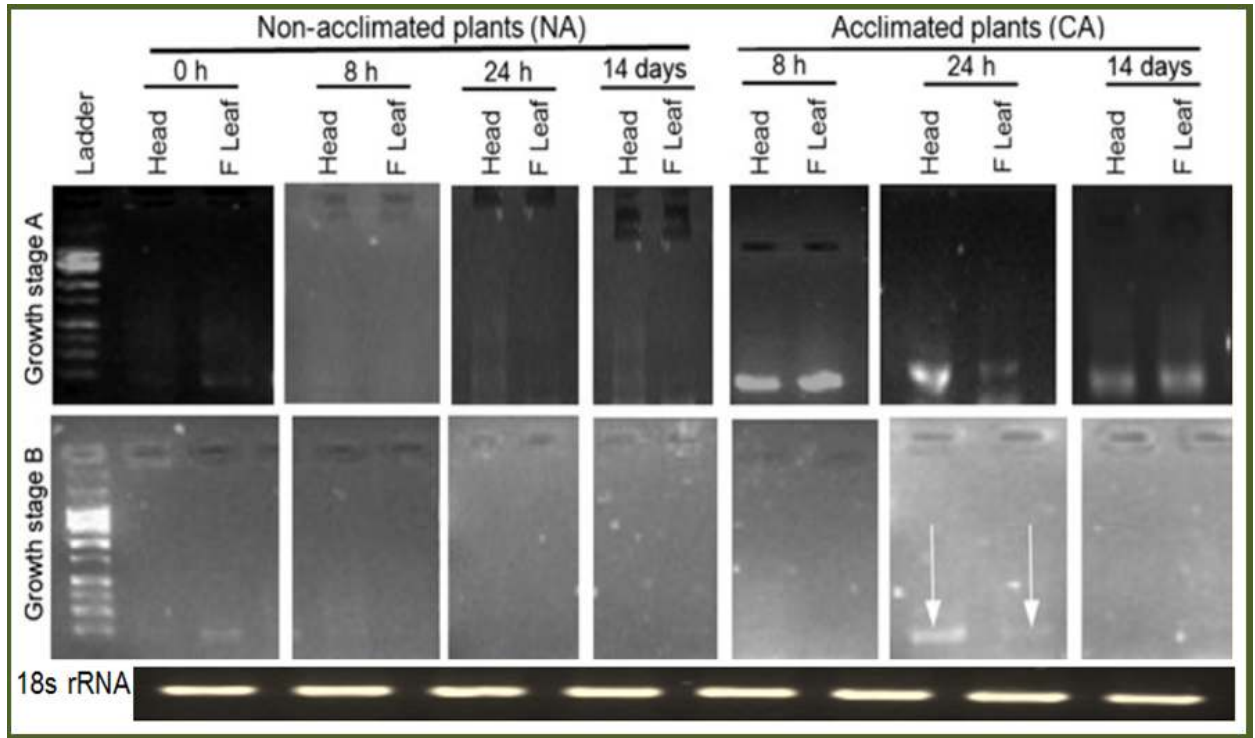


**Figure 10.** The proportional band intensity of *Cbf14* and 18s rRNA in European and Iraqi wheat of acclimated and non-acclimated plant (n=3, means + se).

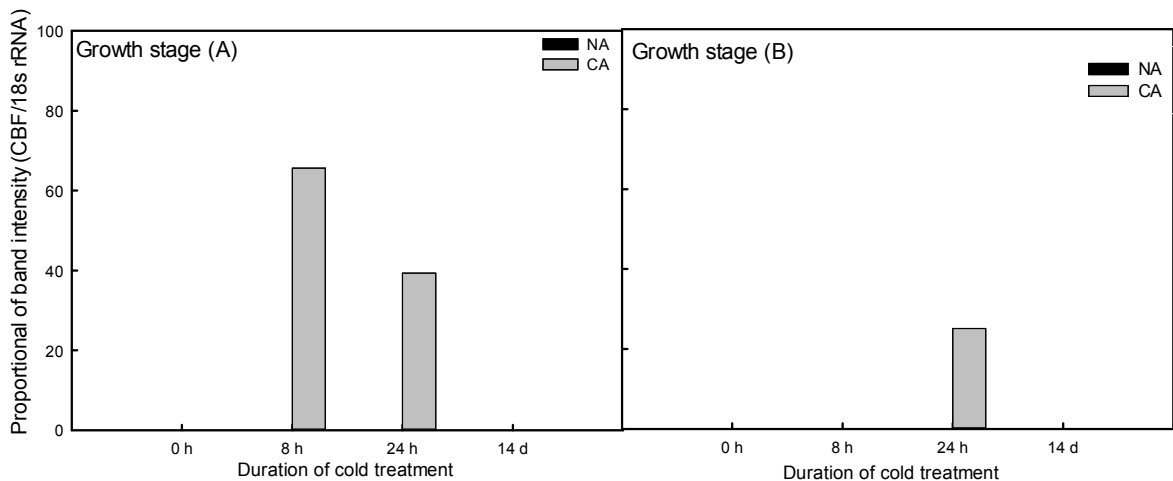
### 6.3.1.2 CBF expression in wheat during reproductive stage.

From the two growth stages that have been studied, the results clearly showed that *Cbf14* was not expressed in non-acclimated European wheat but did show some expression in both flag leaves and spikes especially at the earlier growth stage A (Z51 - Z59), while the expression of *Cbf14* was much less at growth stage B (Z60 - Z69). The expression pattern of CBF genes during acclimation in the reproductive stage seems to take the same pattern as the vegetative stage where it was increased after 8 h and then started to decline after one day especially in early growth stage of spike emergence. The highest expression was found after 8 h exposure to LT in growth stage A and then decreased after 24 h. In growth stage B, the *Cbf14* seemed

to be only expressed after 24 h of acclimation and then disappeared by 14 days. No *Cbf14* expression was found in non-acclimated plants (Plate 16 and Figure 11). *Cbf14* expression was optimized with 18s rRNA endogenous control.

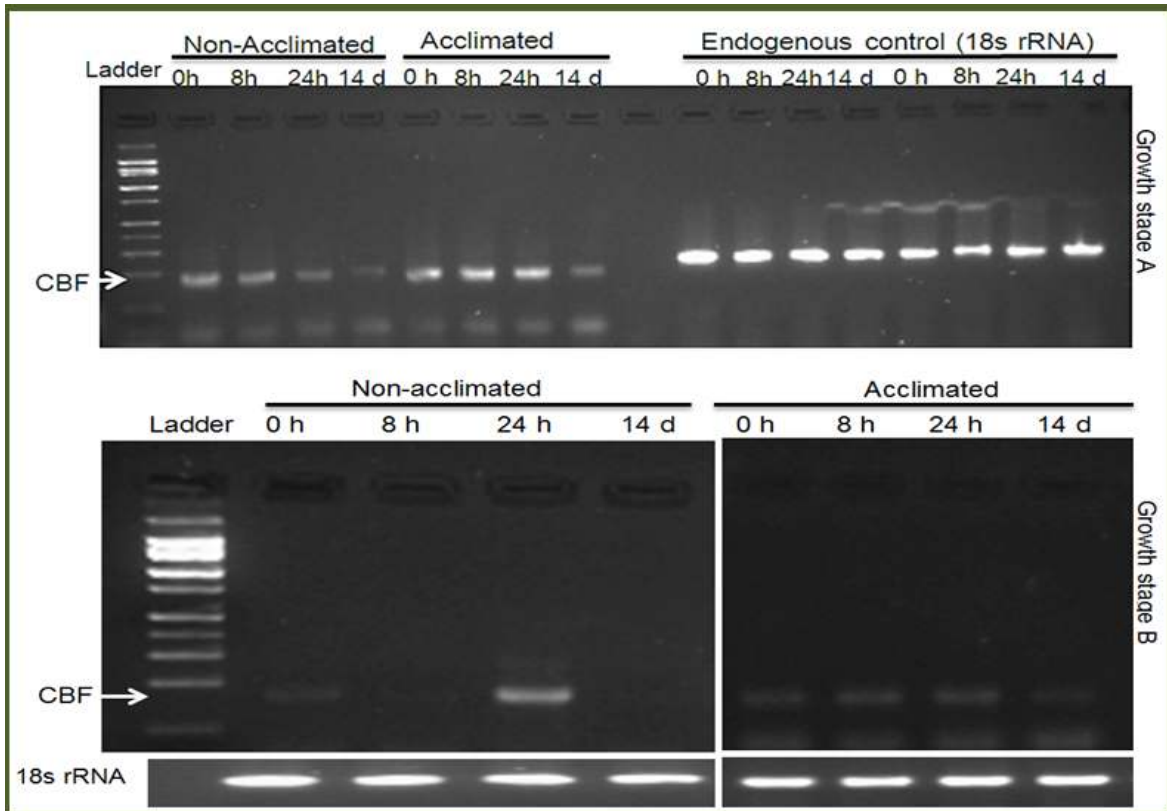


**Plate 16. *Cbf14* transcripts level in European wheat (Claire) at growth stage (A, B) during spike emergence in acclimated and non-acclimated plants (NA & CA) after 0, 8, 24 h and 14 days.**

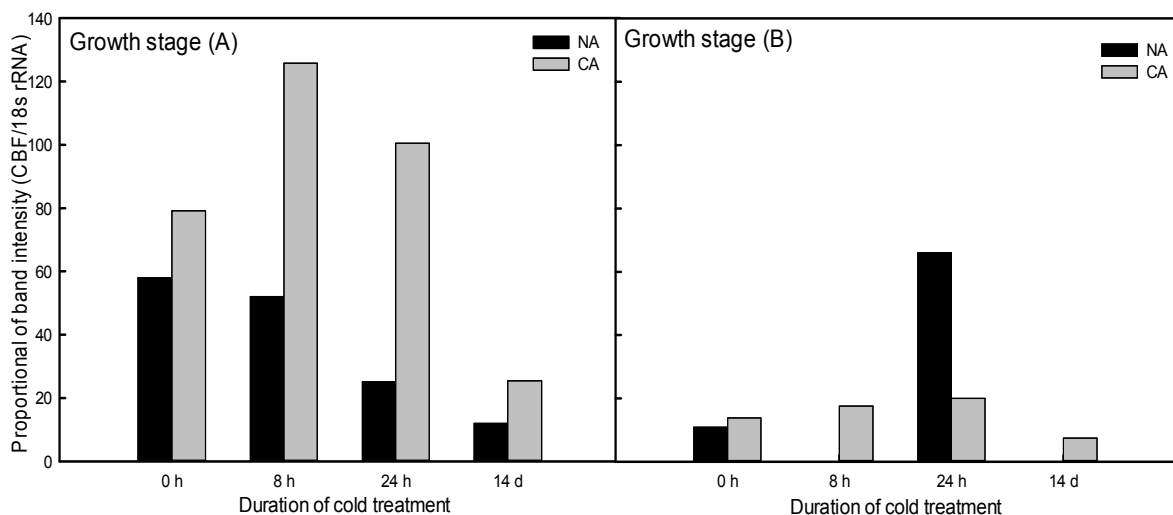


**Figure 11. Proportional band intensity of *Cbf14* to endogenous control (18s rRNA) in acclimated (CA) and non-acclimated (NA) plant of European wheat after 0, 8, 24 hr and 14 days from cold treatment in two spike growth stages of European wheat.**

Data presented in Plate 17 and figure 12 showed that the Iraqi wheat behaved in a slightly different manner compared to the European wheat after late developmental stages especially in non-acclimated plants. In acclimated Iraqi plants at growth stage A *Cbf14* was apparently expressed at 0 h and then subsequently the pattern of *Cbf14* expression was same as its pattern in European wheat (figure 11, plate 16). The highest expression was after 8 and 24 h of the cold treatment and then decreased after 14 days. In growth stage B, cold treatment up-regulated *Cbf14* expression in acclimated plants but the expression was very low compared to its expression in growth stage A. However, it has been found that there was up regulation of CBF genes in the non-acclimated control group of plants and the expression declined after 8, 24 h and 14 days. This expression gradually decreased at 22°C from 0 h to 14 days. In growth stage B *Cbf14* showed little expression only at 0 h and a bit higher after 24 h of cold treatment (Plate 17 and figure 12).



**Plate 17.** *Cbf14* expression in Iraqi wheat (Abu-Ghariab) at growth stage (A, B) during spike emergence in acclimated and non-acclimated plants (NA & CA) after 0, 8, 24 h and 14 days.

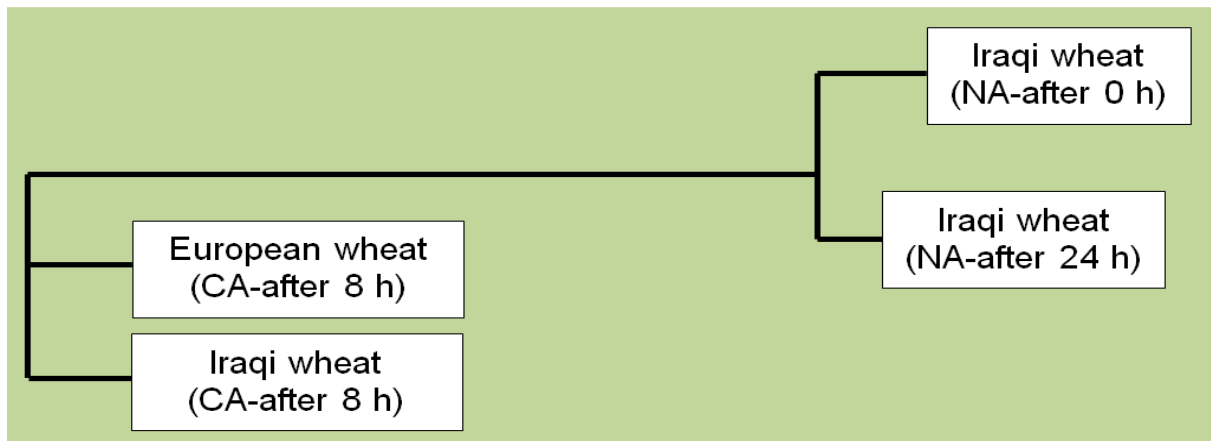


**Figure 12.** Proportional band intensity of *Cbf14* to endogenous control (18s rRNA) in acclimated (CA) and non-acclimated (NA) plant after 0, 8, 24 h and 14 days from cold treatment in two spike growth stages of Iraqi wheat.

In order to make sure that the *Cbfl4* expression that was found in the non-acclimated plants was *Cbfl4* or a different gene, the nucleotide sequence of the cDNA was compared with the CBF gene sequences reported in GenBank. The nucleotide sequence of cDNA isolated from flag leaves of both varieties and under different treatment was determined and compared with each other (Table 12 and plate 18). The data showed high scores of similarity between the CBF that were isolated from non-acclimated Iraqi wheat after 0 and 24 h of treatment and between acclimated Iraqi and European wheat after 8 h of cold treatment.

**Table 12. Scores of the sequence conformity between CBF cDNA isolated from wheat plants under different treatment.**

Seq A	Name	Length	Seq B	Name	Length	Similarity Score (%)
1	Iraqi wheat (NA- after 0 h)	179	2	Iraqi wheat (NA- after 24 h)	178	98.0
1	Iraqi wheat (NA- after 0 h)	179	3	Iraqi wheat (CA- after 8 h)	163	56.0
1	Iraqi wheat (NA- after 0 h)	179	4	European wheat (CA-after 8 h)	163	56.0
2	Iraqi wheat (NA- after 24 h)	178	3	Iraqi wheat (CA- after 8 h)	163	57.0
2	Iraqi wheat (NA- after 24 h)	178	4	European wheat (CA-after 8 h)	163	57.0
3	Iraqi wheat (CA- after 8 h)	163	4	European wheat (CA-after 8 h)	163	98.0



**Plate 18. Phylogenetic relation of the *Cbf14* that was expressed in non-acclimated Iraqi wheat and compared to *Cbf14* that were expressed in acclimated Iraq and European wheat.**

These sequences were also matched with CBF sequences reported in the GenBank (BLAST-NCBI) in order to determine the similarities of the isolated *Cbf14* with CBFs isolated and sequenced by other researchers (Table 13). The results revealed that all isolated *Cbf14* showed significant resemblances which were between 96-100% sequences consensus with the CBF in *Triticum aestivum* cultivar Chinese Spring TaCBF14 (*Cbf14*) mRNA where the primers were designed from. The maximum identity score of cDNA isolated from non-acclimated Iraqi wheat after 0 and 24 h was 96% in both treatments while the conformity were 100% in acclimated Iraqi and European wheat after 8 h of cold treatment.

**Table 13. Alignment of cDNA sequences of CBF gene isolated from flag leaves of wheat under different treatments in nucleotide database.**

<b>A- The closest sequences from non-acclimated Iraqi wheat at 0 h of treatment</b>						
Accession	Description	Max score	Total score	Query coverage	E value	Max ident.
JF758498.1	Triticum aestivum clone BAC 210D22, complete sequence	267	267	90%	3e-68	96%
EF028777.1	Triticum aestivum CBFIVc-14.1 mRNA, complete cds	267	267	90%	3e-68	96%
EU076382.1	Triticum monococcum CBF14 gene, complete cds	261	261	90%	3e-66	96%
EF028779.1	Triticum aestivum CBFIVc-14.3 mRNA, complete cds	261	261	90%	3e-66	96%
AY785901.1	Triticum aestivum cultivar Chinese Spring TaCBF14 (CBF14) mRNA, complete cds	255	255	90%	3e-65	96%
<b>B- The closest sequences from non-acclimated Iraqi wheat at 24 h of treatment</b>						
JF758498.1	Triticum aestivum clone BAC 210D22, complete sequence	276	276	90%	4e-71	98%
EF028777.1	Triticum aestivum CBFIVc-14.1 mRNA, complete cds	276	276	90%	4e-71	98%
JF758494.1	Triticum aestivum clone 1179D19 genomic sequence	276	270	90%	2e-69	97%
AY785901.1	Triticum aestivum cultivar Chinese Spring TaCBF14 (CBF14) mRNA, complete cds	265	265	90%	9e-68	96%
AY572831.1	Triticum aestivum C repeat-binding factor 2 mRNA, complete cds	265	265	90%	9e-68	96%
<b>C-The closest sequences from acclimated Iraqi wheat at 8 h of treatment</b>						
EF028778.1	Triticum aestivum CBFIVc-B14 mRNA, complete cds	145	270	94%	1e-31	100%
AY785901.1	Triticum aestivum cultivar Chinese Spring TaCBF14 (CBF14) mRNA, complete cds	145	270	94%	1e-31	100%
AY572831.1	Triticum aestivum C repeat-binding factor 2 mRNA, complete cds	145	270	94%	1e-31	100%
JF758498.1	Triticum aestivum clone BAC 210D22, complete sequence	141	270	96%	1e-30	96%
EF028777.1	Triticum aestivum CBFIVc-14.1 mRNA, complete cds	141	270	96%	1e-30	96%
<b>D- The closest sequences from acclimated European wheat at 8 h of treatment</b>						
EF028778.1	Triticum aestivum CBFIVc-B14 mRNA, complete cds	145	270	94%	1e-31	100%
AY785901.1	Triticum aestivum cultivar Chinese Spring TaCBF14 (CBF14) mRNA, complete cds	145	270	94%	1e-31	100%
AY572831.1	Triticum aestivum C repeat-binding factor 2 mRNA, complete cds	145	270	94%	1e-31	100%
JF758498.1	Triticum aestivum clone BAC 210D22, complete sequence	141	270	96%	1e-30	96%
EF028777.1	Triticum aestivum CBFIVc-14.1 mRNA, complete cds	141	270	96%	1e-30	96%

The nucleotide sequence of cDNA isolated from flag leaves of wheat was compared with CBF gene sequences reported through different researchers in wheat and other cereal species. The results in Figure 13 showed significant resemblances with up to 100% sequence consensus found with sequences from *Triticum aestivum* CBFIVc-B14 mRNA (GenBank: EF028778.1, 863 bp gene, (Badawi et al., 2007)), *Triticum aestivum* C repeat-binding factor 2 mRNA (GenBank: AY785901.1, 1049 bp gene (Skinner *et al.*, 2005)) and *Triticum aestivum* C-repeat-binding factor 2 mRNA, (GenBank: AY572831.1, 645 bp gene (Singh,N.K., Chinnusamy,V. & Bansal,K.C, Unpublished). While the 94% consensus was found with *Secale cereale* cultivar Lo152 Cbf14 gene (GenBank: HQ730768.1, 560 bp gene (Li et al., 2011)). The % was obtained by the number of identical nucleotides in all sequences divided by the total nucleotide sequence isolated and then multiplied by 100. All multiple alignments were made using ClustalW2.EMBL-EBI (Larkin et al., 2007).

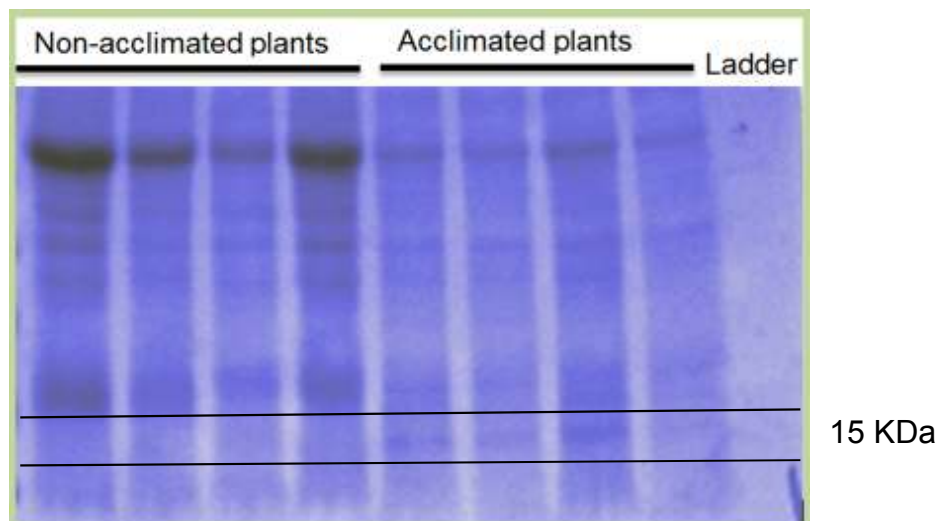




### 6.3.2 Detection of COR15a protein in wheat.

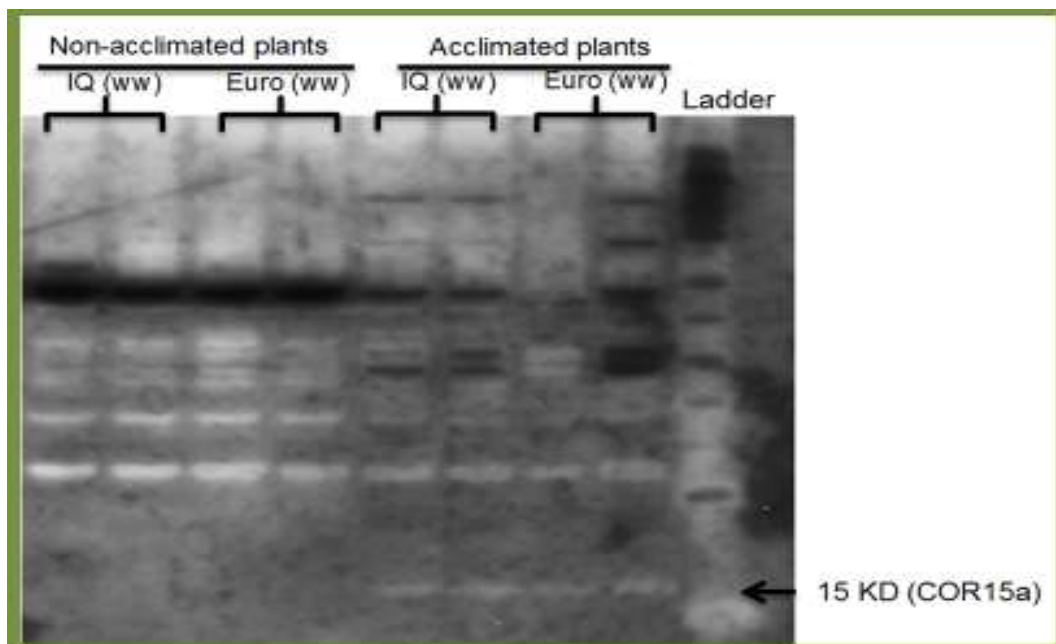
#### 6.3.2.1 COR15a expression in vegetative wheat

COR15a is one of the most best characterised Arabidopsis COR proteins and is described as being cold and drought induced. Low temperature induces accumulation of COR protein in the soluble chloroplast stroma fraction shortly after expose plant to cold or dehydration stress. These soluble proteins were extracted from non-acclimated and acclimated plants and then were fractionated by SDS-PAGE. For each investigation there were two gels, one was stained (Plate 19) and the other was used in the western blotting system in order to transfer proteins to the PVDF membrane for COR15a detection (Plate 20).



**Plate 19. SDS-PAGE gel separation of protein samples extracted from acclimated and non-acclimated plants of Abu-Ghariaband Claire wheat during reproductive stages.**

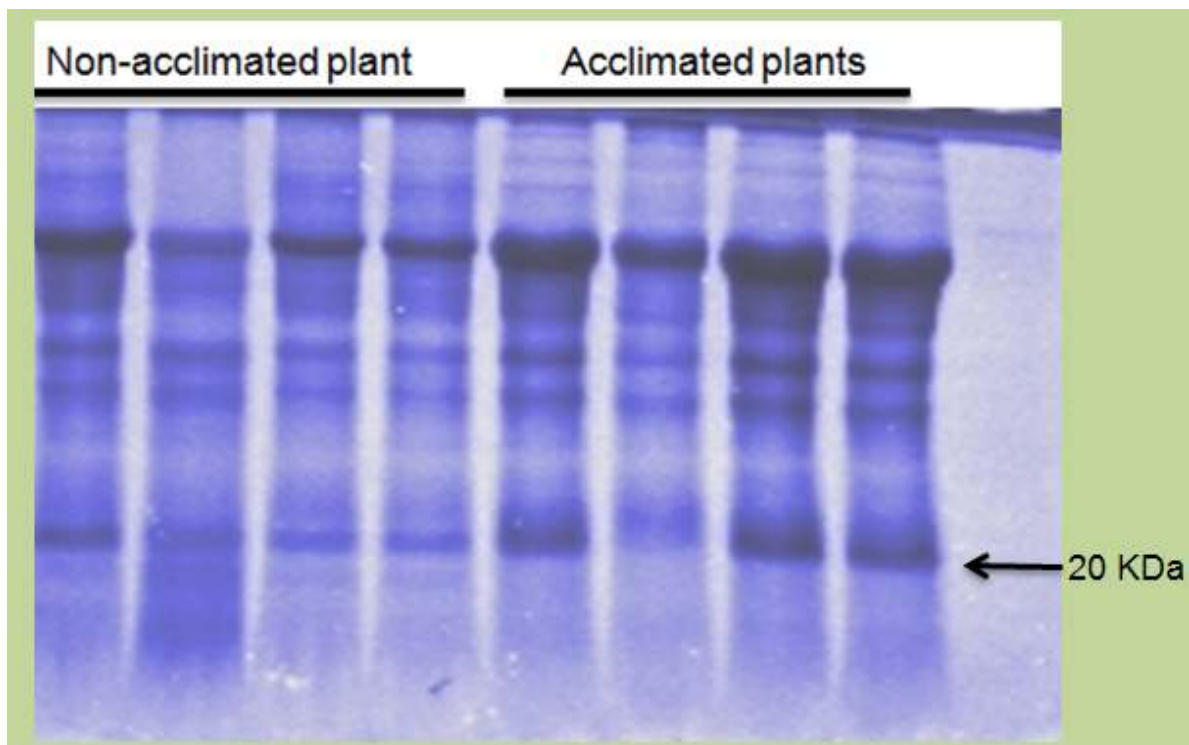
Acclimated plants of the two varieties as they showed bands near to 15 KDa according to Bioline ladder that has been used (detected under UV light). The results of the western blotting system confirmed that acclimated vegetative wheat plants contain COR15a protein under low temperature acclimation. Therefore the results here clearly showed that there was an effect of the acclimation treatment on the expression of COR15 gene due the presence of the COR15a proteins in acclimated plants. There was no expression of the COR15a proteins in non-acclimated plants of both varieties (Plate 20) although there were many cross-reacting bands of higher molecular weight than COR15a KDa.



**Plate 20. Western blot analysis for the detection of COR15a protein in wheat under non-acclimated and acclimated conditions with Abu-Ghariab (IQ WW) and Claire (Euro WW) wheats.**

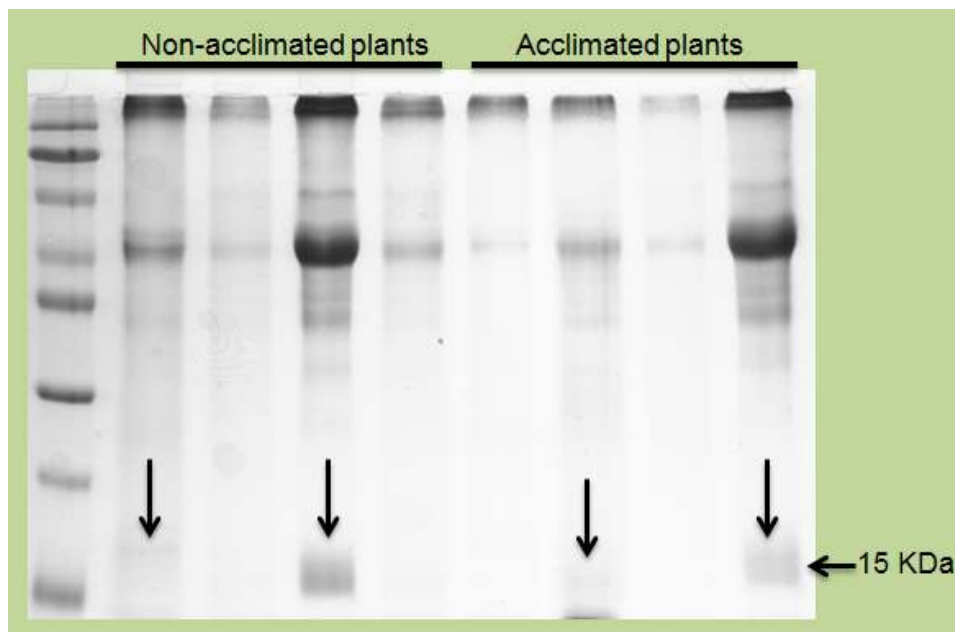
### 6.3.2.2 COR15a expression in wheat during reproductive stage.

COR15a protein was detected just in growth stage A in both Iraqi and European wheat. Many runs had to be carried out in order to optimise every step in the analysis of the COR15a protein expression since there was evidence that COR15a expression should follow the up regulation of *Cbf14*. In some of these runs 12 and 14% acrylamide gel was used and in others a gradient (4 - 20%) ready gel was used as referred to in the materials and methods. Also the concentration of protein samples was manipulated where different protein concentrations were used to get clear bands of the protein of interest. Protein bands were nicely separated when 12 µg/Lane used, but there was no band close to 15 KDa which is the expected location of the protein of interest (Plate 21).



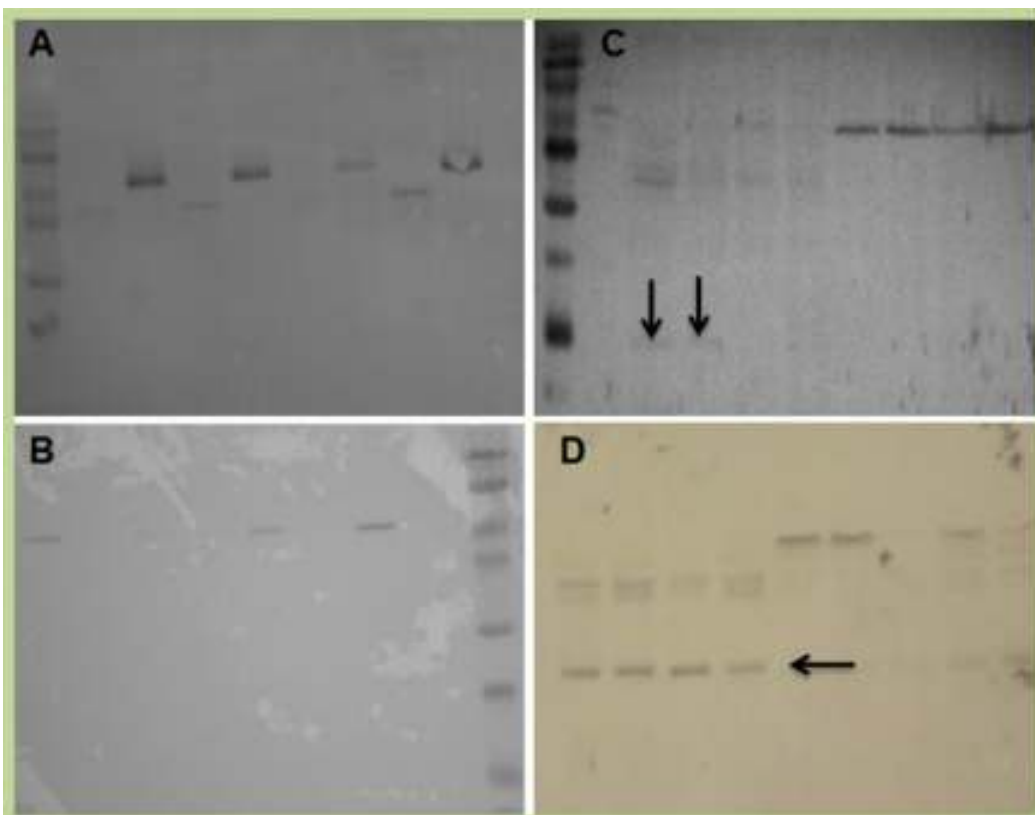
**Plate 21. SDS-PAGE analysis of protein samples (12 µg/Lane) extracted from acclimated and non-acclimated plants of Abu-Ghariab and Claire wheat during the reproductive stage**

It seems that a high amount of the protein is stuck at a higher molecular weight position. When the concentration and the amount of the proteins were reduced in each well to 10 µg/Lane the results in the plate (22) revealed that there were some bands close to 15 KDa molecular weight (arrowed).



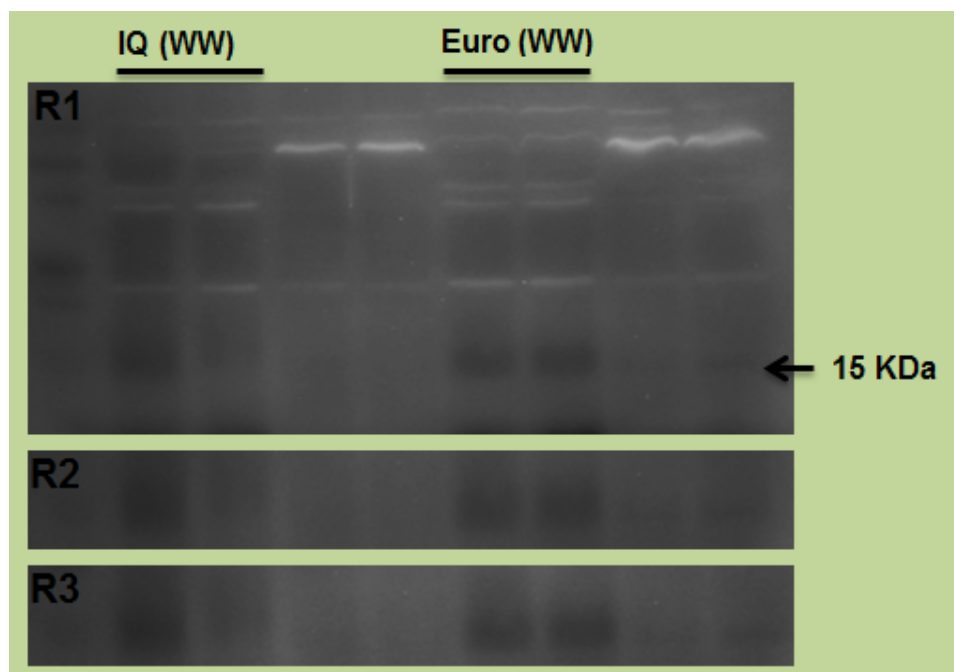
**Plate 22. SDS-PAGE analysis of protein samples (10 µg/Lane) extracted from acclimated and non-acclimated plants of Iraqi and European wheat during the reproductive stage (GSA, 10% of spike visible (spike peep) to: whole spike visible).**

Western blotting was repeated many times using different types of acrylamide gels in an attempt to get nice and clear bands close to 15 KDa. Data in plate 23 showed that most of the investigations revealed bands in different places other than at 15 KDa, and the only gel which showed that there were bands at 15 KDa molecular weight was the gradient gel (4-20%) (Plate 23 - C).



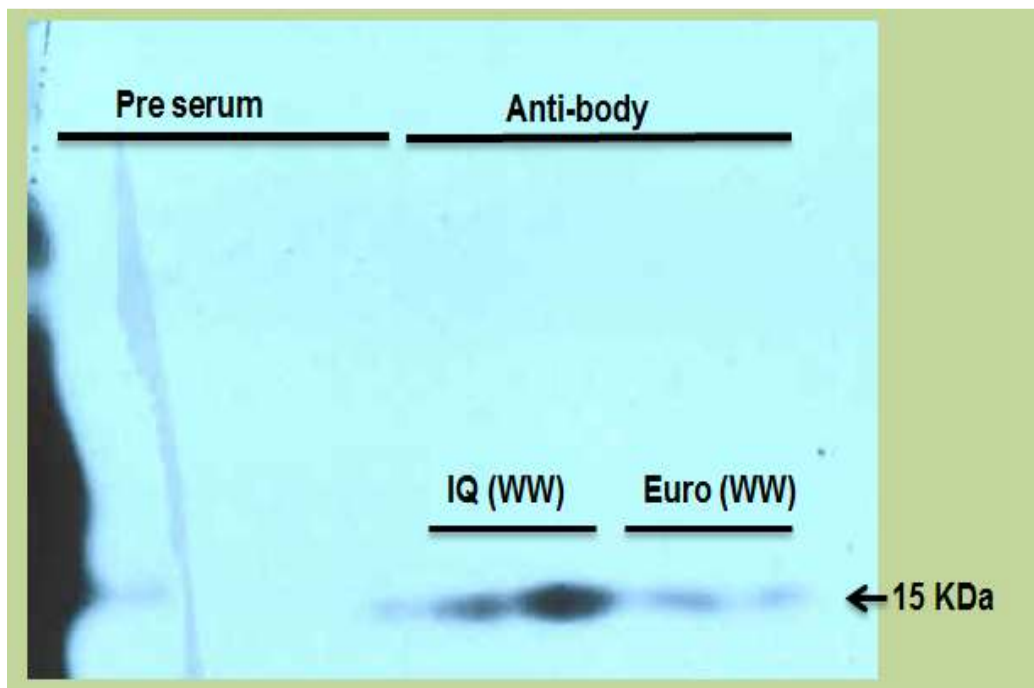
**Plate 23. Western blotting analysis for the detection of COR15a protein in wheat under non-acclimated and acclimated conditions of the Iraqi and European wheat during reproductive stage. A- The concentration of the protein was 12  $\mu\text{g}/\text{lane}$ , no 15 KDa bands were detected, B- the concentration of protein was 10  $\mu\text{g}/\text{Lane}$  no 15 KDa bands were detected, C- gradient gel was used, little bands were detected in the first two lanes, chromogenic reaction was used in A, B and C. D- the concentration of the protein was 10  $\mu\text{g}/\text{Lane}$  20 KDa bands were detected using Luminata Western HRP Substrates as a developing solution.**

Difference results that were obtained by the use of different reagents and different acrylamide gel concentrations finally gave a positive impression for the presence of COR15a protein expression in flag leaves of acclimated plant. According to the results presented in plate 24, it was found that using a gradient gel (4-20%) revealed first positive results of showing small bands close to 15 KDa (plate 23-C) and also using the Luminata Western HRP substrates as a developing solution revealed clear bands (plate 23-D). A combination of these was used in subsequent runs and the result showed that COR15a protein was found in both Abu-Ghariab and Claire wheat at growth stage A (Z51-Z60) (Plate 24). This expression must have followed the expression of the CBF gene indicating that the COR15a protein is part of the regulon of the *Cbf14* gene. This was repeated three times in order to confirm these findings.



**Plate 24. Western blotting analysis for the detection of COR15a protein in wheat under non-acclimated and acclimated conditions of the Iraqi and European wheat during reproductive stage using gradient gel (4-20%), Luminata Western HRP Substrates as a developing solution and the ordinary gel doc system.**

A completely different system for western blotting was also used and also gave a positive result of the COR15a protein expression. Using Immunoprecipitation Reagent (protein A/G Plus agarose) with both the antibody of the COR15a protein and pre-serum showed that COR15a was expressed in samples treated with the antibody and not in the samples treated in pre-serum (Plate 25). The result in plate 24 and plate 25 were consistent therefore and accordingly it can be confirmed that COR15a protein can be produced during reproductive development.



**Plate 25. Western blotting analysis for the detection of COR15a protein in wheat under non-acclimated and acclimated conditions of the Iraqi (IQ-Abu-Ghariab) and European (Euro-Claire) winter wheat (WW) during reproductive stage using gradient gel (4-20%), Immunoprecipitation Reagent (protein A/G Plus agarose), Luminata Western HRP Substrates as a developing solution and the sensitive X-ray imaging system.**



## 6.4 Discussion

### 6.4.1 The expression of the CBF and its pattern in wheat

#### *Cbf14 expression in vegetative and after vegetative/reproductive transition*

The expression of *Cbf14* in the vegetative wheat of both varieties normally increased in the first day of exposing plants to low temperature and then starts to be decreased in the few next days. This is the normal pattern of the CBF gene expression. Jaglo et al (2001) confirmed this pattern when he grew winter wheat (Norstar) at constant low temperature (4°C) in controlled environment. Then results revealed that CBF gene expression increased within first day of exposing wheat plants to low temperature and then decreased during the next several hours. Transcripts of CBF have been reported to start to be accumulated rapidly after 15 minutes upon transferring plant to low temperature conditions (4°C). These transcripts normally remain elevated for weeks as long as plants are maintained under low temperature conditions. However these transcripts also show a quick decrease (within hours) when plants are returned to warm conditions (20°C) (Thomashow, 1999, Matthew, 2005). Vágújfalvi et al (2005) have stated that the highest transcripts were observed for TaCBF14 (which used in current study) and TaCBF15 when plants were exposed to 2 °C for 2 h when they used quantitative PCR system in order to study the transcription profile of different wheat cold induced genes. In addition the northern blot analyses have revealed same pattern of CBF expression (Denesik, 2007). However, the WCBF1 expression has gradually increased early in the first hours of cold treatment and then starts to decline, although it still can be detected after 24 h of cold treatment. Furthermore, in spite of the fluctuation in the field temperatures, the case of increase

and decrease of CBF expression within the first day also was observed in all genotypes under study (Denesik, 2007)

Frost tolerance in wheat during vegetative stage is highly maintained since *Cbfl4* was easily up-regulated in response to low non-freezing temperature during this stage. Therefore wheat varieties can tolerate low temperature stress during winter months. Fowler and Limin (2007) have stated that the full expression of the frost tolerance only occurs during vegetative stage since the genetic analysis at the whole plant level showed that there was a developmental regulation of frost tolerance gene response. Fuller et al (2007) also have confirmed that wheat can only tolerate freezing temperature during vegetative stage while it loses its frost tolerance in reproductive stage. Frost tolerance greatly varies between wheat varieties. Although the two varieties that have been used in current study are considered to be winter wheat there was some superiority of European winter wheat over the Iraqi wheat. However *Cbfl4* expression in both varieties was corresponded to their cold tolerance where they showed  $LT_{50}$  of  $-8.07$  and  $-8.01^{\circ}\text{C}$  for European and Iraqi wheat respectively under acclimated temperature, and they show similar cold tolerance under normal conditions where they showed  $LT_{50}$  of  $-5.93$  and  $-5.8$  in European and Iraqi wheat respectively (Table 5, chapter 3). In spite of that the two varieties were selected under totally different environments, it has been found that those two varieties showed similar cold gene expression and consequently they also showed similar cold tolerance.

The genetic analysis at the level of whole plant showed there was a developmental regulation of low temperature tolerance genes response. It revealed that the full expression of the cold hardiness genes only occurs during vegetative stage and the transition from vegetative to reproductive growth stages is a critical switch, in turn it

may limit the ability to cold acclimate. Therefore it is thought to be a master switch that can initiate the down-regulation of low temperature tolerance genes (Fowler and Limin, 2007). Nevertheless also has been mentioned that both winter and spring growth habits genotypes can cold acclimate after reproductive transition, but before heading. Therefore, it demonstrating that the vegetative/reproductive transition does not act as an off switch for cold acclimation genes (Fowler, 2007). The results here have also confirmed this and *Cbf14* was expressed early after reproductive transition (e.g. GSA) but with much lower or no expression while spike progressing in growth (e.g. GSB & C) in both Iraqi and European wheat (Al-Issawi et al., 2012). Mahfoozi et al (2001) and Fowler et al (2007) have suggested that once the vernalisation requirement has been fulfilled in wheat, the expression of genes for cold acclimation are switched off, claiming that genes for vernalisation are the master switch for cold acclimation genes and wheat would only be able to resist frost to its constitutive frost resistance level, which appears to be around -5°C. Fuller et al (2007) and Fuller et al (2009) also proposed that acclimation cannot be triggered in wheat plants at the spike emergence stage and the plant is insensitive to acclimation temperatures. This is largely upheld in this study although there is limited evidence of acclimation in flag leaves but not in spikes themselves. The molecular analysis (*Cbf14* up-regulation) partially upholds this hypothesis. As spike emergence progressed, the ability to up-regulate *Cbf14* declined but at an early stage of spike emergence *Cbf14* could still be up-regulated. As the early spike emergence stage is several months after vernalisation is fulfilled, it is unlikely that vernalisation provides the 'Master switch' for acclimation gene regulation via *Cbf14* control. However, despite the cold-induced up-regulation of *Cbf14* at early spike emergence, acclimation did not occur suggesting that the control of frost resistance by vernalisation is either downstream of the CBF

transcription factor or on a different path to that in which *Cbf14* operates. The most highly induced genes during acclimation are COR, KIN, RD, LTI and ERD (Thomashow, 1998) and some of these genes act directly to stimulate the production of cryoprotective polypeptides (e.g. COR15a), but generally the proteins encoded by these genes are extremely hydrophilic, and they could be members of the dehydrins (Close, 1997; Wise and Tunnacliffe, 2004) Protein families (Fowler et al., 2007). It is possible that despite *Cbf* induction its regulon is not induced. Further work is still necessary to determine the exact point of breakdown of gene up-regulation for acclimation during spike emergence in wheat.

It is worth mentioning that there is a strong link between vernalisation and frost tolerance especially in winter wheat. However, it has been found that VRN1 is released by vernalisation which in turn initiates the transition of the vegetative apex to double ridge stage and down-regulate the CBF/COR pathway in winter wheat (Galiba et al., 2009). Finally, non-molecular studies have shown that chromosomes involved in cold acclimation. According to these studies it has been generally anticipated that large number of genes with small effect and complex interactions determine the phenotypic expression of low temperature tolerance. Therefore, molecular mapping studies have only succeeded in locating low temperature tolerance genes which are linked to the vernalisation genes in the fifth group of chromosomes in wheat (Storlie et al., 1998, Galiba et al., 1995, Fowler and Limin, 2007). Generally it was found that the up-regulation of the CBF is important to increase the frost tolerance in plant, therefore it is important to shed light on how the transcript level of CBF could be elevated during the low temperature.

### **CBF pathway and signal transduction**

As previously mentioned, wheat varieties vary greatly to survive cold temperature and an also *Cbf14* pathway is a component of cold tolerance in wheat. An important question that then arises is how CBF pathway could contribute to increase cold tolerance in wheat. Before addressing this question the cold signal perception should be known. In addition, and according to the fact that low temperature stress shares some characteristics with drought and salt stress, therefore it is important to separate the events that take place through cold stress signal transduction from those related to other environmental stresses. However, the response of plants to any of environmental stresses is mediated by a series of reactions, cooperatively known as signal transduction (Heidarvand and Maali Amiri, 2010). Low temperature requires cells recognition and signalling process in order to activate cold responsive genes and eventually allows that plant to survive low temperatures (Denesik, 2007). Basically, the CBF pathway is consisting of two essential regulatory steps. The first step is the CBF genes are activated rapidly in response to low temperature stress. The second step is that the activators that have produced by CBF genes induce expression of genes containing CRT/DRE regulatory elements. The expressions of the CBF regulon of genes, which are responsive to CBF expression, are able to increase the frost tolerance in plants (Next section) (Matthew, 2005, Jaglo-Ottosen et al., 1998, Liu et al., 1998, Kasuga et al., 1999). In the first step, low temperature is going to affect the membrane fluidity and as result membrane will become rigid (Denesik, 2007, Sangwan et al., 2002). When the membrane becomes rigid, the phospholipids become more unsaturated (Sangwan et al., 2002, Nishida and Murata, 1996). This state will protect cells during low temperature stress by helping to maintain the cellular shape and prevent cellular component from being loss of their

water. Also rigid membranes might not allow cells to collapse during extracellular freezing by developing a negative pressure in the cells (Rajashekar and Lafta, 1996). It has also been suggested that there is a role of the cell membrane physics state on frost tolerance in the plant (Heidarvand and Maali Amiri, 2010). The microtubules in the cell membrane also will be changed in response to low temperature and became disassembled (Abdrakhamanova et al., 2003). Thion et al (1996) and Dodd et al (2006) also have stated that cytoskeleton might act as low temperature sensor in plants. These cytoskeleton rearrangements might allow the ion channels to be opened and cause influx of ions in the cytoplasm of the cells (Sangwan et al., 2002). Since the microtubules and microfilaments are consider being the downstream of the targets various signalling pathways including cold stress, they appear to have a central role in cold signalling and cold acclimation by opening the Ca<sup>2+</sup> channels (Thion et al., 1996, Orvar et al., 2000).

#### **6.4.2 The expression of COR15a protein in wheat.**

COR15a is a regulon of CBF transcription factor as mentioned previously; therefore it is a downstream of the cold signal transduction pathway in Arabidopsis (Jenks and Hasegawa, 2005). COR15a is a small hydrophilic polypeptide which designated as COR15a protein and targeted to chloroplast after its induction during cold acclimation (Nakayama et al., 2007, Lin and Thomashow, 1992). The first direct evidence was provided that COR15a protein is having role in freezing tolerance is from Artus et al (1996). However, they found that the constitutive expression of COR15a protein in non-acclimated transgenic Arabidopsis plants increased the frost tolerance in both chloroplast in situ and isolated leaf protoplast. COR15a found to be the protein that can increase frost tolerance in plant. Winter wheat can survive freezing temperature

while it in vegetative stage and this is due to the up-regulation of CBF gene and its downstream regulon (COR15a). Thomashow (1999) confirmed the positive effect of COR15a when the protoplast number was taken into account within a range of -4 to -8 °C. Wheat during reproductive growth became more sensitive to temperature. It is thought to be because of the failure in cold acclimation process, since there is no low temperatures precede freezing events. In current study, plants during reproductive growth were exposed to low temperature in order to investigate the possibility of increasing frost tolerance during this stage. The only stage was investigated is growth stage A which comes directly after vegetative/reproductive transition. In this stage both CBF genes and COR proteins were able to be up-regulated. Frost tolerance also increased in early stage of the transition (GSA) but not later (GSB & C). Therefore, results here supported the findings in the literature that COR15a increases frost tolerance in plant whenever it is up-regulated. The functional activity of COR proteins is not well known and remains speculative (Jenks and Hasegawa, 2005). However, COR15a protein mainly helps in stabilising membranes against freezing injury (Artus et al., 1996). The COR15a genes encode a 15-KDa polypeptide (COR15a) which has been located in cell chloroplast (Lin and Thomashow, 1992). Steponkus et al (1998) have stated that COR15a act as cryoprotective polypeptide since it decreased the propensity of the membranes to form the hexagonal II phase (Inverted hexagonal phase: a deleterious non-bilayer structure that occurs due to the cellular dehydration which correlated with freezing injury). It also has been reported that this protein is able to protect other proteins against freeze-thaw inactivation *in vitro* (Bravo et al., 2003, Hara et al., 2003) in addition to membrane stabilisation. Furthermore, COR15a protein as well as LEA and Dehydrin proteins can also act as hydration buffer so they are sequestering ions and renaturing unfolded proteins

(Bray, 1993). However, Wise and Tunnacliffe (2004) thought that these proteins are possible to take up a folded conformation upon dehydration stress or could bind to a molecule target. Recently Thalhammer et al.(2010) reported that *COR15a* and *COR15b* genes are both highly cold induced and encode proteins COR15a and COR15b respectively which belong to LEA-4 (group 3) of late embryogenesis abundant proteins. They also stated that both proteins are intrinsically disordered proteins (IDPs). They are predominantly unstructured and mainly  $\alpha$ -helical after drying and also they both have same effect on the thermotropic phase behaviour of dry liposomes (Thalhammer et al., 2010). Although, great explanations were made by Thalhammer et al (2010) and Hinch and Thalhammer (2012) the membrane binding of these proteins and their folding in the hydrated state still to be cleared.

It can be concluded that COR proteins might increase cold tolerance in wheat at early stage after vegetative transition as well as in vegetative growth stage in both varieties under study. Also it has been confirmed that COR genes are highly cold-induced genes which encode COR proteins (e.g. COR15a), thus they impart plants tolerance to low temperature.



**Chapter 7: The effect of Molybdenum application on the development of frost tolerance of wheat.**

## 7.1 Introduction

Molybdenum (Mo) is an essential micronutrient which exists in a wide range of metalloenzymes (molybdoenzymes) in plant, fungi, algae and animals and is normally a part of the active sites of these enzymes (Mendel and Bittner, 2006). Mo requirements and concentrations in higher plants are very low but it plays a vital role (Hu et al., 2002). Molybdenum is biologically inactive and cannot catalyse the biological system unless it is combined with special co-factors (Sun et al., 2006). More than 40 molybdoenzymes are available in all organisms, but only four of these are available in plants (Schwarz and Mendel, 2006). These enzymes are nitrate reductase (NR), xanthine dehydrogenase (XDH), sulphate oxidase (SO) and aldehyde oxidase (AO) (Mendel and Hänsch, 2002). These enzymes participate in many metabolic processes, basically in redox reactions such as nitrate assimilation, phytohormone synthesis, purine catabolism and sulphate detoxification in plants (Mendel and Hänsch, 2002, Sun et al., 2009). Aldehyde oxidase (AO) is the enzyme that can catalyse the final conversion of indole-3-acetaldehyde to indole-3-abscisic acid (IAA) and also the oxidation of abscisic aldehyde to abscisic acid (ABA) (Kaiser et al., 2005). Therefore molybdenum is implicated in the regulation of ABA synthesis via AO and the phytohormone ABA in turn has been implicated in mediating the expression of COR genes (Sun et al., 2009). There is therefore a link between Mo and cold hardiness.

During the acclimation process in plants, many changes occur such as the accumulation of osmoprotectants like soluble sugars (Uemura et al., 2003), amines, and compatible solutes such as polyols, proline and betaine (Naidu, 1998, Allard et al., 1998), via activation of low temperature signal transduction pathways which eventually lead to membrane stability and altered gene expression to provide the

tolerance at all levels (Thomashow, 2010). Recent studies have reported that gene expression for wheat might be changed not just through low temperature (4°C), but following exposure to some chemicals used for seed priming e.g. Molybdenum (Mo), which can increase CBF expression (Sun et al., 2009). Moreover, this study has also showed that this chemical increased the plant ability to tolerate low temperature through a regulation of abscisic acid (ABA) biosynthesis via Aldehyde oxidase (AO). Meanwhile, Sun et al (2009) reported that the AO activity, ABA content, and Indole acetic acid (IAA) content increased in Mo treated wheat leaves.

In order to improve our understanding of the molecular basis of cold stress enhanced by Mo application under normal and low temperature, the present work studied frost damage to wheat genotypes in relation to CBF14 gene and COR15a protein expression in response to Mo treatments and low temperature.

## **7.2 Materials and methods**

### **7.2.1 Pilot studies – Experiments 1 & 2.**

A pilot study was carried out prior to the main experiment in order to make sure that Molybdenum really can be used as CBF inducer with or without acclimation. Two methods of Mo application were used. First (Expt 1), Mo was used as a foliar application at a concentration of 15 PPM (~0.022 mM). Plants were sown in pots with 9 plants per pot. When plants reached the 3-4 leaves stage, pots were divided into two groups. One group was sprayed with Mo solution and the other group was sprayed with distilled water. Then each group of these two groups was sub-divided into two groups: The first group was moved to a warm growth cabinet (20°C) and the

other group was moved to cold store (4°C). After one day, samples were collected from each treatment and stored at -80°C for later molecular analysis.

The second method (Expt 2) of Mo addition used seed lots divided into two groups. The first group was soaked in a solution of 1% Mo (~8.13 mM) for 8 h at room temperature, and the second group was soaked in distilled water. Seed of both treatments were then dried back until they reached their original moisture content (14 %). Then the seeds were sown in pots with 9 seeds per pot and left to grow until they reached the 3-4 leaf stage. Mo treated and non-treated seedlings were then subdivided into two groups, one was moved to a warm growth cabinet (20°C) while the other was moved to the cold store (4°C). After one day samples were collected and kept at -80°C for later molecular analysis.

### **7.2.2 Seed treatment with Mo and plant materials (Experiment 3).**

Seed of two varieties were used, European wheat cv. Claire and Iraqi wheat cv. Abu-Ghraib. Two treatments (+Mo and -Mo) were applied to seeds of both varieties. Half of the seeds from each genotype were soaked in a solution containing 1% molybdate [(NH<sub>4</sub>)<sub>6</sub>Mo<sub>7</sub>O<sub>24</sub>.4H<sub>2</sub>O] (0.008 M) for 8 h at 20°C (Jafar et al., 2012, Wang et al., 1999), and the other half were soaked in distilled water for the same period (hydro priming) and then air dried until they reached their original moisture content (14%) (Ahmadi et al., 2007). Seeds were then sown in trays in a growth cabinet (Snijder scientific) in order to obtain seedlings at the same physiological age. Seedlings were transplanted into pots (127 ×127 ×152.4 mm) with 9 seedlings per pot, containing John Innes N°. 1 compost. Seedlings were left to grow in a semi-controlled greenhouse (~20°C) for 30 days until the 3-4 leaf stage (GS13-14) (Zadoks et al., 1974). Pots of each treatment were subdivided into two groups (n=7), and placed

either into an acclimation chamber at 4°C, 8 h photoperiod (PAR 177  $\mu\text{mol m}^{-2} \text{sec}^{-1}$ ) for 14 days, or a growth cabinet at 20°C at the same photoperiod and PAR. Samples for both frost tolerance assessment and molecular analysis were taken after 0, 8, 24 hours and 14 days from transferring plants to the new conditions. Samples for molecular study were stored at -80°C until analysis.

### **7.2.3 Frost tolerance assessment.**

Frost damage was estimated as Relative Electrical Conductivity (REC%) (see chapter 2). Leaves were cut and put singly into labelled boiling tubes (75 mL) and then exposed in stepwise intensities of sub-zero temperature stress (0, -4, -6, -8, -10°C) with a 2h hold at each temperature after which samples were removed.

### **7.2.4 Total mRNA extraction, cDNA synthesis and quantitative PCR (RT-PCR).**

Extracted RNA (see chapter 2) was quantified using the Nano drop 1000 technique to estimate its concentration and then stored at -80°C. The first strand cDNA was obtained by using M-MLV Reverse Transcriptase (Sigma: M1302) in 20  $\mu\text{L}$  volume. Forward and reverse PCR primers were designed for the *Cbf14* gene. The wheat 18s rRNA was used as an endogenous control. Primers for the genes were designed with gene sequences obtained from Blast software to give the following primer templates: forward primer *Cbf14*-int-F 5'-CCGTTCAGCACCGCCAAGGA-3', reverse primer *Cbf14*-Int-R 5'-CCATGCCGCAAACCAGTGC-3', forward 18s rRNA 5'-TGTGCCTAACCCGGGGGCAT-3' and reverse 18s rRNA 5'-GAGCGTGT TTTGGCGTGACGC-3'. All primers were obtained from Eurofins MWG. cDNA was used as a template for the RT-PCR detection system. The cDNA for the samples was used as template for the real time PCR reaction (Applied Biosystem,

StepOne Plus) with SYBR Green JumpStart Taq ReadyMix (Sigma kit Cat. # S4438-100RXN). The PCR thermal cycle was optimised to be as follow: 10 min denaturation at 95°C and then 40 cycles of 15 sec at 95°C and 1 min at 60°C. The melting curve was set up at the end of the 40 cycle for 15 sec at 95°C, 1 minute at 60°C and 15 sec at 95°C in order to be sure that only the gene of interest and the control gene were amplified.

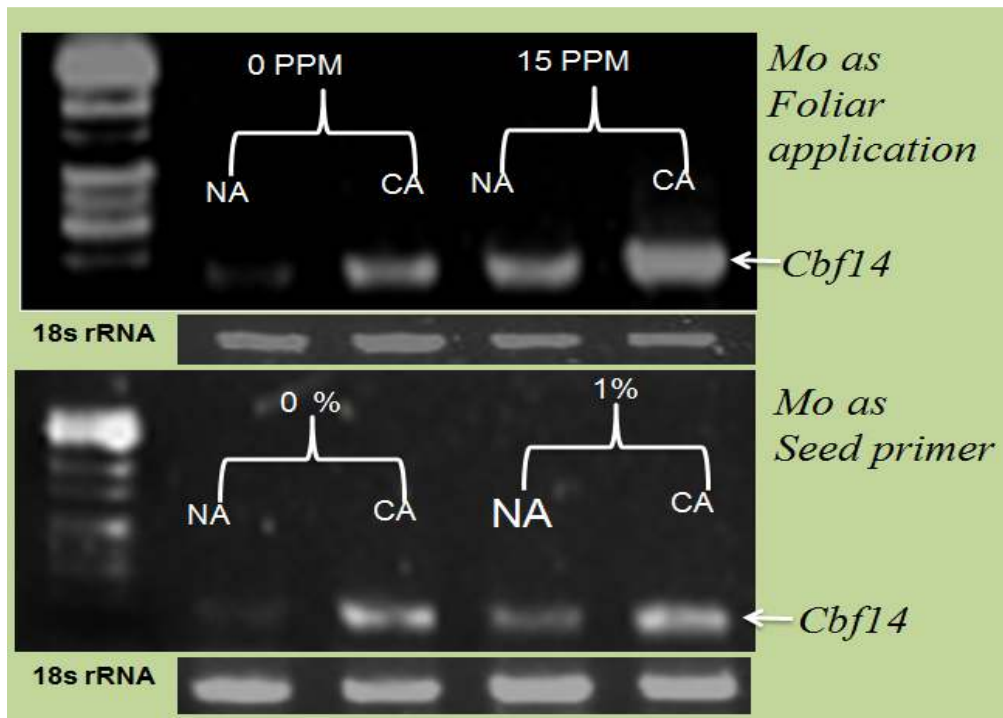
### **7.2.5 COR15a detection**

Samples for COR15a protein investigations were taken from each treatment simultaneously with the samples of 14 days of *Cbf14* expression and stored at -80°C. Protein was extracted and estimated from these samples according to standard operating procedures (see Chapter 2). The detection of this protein by the Western blotting technique (see Chapter 2).

## **7.3 Results**

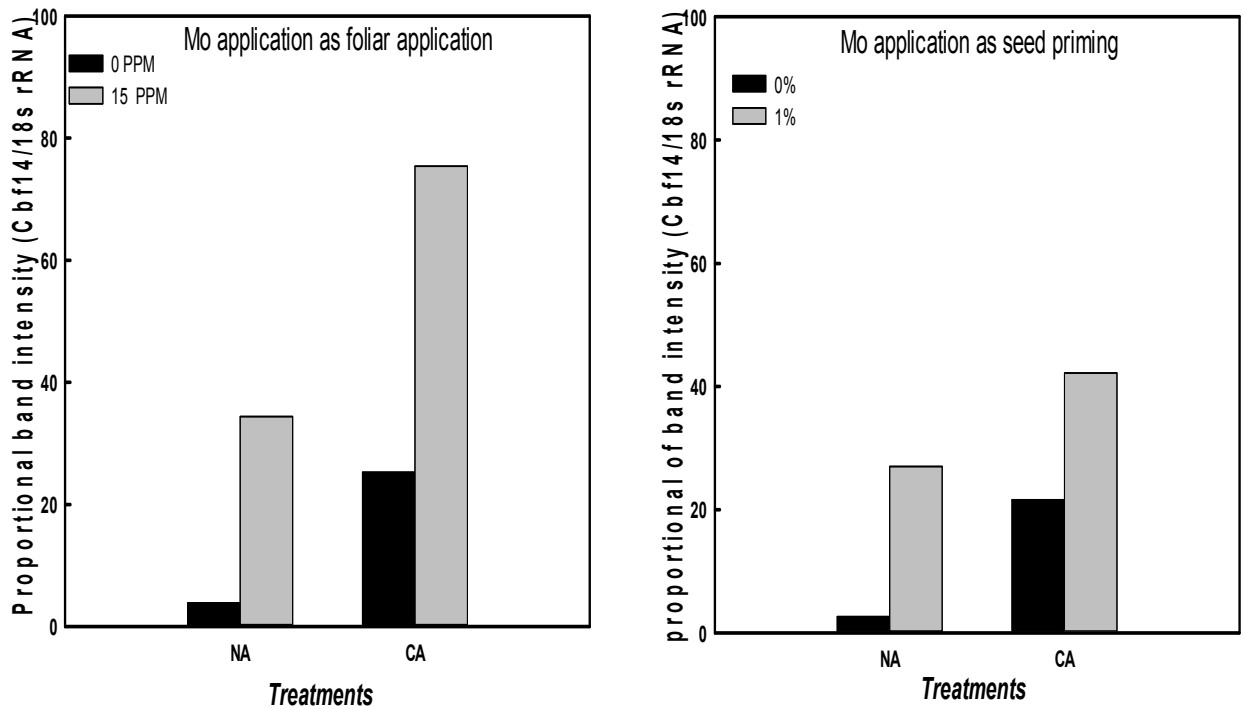
### **7.3.1 CBF expression in two different ways of application as a pilot study**

The pilot study showed promising results and gave an incentive to extend the investigations of the Mo effect on the *Cbf14* and COR15a expression in wheat. Regardless of the way of adding Mo to plants, Mo was found to increase the up-regulation of frost tolerance genes and increase the plants ability to withstand to low temperature (Plate 26; figure 14). Whilst it was found that Mo increased *Cbf14* expression in both acclimated and acclimated plant the combination of Mo and low temperature showed a combined effect with even higher expression of *Cbf14*.



**Plate 26.** The expression of *Cbf14* under two different methods of Mo application and two temperature treatments (Expt 1 above; Expt 2 below).

The most interesting result recorded here was that Mo increased the expression of *Cbf14* in non-acclimated plants when compared with un-treated plants. Practically this finding gives a very good motivation that the application of Mo has the potential to increase the ability of plants to tolerate low temperature or any abiotic stress that can share a network with it. Also it has been observed that the combination of both Mo application and LT can increase the expression of *Cbf14* much higher than either on their own (figure 14). These findings gave the stimulus to continue with these investigations.

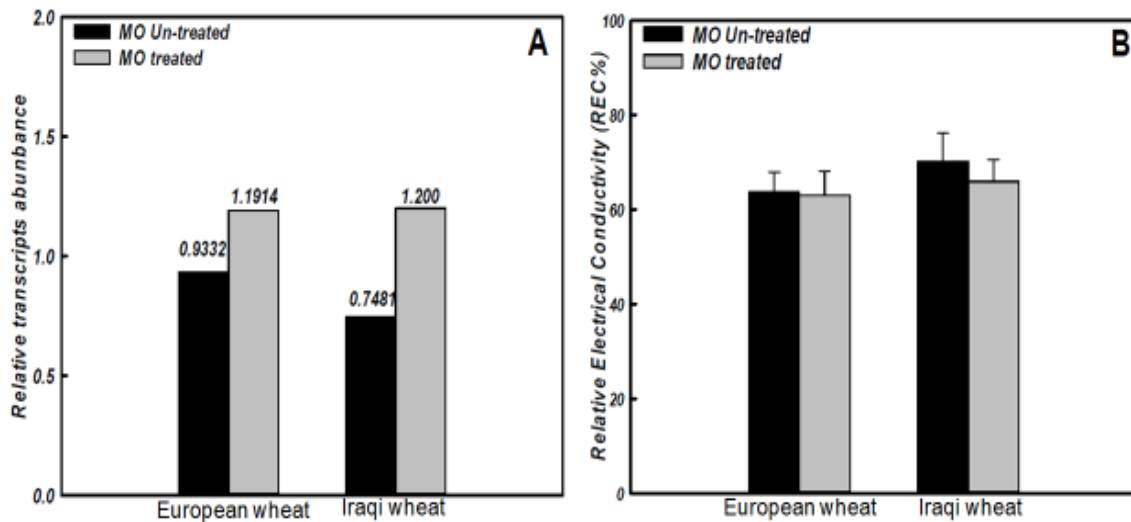


**Figure 14. Band intensity proportion of Cbf14 expression relative to the endogenous control gene 18s rRNA, (Expt 1 left; Expt 2 right).**

### **7.3.2 Effects of Mo on Cbf14 expression and frost damage to wheat.**

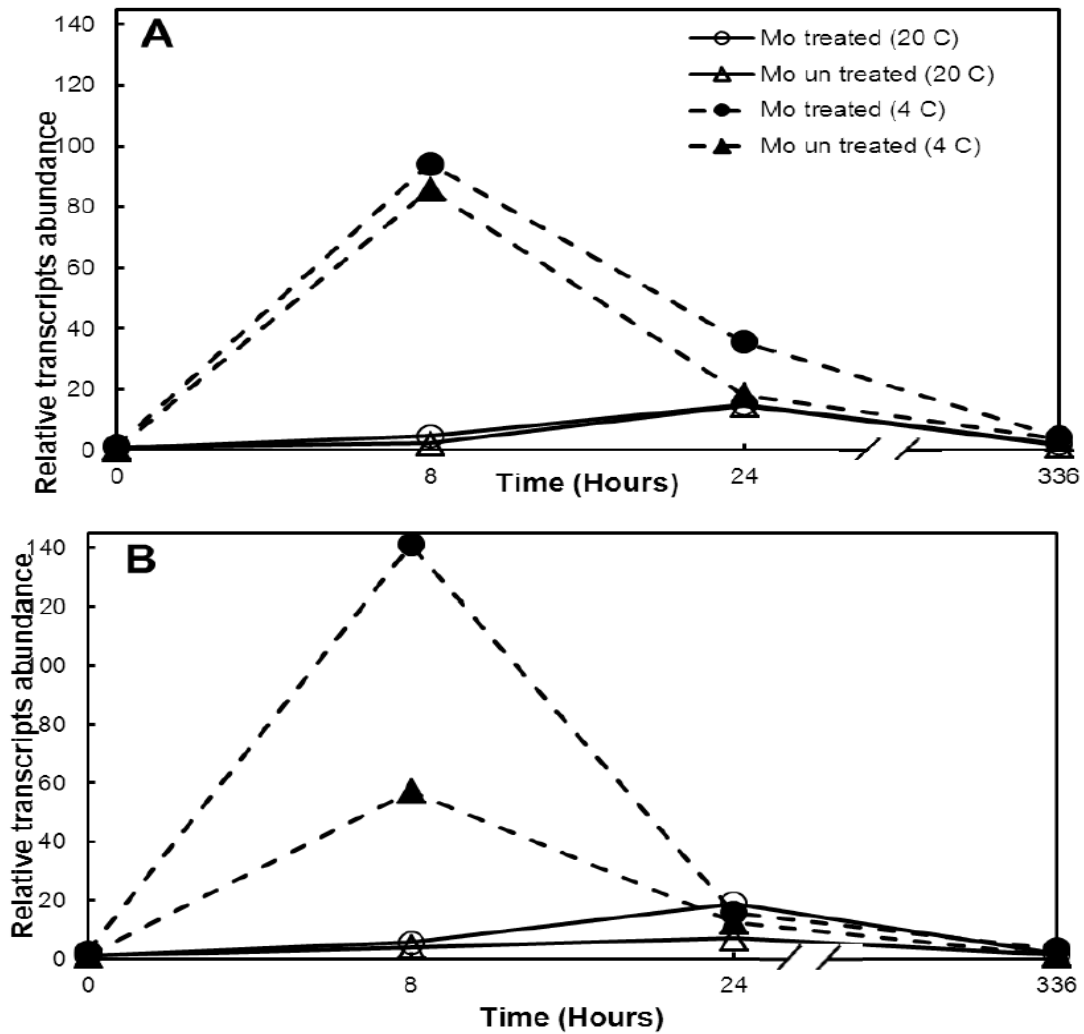
The *Cbf14* expression under +Mo treatment in both genotypes was significantly higher ( $P < 0.001$ ) than –Mo after 30 days from sowing and before exposing plants to low temperature (Figure 15-A). This increase suggests that Mo can induce the transduction pathway for CBF expression in wheat even without exposing plants to acclimating temperatures. However, there were no significant differences in the physiological response of either genotype in response to Mo in terms of frost resistance in the absence of acclimating temperature exposure (Figure 15-B).





**Figure 15. Effects of molybdenum on *Cbf14* expression (A n=2) and frost damage (B n=7), after 30 days from sowing wheat genotypes and before transfer plants to low temperature (4 °C) (Expt 3).**

Transcripts levels of *Cbf14* were increased markedly after 8 hours exposure to acclimation temperature (4°C) in both genotypes (Figure 16) and there was significantly higher transcript abundance in +Mo treated and acclimated plants in both varieties (Figure 16). The added response to +Mo was proportionately higher in the Iraqi variety than the European variety. After 24 hours the transcript level declined to less than the acclimated only treatment in the Iraqi variety (Figure 16-B) and also declined in the European variety but was maintained at a significantly up-regulated level compared to the acclimated only treatment (Figure 16-A). The expression of *Cbf14* in Mo treated plants under warm conditions (20°C) in comparison to acclimating temperatures was very low in both genotypes but there was some significant up-regulation with +Mo in the Iraqi variety at 24 h. It was clear that for wheat of both varieties grown under warm conditions (~20°C) that without +Mo *Cbf14* was not expressed.



**Figure 16. The effect of molybdenum (solid and open circles) on Cbf14 transcript abundance under normal (solid lines) and acclimating (dotted lines) temperature conditions in (A) Claire and (B) Abu-Gharaib.**

Frost tolerance increased as a result of acclimation of more than 24 h (Figure 17) and molybdenum (+Mo) enhanced this effect. Frost damage under acclimation was similar in both varieties, but when plants were exposed to low temperature stress, European wheat demonstrated significantly improved tolerance (ANOVA  $p < 0.05$ ) to frost damage (REC 22.58 %) compared with Iraqi wheat (REC 40.68 %) (Figure 17).

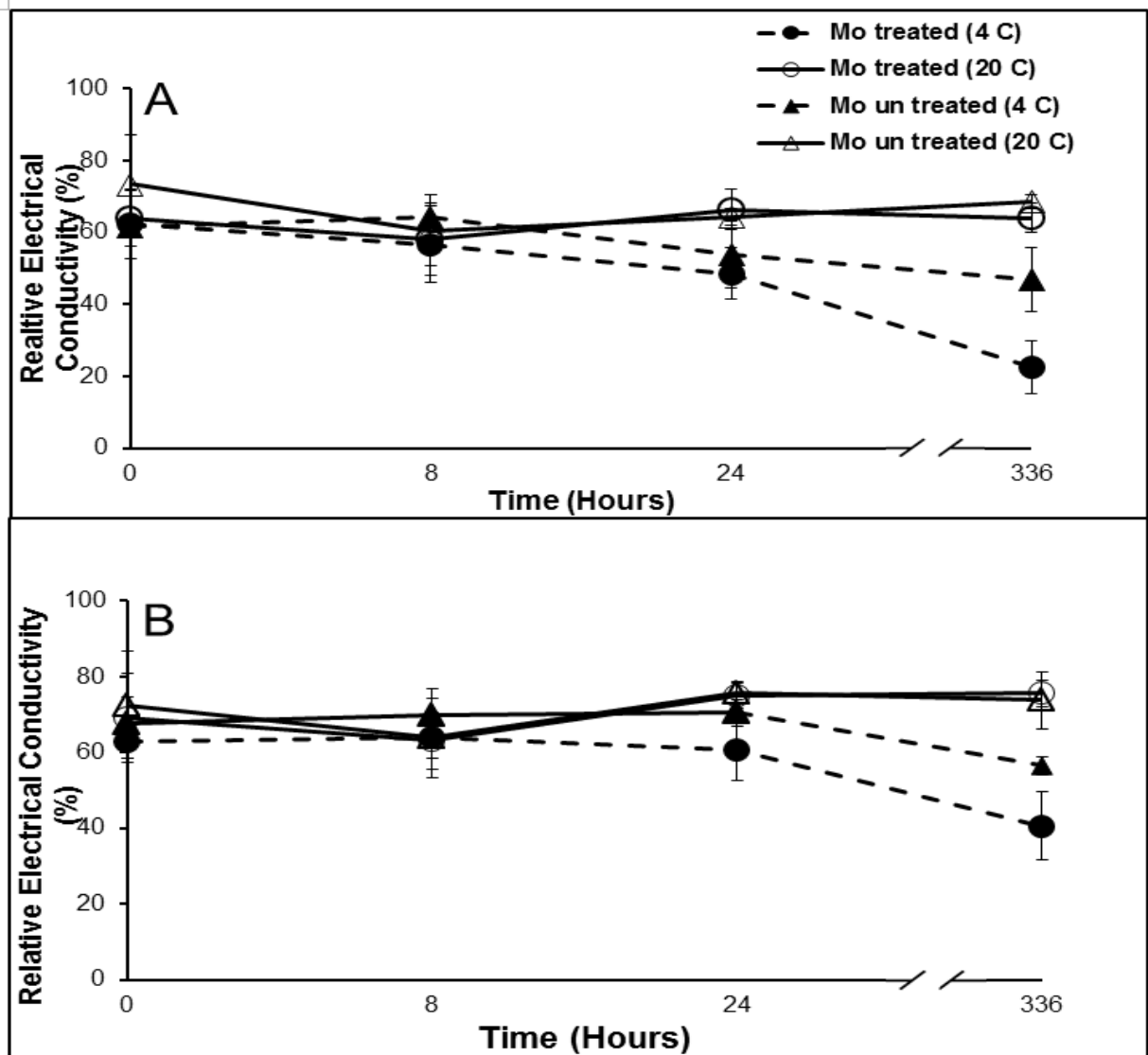


Figure 17. The effect of molybdenum (solid and open circles) on frost damage under normal (solid lines) and acclimating (dotted lines) temperature conditions in (A) Claire and (B) Abu-Ghariab (means + se).

### 7.3.3 Effects of Mo on frost Hardiness (LT50) in wheat genotypes

The application of molybdenum resulted in an increase in frost tolerance in both wheat varieties and the two varieties showed similar patterns in their response to the treatments. Acclimation clearly enhanced frost tolerance in wheat after 14 days exposure to low temperature and the REC% was significantly ( $p < 0.000$ ) less in acclimated plants with and without Mo application. Mo treated plants were more tolerant to freezing temperatures compared to acclimation alone. In general,

European wheat had consistently more frost tolerance compared with the Iraqi wheat.

LT50 values in acclimated plant were lower than those growing under non-acclimating conditions for both varieties. There were no significant differences between Mo treated and untreated plants under non-acclimating conditions (Table 14). European wheat showed a higher capacity (-8.14°C) to be acclimated than Iraqi wheat.

**Table 14. LT50s for the Molybdenum (+Mo) treated and untreated (-Mo) plants in acclimated (CA) and non-acclimated (NA) state.**

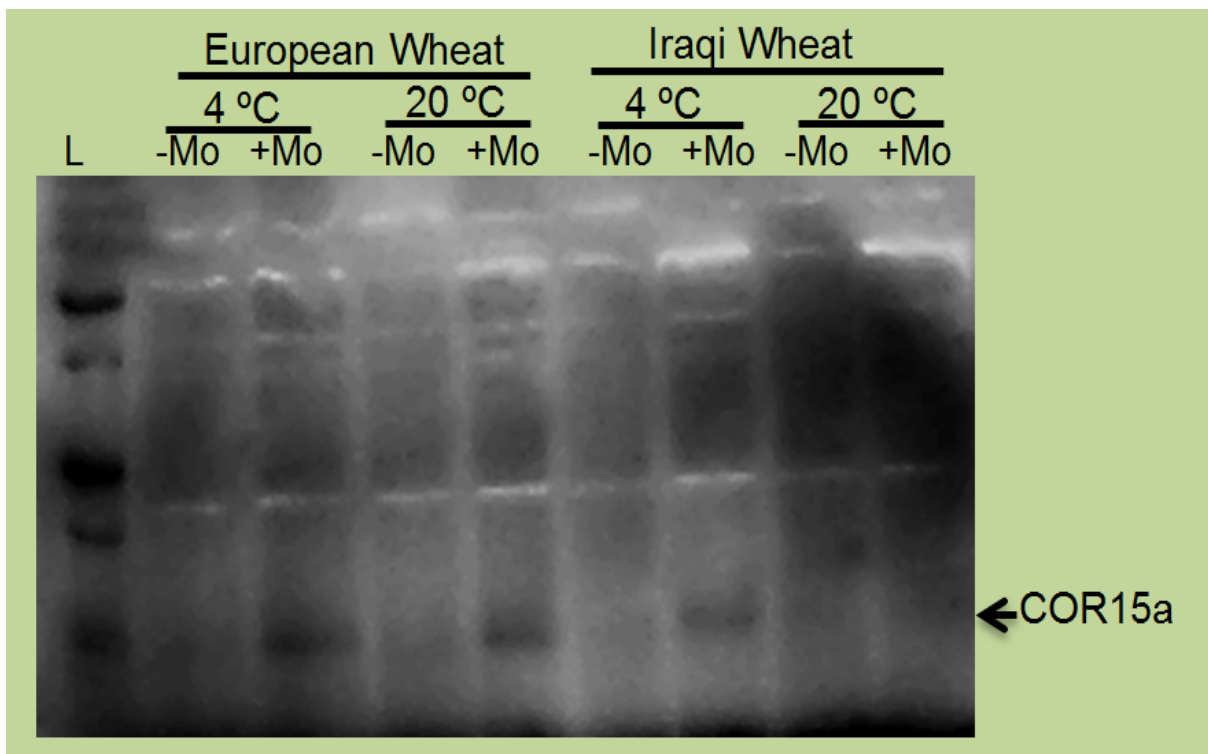
Genotypes	Mo	NA	CA	$\Delta$ LT <sub>50</sub> Due to acclimation	$\Delta$ LT <sub>50</sub> Due to Mo addition	
					NA	CA
European	+Mo	-5.43	-8.14	-2.71	-0.27	-1.07
	-Mo	-5.16	-7.07	-1.91		
Iraqi	+Mo	-5.53	-7.40	-1.87	-0.51	-0.43
	-Mo	-5.02	-6.97	-1.95		

Acclimation lowered the LT50 by approximately 2.0 °C in both the European and Iraqi wheat while the Mo application lowered the LT<sub>50</sub> further by 1.07°C and 0.43°C in European and Iraqi wheats respectively.

#### **7.3.4 Effects Mo on COR15a protein expression during vegetative stage in Iraqi and European wheat.**

COR15a protein was investigated in molybdenum treated and non-treated wheat under acclimated (4°C) and non-acclimated (20°C) temperatures. Results revealed that no bands were detected in –Mo plants of the two varieties grown under warm conditions (20°C) (plate 27). However, there was COR15a protein expression when

plants were acclimated (4°C) whether they treated with Mo or not. The most important result was that the COR15a was detected in Mo treated plant under normal condition in European wheat but not in Iraqi wheat. However the expression of COR15a protein in acclimated and treated plant was higher than its expression in Mo only treated plants. The results of COR15a protein expression were consistent with the findings of *Cb1f* expression especially in the European wheat.



**Plate 27. Western blotting analysis for detection of COR15a in Mo treated and non-treated under two temperature treatments (4 and 20°C) for both Iraqi and European winter wheat.**

## 7.4 Discussion

Molybdenum is an essential micronutrient for plants (Kaiser et al., 2005) and since it is a transition element, it can be presented in several oxidation states. It has previously been proposed that molybdenum can be involved in the amelioration of frost damage (Li et al., 2001, Du et al., 1994) and improvement of freezing tolerance in wheat (Sun et al., 2006, Wang et al., 1995) but the mechanism for this improvement has not been definitively determined. Mo is not free in cells, since it is very unstable and therefore it would be expected to be in a tight complex with proteins. It is stored in this status and utilises Mo carriers (Vunkova-Radeva et al., 1988, Rajagopalan and Johnson, 1992) when cells require it. There are several possible ways by which Mo could enhance the development of freezing tolerance in plant cells. 1. Mo may increase the anti-oxidative defence by increasing the activity of the anti-oxidative enzymes SOD, CAT and POX (Yu et al., 1999). Frost stress imposes induced oxidative stresses by producing Reactive Oxygen Species (ROS), which reacts very rapidly with DNA, Lipids and protein and then cause cellular damage (Sattler et al., 2000). Sun et al (2006) claimed that Mo application enhanced the adaptation of plants to low temperature stress by increasing the ability of scavenging the ROS thus alleviating membrane damage in winter wheat under low temperature. 2. Alternatively, Mo may increase the activity of AO and thereby the ABA content and it has been found that these were higher in Mo treated plants compared with Mo untreated plants. ABA biosynthesis through AO can trigger bZIP and the up-regulation of the ABA dependent COR gene expression pathways (Sun et al., 2009) and thereby protect against frost damage. 3. A third mechanism could be through nitrogen reductase (NR) and enhancing nitrate assimilation (Hamdia et al., 2005) as it has been found that NR activity was significantly increased in Mo-treated

wheat (Hale et al., 2001, Stallmeyer et al., 1999., Yu et al., 1999) and in addition K<sup>+</sup> ions increased and this was associated with amino acid accumulation (Hamdia et al., 2005). One of these amino acids could be proline since it has been found that this amino acid was increased under low temperature stress (van Swaaij et al., 1985) and water stress (Stewart and Voetberg, 1985). 4. The results presented in the current study provide evidence for a fourth pathway demonstrating that Mo can influence the up-regulation of the CBF pathway and this in turn can lead to COR up-regulation increases protein content in cells and decreases the damage caused by freezing temperatures (Thomashow, 2010, Jaglo-Ottosen et al., 1998b, Stockinger et al., 1997). It is speculated that exogenous Mo application leads to overexpression in *Cbf14*, which then led to feedback inhibition. This was confirmed after 24 hours exposure to acclimation where *Cbf14* transcripts dramatically declined in Iraqi wheat compared to European wheat, which responded to Mo application in a more consistent manner. The results suggest that Mo can increase the transcripts levels of *Cbf14* at 20°C (non-acclimating) in both wheat varieties and that Mo treatment applied as seed priming can increase the constitutive expression of abiotic stress genes (e.g.*Cbf14*) and when combined with acclimating temperatures and can significantly improve frost stress more than low temperature alone. It is well documented that wheat can be acclimated and re-acclimated to low temperature while it is still in the vegetative phase of growth (Mahfoozi et al., 2001) and LT50 results presented here confirmed that European wheat was more tolerant to freezing when acclimated than Iraqi wheat (Table 14).

The findings here with Mo may offer an opportunity to use Mo to up-regulate the CBF pathway and stimulate acclimation in crops threatened with late season frosty weather.

Results in this chapter showed a strong association between *Cbf14* expression and frost damage in European wheat, while it was much less in the case of Iraqi wheat. This suggests that *Cbf14* might not be followed by activation of *COR* genes in Iraqi wheat and the improvement in *Cbf14* expression was because of another pathway. Further studies are needed in order to clarify this molecular response in Iraqi wheat. Prior to acclimation, Mo was able to increase *Cbf14* expression, but this increase was small (Figure, 15). However, in the presence of acclimating temperatures, Mo significantly increased transcripts levels of *Cbf14* and also enhanced frost tolerance. Whilst the results presented here do not fully explain the effects of Mo in frost tolerance they do confirm that Mo can help to up-regulate the important transcription factor *Cbf14* of cold acclimation and thereby enhance frost tolerance in wheat. However, in the absence of acclimating temperatures this increase did not lead to an increase in frost tolerance. When acclimating temperatures were imposed, Mo treatment enhanced the development of frost tolerance in both varieties but not by the same level. Iraqi wheat responded more quickly to the application of Mo at the level of *Cbf14* expression however, it also dropped away more quickly. It has been reported that the constitutive expression of *COR15* enhances the frost tolerance of the chloroplast and reduces the damage to photosystem II in non-acclimating plants (Artus et al., 1996). *COR15a* is transported into the chloroplast and accumulates in the stromal compartment (Shimamura et al., 2006). Sun et al (2009) thought that Mo maintained the activity of the photosynthetic system through the regulation of the redox state in wheat chloroplasts (Takumi et al., 2003). Sun et al (2006) and Yu et al (2006) also found that low temperature stress and deficiency of Mo decreased the rate of net photosynthesis (*P<sub>n</sub>*), and the application of Mo enhanced frost tolerance as well as photosynthesis through the regulation of ABA-independent *COR* gene



expression. Further studies to investigate the use of different concentrations of Mo and different methods of application will be useful on both genotypes in order to see whether the Mo enhancing effect can be optimised further. Furthermore, it would be useful to investigate the effect of Mo application to wheat genotypes at later growth stages to investigate whether it can be used to overcome the development limited expression of frost resistance in wheat.

## **Chapter 8: General Discussion**

## 8.1 introduction

Whilst there is generally a predicted rising in temperature associated with global warming there are still predicted to be risks associated with low temperature for temperate crops. One problem is where plants can grow faster and achieve the susceptible growth stages earlier in the season when radiation frosts are still a risk. This scenario could apply to wheat. When wheat is exposed to frost during the flowering stage huge economic losses can result and this problem frequently occurs in key production countries including Australia, South America and Mediterranean areas (Frederiks, 2005) and China (Zhong et al., 2008).

### **Low temperature is a problem in wheat during flowering?**

Across all the results presented in this study it has been confirmed that wheat is unable to avoid serious damage from subzero temperatures below  $-5^{\circ}\text{C}$  during flowering. In this study it was confirmed that wheat in a vegetative stage can tolerate to around  $-5^{\circ}\text{C}$  whilst this is lowered to  $-7.47$  and  $-6.41^{\circ}\text{C}$  for European and Iraqi wheat respectively after 14 days acclimation treatment. Although there was evidence that acclimation as indicated by *Cbf14* can be switched on early in the flowering stages, such acclimation temperatures are not expected during the flowering stage in most agroclimatic zones for wheat during such growth stages. Therefore in a zone where frost during heading are common, it could be a better tactic to keep acclimation constitutively expressed than delaying planting date to avoid frosts as this contributes to reducing yield potentials as heading is delayed. Frederiks et al (2008) have reported that growers in Australia are routinely delaying the planting date so that flowering will also be delayed, thus avoiding potential risk of radiant frost in spring; however, it seems that flowering would not be much affected in this delay

since it strongly depends on vernalisation requirements and photoperiods. In addition any delay in the sowing date might expose plants to high temperatures and diminishing water supplies, late in the season, or as is the case in Australia, push ripening into the wet season which is equally unacceptable agronomically.

It has been postulated that low temperature during flowering mainly kills the male parts of the flowers and causes sterility (Reynold et al., 2001). Flowering in wheat spikes usually starts from the centre and moves towards the top of the spike first and then down to its base, it is feasible therefore that exposure to transient sub-zero temperatures during this period might cause either partial or complete sterility. In the study here, it was noted that where variable sterility occurred due to frost, grain set was normal in the centre of the spike while the aborted florets were either at the top or the bottom. It was also observed that spikes at an early growth stage were more prone to be completely damaged, while spikes at a more advanced age were only partially damaged. From the infrared work it is postulated that this is because the ice front is delayed at the rachis nodes. Single (1964) also postulated that the ice might be internally formed in young spikes causing their complete death while mature spikes might be partially damaged since the ice might be halted at the nodes along the rachis. Also he stated that when unprotected spikes emerge from their leaf sheaths, direct inoculation of the spike by ice nuclei in the atmosphere promotes greatly increased risk of spike damage. In this important stage of wheat growth, it is possible to attempt to increase plant tolerance to frost by increasing expression of cold acclimation genes. Alternatives would be useful to increase the ability of wheat to be acclimated to low temperatures, specifically during flowering. Based on the finding that various abiotic stresses share some components of their signal transduction, plants might be able to be adapted to tolerate sub-zero temperatures if

they undergo some other types of pre-conditioning abiotic stress. It was shown here that the expression of cold acclimation could also be increased by treating plants with Molybdenum and this could be a useful intervention tool for farmers if it could be shown to have effect in the field situation. Otherwise, genetic transformation remains the only route to keep cold acclimation genes constitutively expressed in plants in vulnerable growth stages.

REC % results revealed that the frost damage of European wheat was significantly less ( $P= 0.003$ ,  $n= 12$ ) in acclimated plants as compared to acclimated Iraqi wheat ( $P= 0.296$ ,  $n= 12$ ) in growth stage A. It was also confirmed through this study that the tolerance occurred because of the expression of the cold acclimation in this specific growth stage. This transcription factor (*Cbf14*) was found to be expressed during this stage in both varieties, but the expression was much higher in the European cultivar as compared to the Iraqi cultivar, and this significantly affected the frost damage. Kume et al (2005) have reported that cold acclimation can be upregulated two times with the first activation occurring within the first four hours from exposing plants to low temperature, which in turn corresponds to the rapid response to low temperature. The second activation was observed after 2-3 weeks of cold acclimation treatment. After the second upregulation the amount of CBF transcripts increased more in winter wheat than spring wheat (Kume et al., 2005). They conclude that the maintenance of high level of *wcbf2* transcripts might represent the long term effect of cold acclimation and this gave an indication that cold acclimation can be triggered after vernalisation fulfilment and is contrary to previous hypotheses. It was suggested

that vernalisation gene (VRN1) can down-regulate the CBF/COR pathway in cereals (Limin and Fowler, 2006). Also Liu et al (2002) have hypothesised that CBF is involved in the vernalisation cold signalling and CBF could substitute for vernalisation and abbreviate the vegetative phase in winter annual cereals. But their findings indicated that vernalisation involves a pathway that is completely different from cold acclimation (CBF) pathway when they found that flowering time was not accelerated in transformed plants with CBF constitutively expressed. The CBF induced downstream gene COR15a was also expressed in the experiments reported here at growth stage A (GSA), and it is known that this is partially responsible for imparting tolerance to freezing temperatures (Lin et al., 1990). In contrast, cold acclimation does not seem to be activated in advanced growth stages of spike development (e.g. GS B spikes 75% emerged) and CBF expression was not evident at this growth stage. It was clear from these findings that inductions of CBF genes are responsible for cold tolerance induction in wheat. CBF was found to be activated by a low temperature signal pathway, and subsequently led to the induction of the CBF regulon (as represented by COR15a) (Denesik, 2007).

In the European wheat there was a relationship between the cold acclimation expression and frost tolerance in growth stage A, but not in Iraqi wheat. This suggests that it is not necessary for the CBF expression to correspond to the frost tolerance in all varieties of wheat. Denesik (2007) has reported that not all cold regulated genes corresponded to the level of cold tolerance in all genotypes. He found only that the expression pattern of *Wcs120* followed the pattern of cold tolerance in all genotypes, while *WCBF1*, *Wcor14b* and *Wcor410* only corresponded to the level of cold tolerance in some of the genotypes under study. In general, it can be concluded that cold acclimation can be triggered when just 10% of the spike is visible

on the whole spike, and not later on. Once spikes start to flower (anthesis), the frost tolerance will be more complex due to the sensitivity of male and female organs and the complications of supercooling.

### **Can spikes be supercooled and escape frost damage?**

The results of the infrared study confirmed that wheat spikes have a tendency to supercool at the flowering stage and freezing damage can be avoided by supercooling. This confirms that it is a common fallacy that all plants or plant parts are frozen at the same time if they are exposed to sub-zero temperatures (Barker, 2010, Fuller et al., 2009). It has been shown that when ice nucleators are absent, plants or their parts can escape freezing damage even when temperatures are lowered below the frost tolerance temperature of the plant (Wisniewski et al., 2002, Fuller et al., 2003). Results through this study showed that wheat spikes were commonly found to supercool when they were exposed to temperatures of -3 to -8 and even survived as low as -12°C, well below the acclimated LT<sub>50</sub> tolerance temperature. It was found that less than 25% of the spikes under study were frost damaged, while the rest supercooled, and this confirmed that spikes have a high tendency to supercool which was suggested by Fuller et al., (2007).

Although the studies here show damage to wheat during flowering was associated with freezing, it has been speculated that this may be less to do with freezing and more to do with a chilling injury effect during anthesis (Fuller, 2007). If low temperature is experienced during anthesis then catastrophic economic losses can be expected; however, any freezing temperature after the flowering stage might affect the quality of wheat seeds. Seeds that are slightly exposed to freezing temperatures at the milky-ripe stage might grow to the normal size but not fill properly

at maturity (Shroyer et al., 1995). According to the given findings here, wheat spikes mostly tend to supercool during this stage but the main experiment also acclimated for 14 days at chilling temperatures (4°C) without any consequence on spike fertility and thus no effect of chilling damage was noted. It is also clear however that the supercooling phenomenon in wheat needs to be further investigated as supercooled plants can avoid severe frost damage.

Knowing where and how ice has formed and how it progresses in spikes might give an idea about how to control ice formation through a variation in supercooling. In the literature there are very few studies conducted using an IR camera in wheat spikes (Fuller et al., 2009, Fuller et al., 2007, Al-Issawi et al., 2012) and so this is a very under explored research field. The findings of the IR studies here were consistent with those published earlier and revealed the spikes at this stage are highly likely to escape frost damage through a supercooling mechanism. Spikes were not frozen at the same time and this supports the suggestion that ice formation depends on the availability of ice nucleators and these may not be uniformly distributed on the spikes (Reynolds et al., 2001). It seemed that spraying plants with distilled water was not a very effective way to ensure ice nucleation in these experiments since there were no differences ( $P$  value= 0.99,  $n=6$ ) between sprayed and unsprayed plants and may imply that internal nucleators are important. Fuller et al (2007) reported that using ice nucleators increases the probability of ice nucleation, but that distilled water was only a moderately effective ice nucleator and this is upheld here. It was also clear that the acclimation process did not help to protect ears during freezing ( $P$  value= 0.38,  $n=3$ ) and was supported by the molecular result that *Cbf14* expression cannot be triggered in advanced spikes growth stages. It was also confirmed that acclimation did not influence the degree of supercooling either. Furthermore the IR observations



revealed that freezing events usually start at temperatures around  $-5^{\circ}\text{C}$ , which is almost the same as the inherent level of frost tolerance in as put forward by Fuller et al (2007) and confirmed earlier in this thesis with vegetative plants. The coincidence of these temperatures i.e. constitutive hardiness and ice nucleation point does raise a question of whether in freezing studies ice nucleation is actually achieved above  $-5^{\circ}\text{C}$  in laboratory frost tests. It is now commonplace that lab based freezing tests apply ice nucleating agents such as ice or wet paper towels or distilled water in an attempt to control freezing and prevent supercooling, however it is possible that constitutive resistance of wheat is not  $-5^{\circ}\text{C}$  and that this is only the common ice nucleation temperature. Given the expensive nature of IR cameras it is unlikely that freezing chambers will be routinely fitted with such devices in order to verify ice nucleation but in reality, until they do there will always be a suspicion of supercooling in any frost test. The main precautions against this risk practised here was the inoculation of plants with ice at  $0^{\circ}\text{C}$  and a hold of 2 hours at each test temperature to allow ice formation, its spread and equilibration of water potentials.

Freezing in spikes is likely to be a very complex process due to the existence of the barriers which can resist the spread of ice crystallisation at different temperatures (Single, 1984). Therefore it was not surprising to find many locations of ice nucleation sites and many variations in ice progression in spikes. Ice nucleation events were not uniform along spikes and as a consequence, it has been observed that fertile and sterile florets can be found on the same rachis (Single, 1984, Al-Issawi et al., 2012). Many patterns of ice nucleation and its progression were observed through this study suggesting that there is no uniform pattern for ice nucleation and no specific first site where ice may form in wheat at spike emergence. It may depend on many factors including the growth stage, availability of ice nucleators and availability of apoplastic

“free” water. Barriers to water movement would also be very important, not just in the plant but from surface water on the plant.

Based on the findings in this study, it can be concluded that acclimation is the most likely frost hardiness mechanism used by wheat before anthesis (GS-A) and supercooling the most likely after anthesis (GS-B and GS-C). Anthesis is the most freeze sensitive stage and any small differences in temperature and its duration of exposure might cause large difference in the amount of injury (Shroyer et al., 1995). Therefore, future studies should give more attention to this specific growth stage as it seems that the greatest economic losses coincide with this stage. Even if cold acclimation could be triggered by a low temperature stimulus this is not practical to apply in the field but a treatment which helps up-regulate cold hardiness (such as Mo) could be important.

It was shown that cold acclimation could be up-regulated to a certain extent in vegetative wheat using Mo as a seed primer. Moreover cold acclimation expression at the gene level (CBF) was doubled when Mo was applied in conjunction with low temperature. These findings give an indication that both of these stimuli can increase CBF gene expression independently or when applied together on plants (Sun et al., 2009, Al-Issawi et al., 2013).

The results presented in the current study provide, demonstrating that Mo can influence the up-regulation of the CBF pathway and in the presence of cold temperatures and this in turn can lead to COR up-regulation and improvements in frost tolerance. The effect of Mo was to reduce the LT50 by -1.07 °C in acclimated winter wheat. Under normal growth temperatures (20°C) it seems that this response to Mo did not lead to effective increased frost tolerance. While the results presented

here do not fully explain the effects of Mo in frost tolerance, they do confirm that Mo can help to up-regulate the important transcription factors of cold acclimation and thereby enhance frost tolerance in wheat. However, the findings presented here can be considered to offer an opportunity to use Mo (or any other nutrients) to up-regulate the CBF pathway and stimulate acclimation in crops threatened with late season frosty weather.

## **Chapter 9: Conclusion, Limitations and Future work**

## 9.1 Conclusion

The work presented in this thesis has employed several techniques including REC%, infrared video thermography and molecular techniques in order to investigate frost damage to wheat during flowering. The core of the study, involved a field grown trial to ensure that plants being tested were typical of those grown in the field. Both acclimation and frost stress during flowering had to be simulated in the laboratory and then plant material was characterised using the above mentioned techniques. Iraqi winter wheat (cv. Abu-Ghariab) showed the same moderate frost resistance as the European variety Claire despite having being selected under a totally different set of environmental conditions. Abu-Ghariab responded to acclimation treatment better than other Iraqi wheat cultivars and can be considered to be winter wheat type. The results confirmed that there are differences among cultivars in acclimation capacities in wheat. It was also confirmed that wheat during flowering is able to tolerate freezing temperatures to around  $-5^{\circ}\text{C}$  without suffering damage, which is the same constitutive tolerance as found in the vegetative stage of non-acclimated plants. It was shown that there is a possibility to activate the expression of cold acclimation (*Cbf14*) during spike emergence but not later on. Evidence was consistent with *Cbf14* expression and activation of its regulon (evidenced by COR15a upregulation). Whenever COR15a was expressed plants became tolerant to freezing evidencing that COR15a in wheat is associated with cold acclimation. Chemically induced cold acclimation was also demonstrated with Molybdenum and this offers a promising approach to up-regulating acclimation in the absence of complete acclimation temperature environments.

The use of infrared thermography revealed a random pattern for ice nucleation in spikes whether they were acclimated or not. It did demonstrate that ice nucleation

started at temperatures around  $-5^{\circ}\text{C}$  which coincided with the inherent frost resistance of wheat as found using the REC% method. This raises the question of whether or not the vegetative plants actually froze.

Supercooling could be good alternative to acclimation for wheat during flowering if it can be controlled. Distilled water was not shown to be good ice nucleator in this experiment. The, anthesis stage is the most important stage that should be given more attention in terms of upregulation of cold acclimation. The results here that applied to wheat may apply to the other important cereal crops such as barley and rye or other agronomic plants that are regularly exposed to low temperature stress.

## **9.2 Limitations**

Generally, It was big challenge to grow wheat in pots weighed around 20 kg when it was irrigated and then move them between field and laboratory. Soils in pots have to be at the field capacity to avoid the interaction between drought and low temperature effect and that requires regular irrigation for plants. All pots then had to be moved two ways; to the lab and then returned to field for all experiments in this study which require great physical energy. These pots required great effort to dig and bury them in the soil and then dig them out at the time of treatment and replications increased the workload.

The simulation of natural frosts in the laboratory is not easily conducted and frost chambers and cold stores are difficult to be transferred to the field.

In the experiment of frost hardiness screening, six varieties were used and that required a lot of space in the frost chamber and cold store which meant that sowing had to be staggered as a consequence. Then also they had to be exposed to acclimation treatments also requiring a huge effort and long time, especially when there was an interaction between the treatments among the varieties. The biggest experiment consisted of 216 trays (6 varieties × 6 freezing temperatures treatments × 2 acclimation levels (NA, CA) × 3 replicates.

When plant at flowering stage, it was also a challenge to choose three uniform growth stages of spike emergence and required precise searching in the canopy to recognize, characterize and label the growth stage of each ear in the pot.

In the experiment with IR there was only enough space in the frost chamber for one pot, so experiment need to be set and run for each pot individually. Consistency of frost testing could be levelled as a criticism of this methodology, but it was the

logistical solution and use of a datalogger enabled the checking of freezing temperatures during each run and this reduced this criticism.

Previous use of IR have included a video recording of the events in real-time which could be analysed later using specialist software. However here, a newer digital IR camera was used and recorded on DVDs there was no software available to be used in their analysis. This limited the quantitative analysis of the experiments. This was discussed with the manufacturers but no solution was evident and the very specialist nature of the requirement meant that they were not prepared to work on a provision for this.

Using the Relative Electrical Conductivity technique was challenge in itself. It is a technique that is good for leaf tissues and whole plants but is not so suitable for spikes.

The combination of the limitations mentioned above limited the number of varieties that could be used in this study. Therefore two varieties were chosen for most of the investigations.



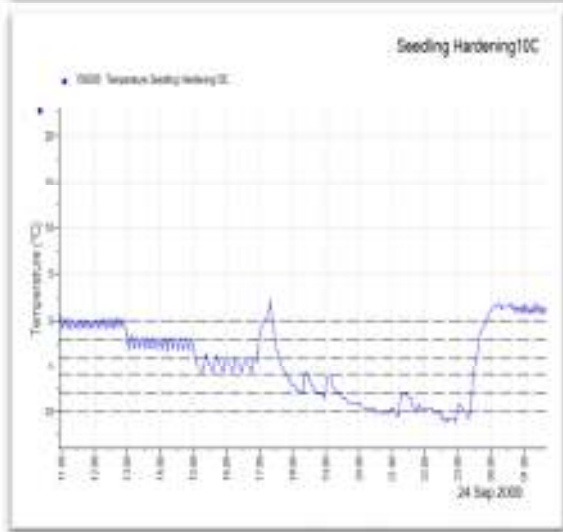
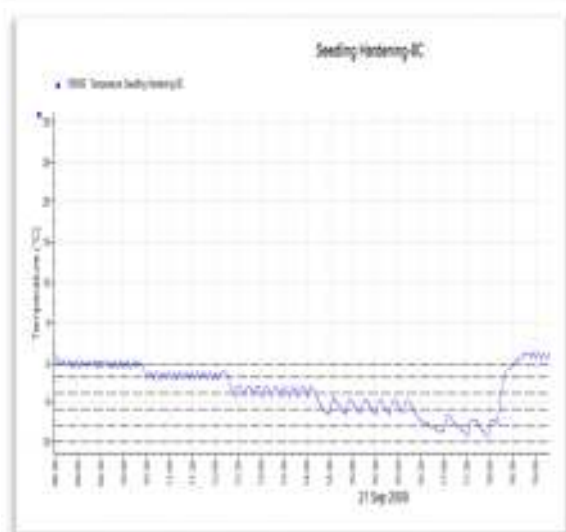
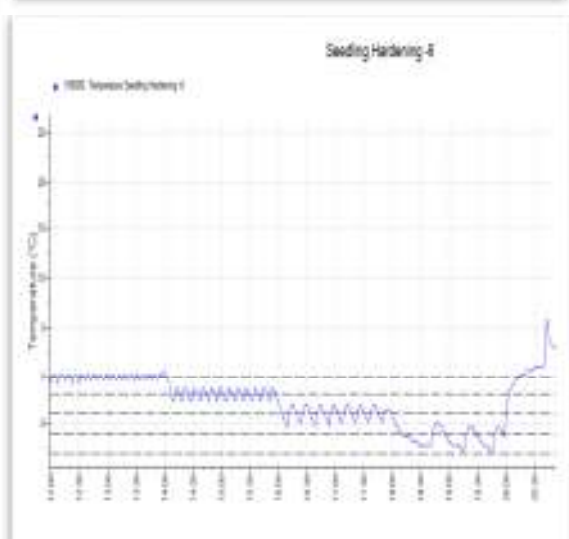
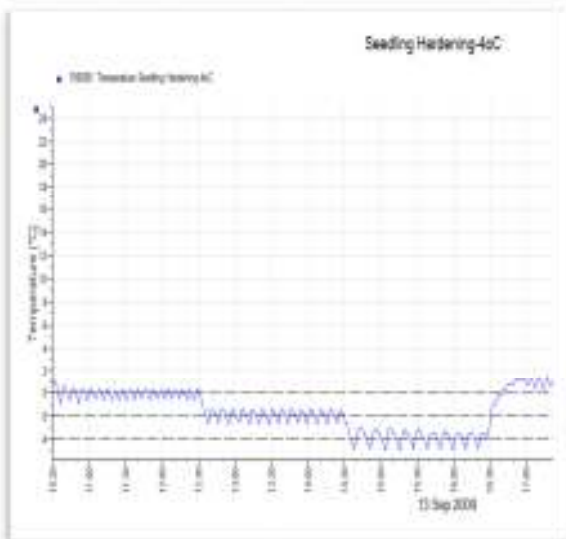
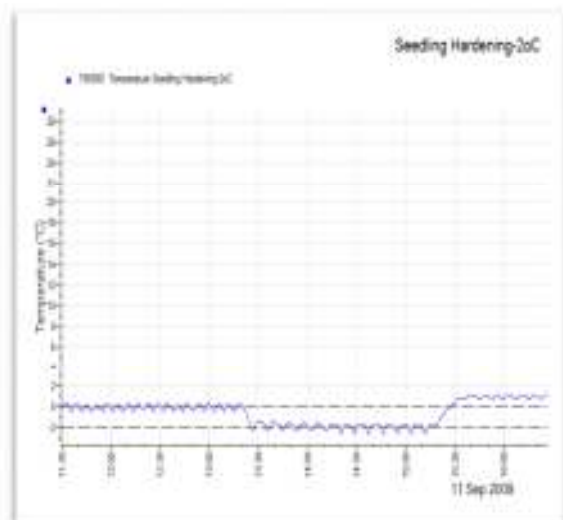
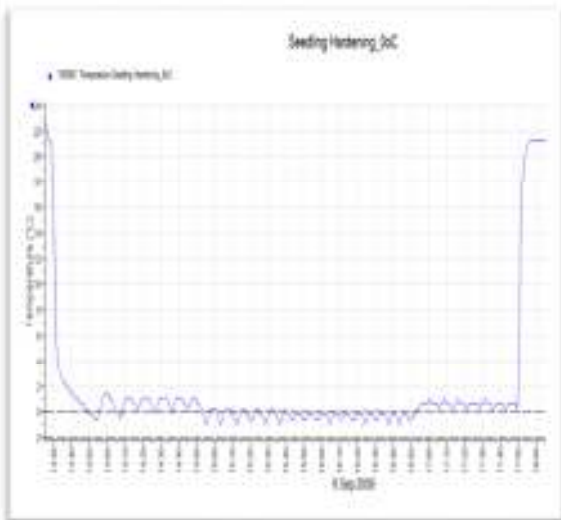
### 9.3 Future work

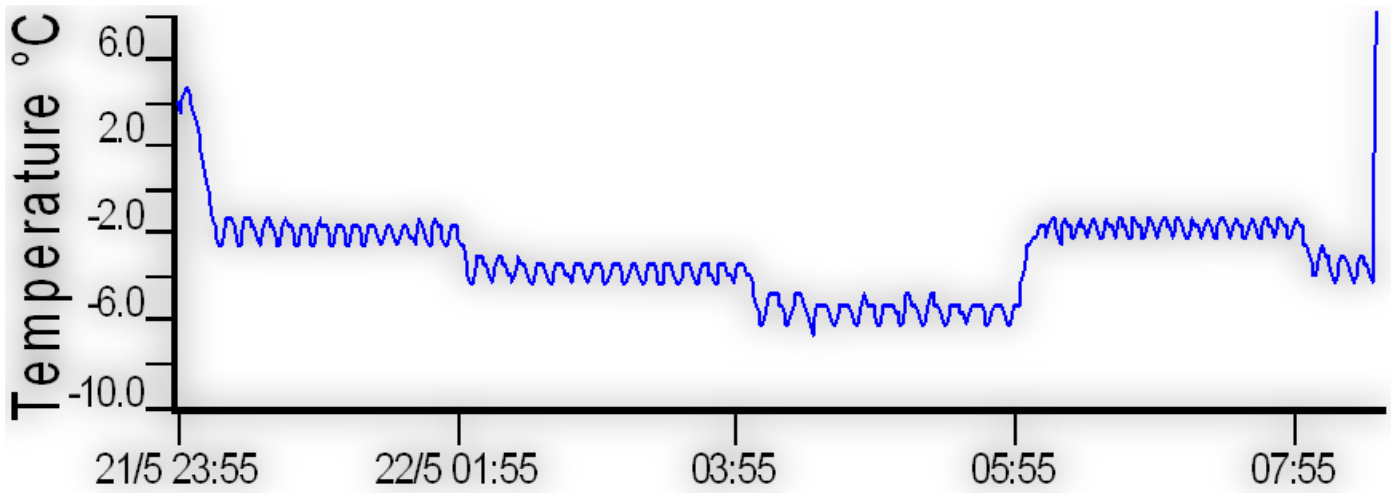
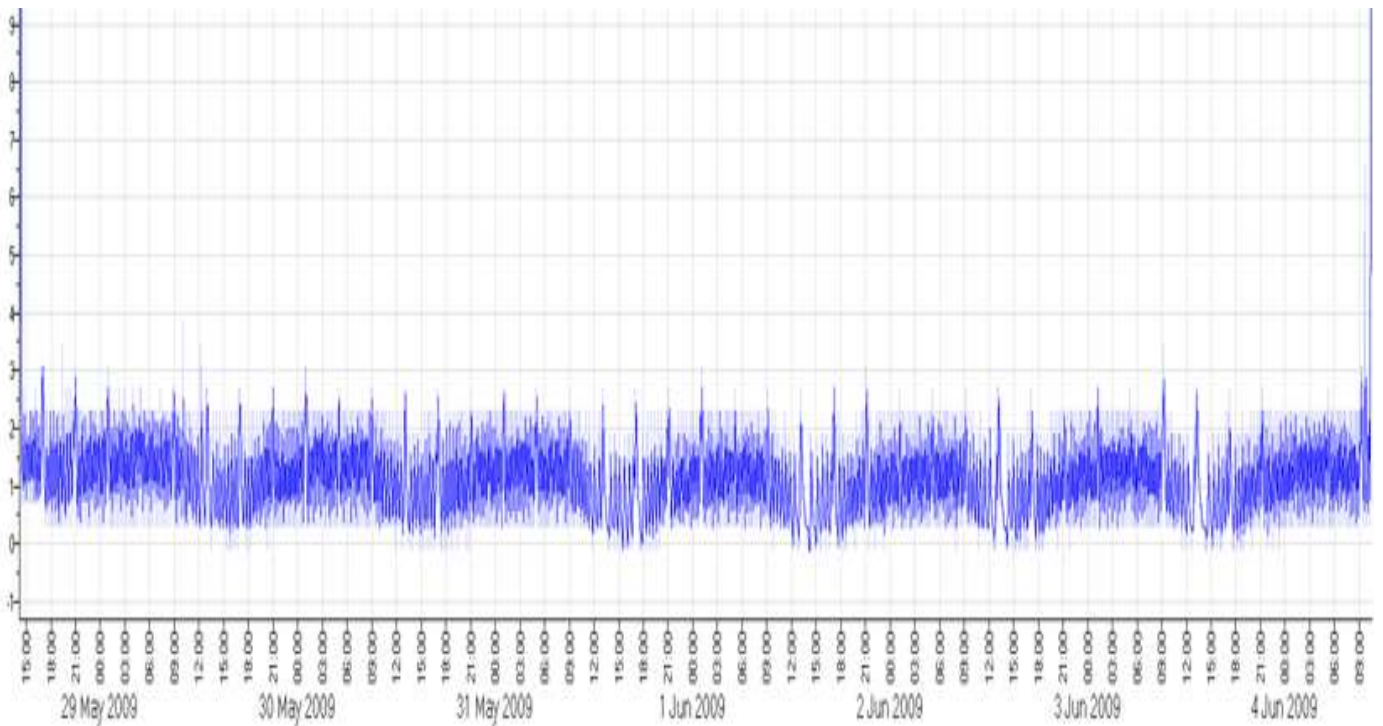
Due to the seriousness of frost damage stress to wheat during flowering in certain production areas, extra effort in characterising and finding suitable solutions to this are imperative. The results of this investigation are not complete answers for the characterisation of low temperatures effect in this important growth stage of wheat but the work demonstrated that there is a possibility to activate cold acclimation after spike emergence. Also results supported the idea that the use of some microelements could be utilised in activation of cold acclimation in plants. Therefore it is suggested that the use different microelement (e.g. Mo, Ca<sup>2+</sup> and B) with different concentrations at various growth stages is assessed. These elements were reported to be implicated during low temperature stress (Dodd et al., 2006, Han et al., 2008, Sun et al., 2009) and if they could contribute in increasing wheat tolerance at any growth stage they could be used to mitigate against damage in the event of a high frost risk at unseasonal times. The effectiveness of using Mo as a foliar application before flowering instead of using it as a seed primer is important to be investigated.

Supercooling shows great potential as a tool to avoid damage in wheat spikes if it can be genetically or physically controlled. Intensive effort through physiology, molecular biology and biochemistry is required to understand this mechanism in wheat varieties may lead to the identification of easily selectable traits. Freezing in wheat during flowering is catalysed by dew or INA bacteria formation on its surfaces and the interaction of water and ice spread into leaves needs greater understanding. Investigation the use of hydrophobic films (e.g. Kaolin (Wisniewski et al., 2002)) which might decrease the probability of ice nucleation *in-planta* and increase the probability of supercooling in this important stage is also worthy of further work.

## 10. Appendices

Appendix (1): Examples of temperature traces of freezing cabinet recorded by data logger for 0, -2, -4, -6, -8, -10 °C.





S/N 156080

Time (starting 21/05/09 23:55:06)

Appendices

Appendix (2): Postgraduate Research Skills and Methods (BIO5124)

Week no.	Sessions		
13	Research ethics J. Eddison		Methodology in science J. Eddison
14	Safety and risk assessment Fieldwork P. Ramsay		Safety and risk assessment Laboratory A. Foey
15	Scientific writing P. Ramsay	Publication strategies P. Ramsay	Critical review of writing Pathway Leaders
16	Research projects Pathway Leaders		
17	Working with literature: electronic resources & advanced searching A. Southam		Working with literature: reference manager software A. Southam
18	Biostatistics 1 M. Franco		Biostatistics 2 M. Franco
19	Project management & the funding environment T. Henry		Careers in biology S. Lane & M. Burns
20	Biostatistics 3 M. Franco		Poster presentations R. Blackshaw
21	Oral presentations P. Calosi		Public communication of science M. Donkin

Appendix (3): ANOVA Score versus Variety, Acclimation, Temperature (LT50).

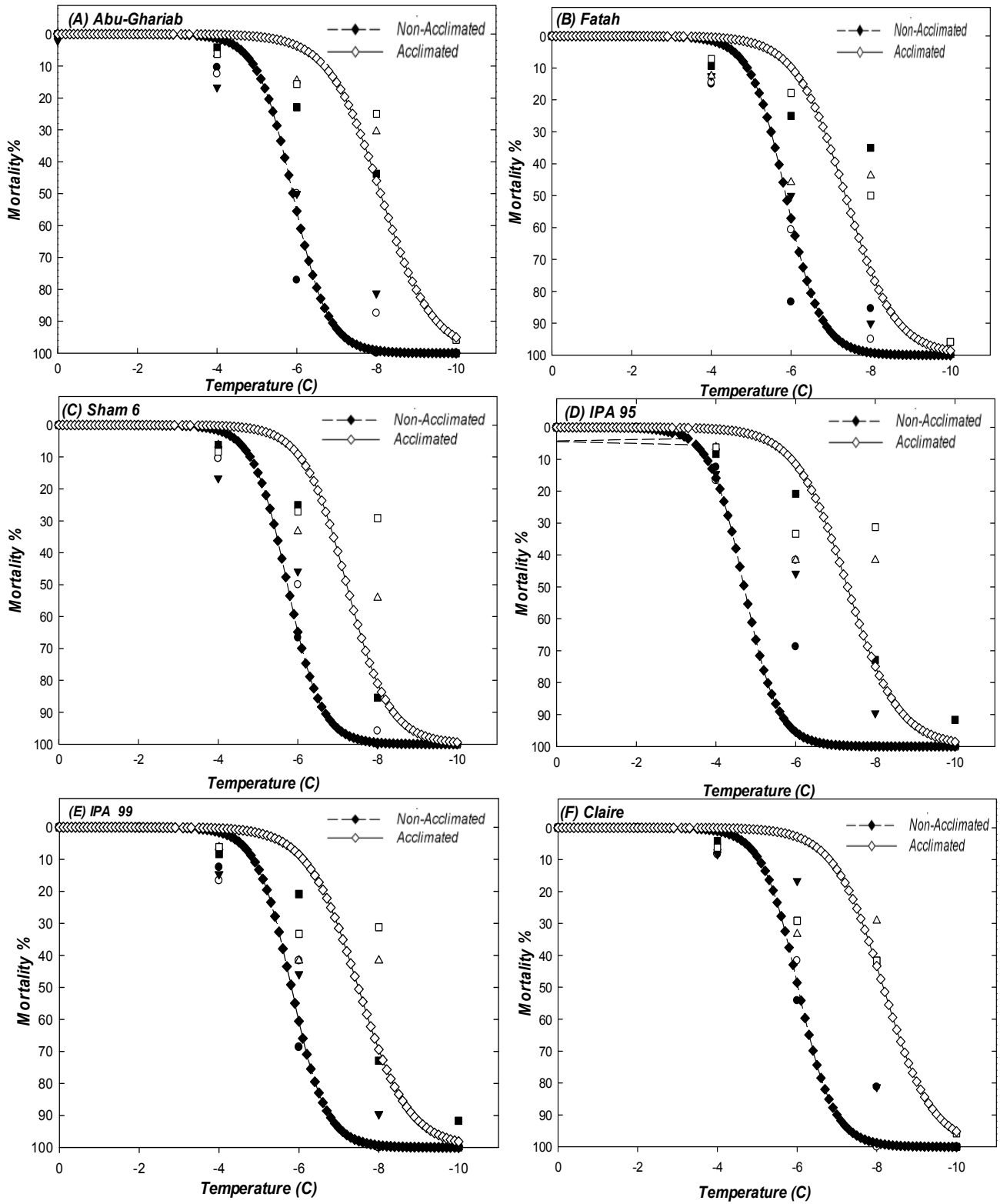
Factor	Type	Levels	Values
Variety	fixed	6	V1, V2, V3, V4, V5, V6
Acclimation	fixed	2	0, 1
Temperature	fixed	6	-10, -8, -6, -4, -2, 0

Analysis of Variance for Score

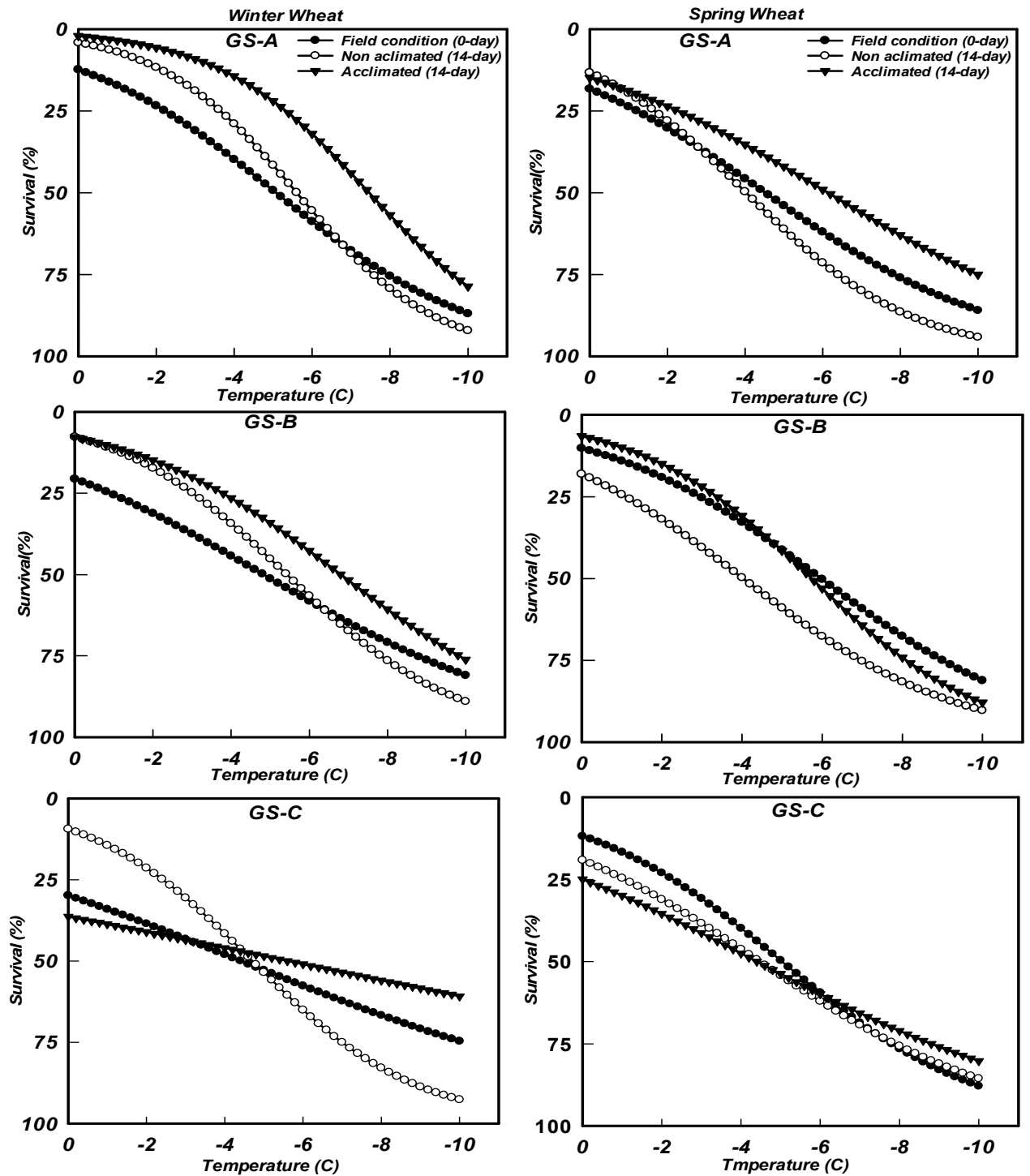
Source	DF	SS	MS	F	P
Variety	5	0.4187	0.0837	1.75	0.027
Acclimation	1	7.8755	7.8755	164.36	0.000
Temperature	5	265.2905	53.0581	1107.28	0.000
Variety*Acclimation	5	0.3810	0.0762	1.59	0.067
Variety*Temperature	25	0.9089	0.0364	0.76	0.787
Acclimation*Temperature	5	12.0633	2.4127	50.35	0.000
Variety*Acclimation*Temperature	25	1.2449	0.0498	1.04	0.422
Error	144	6.9001	0.0479		
Total	215	295.0830			

S = 0.218900 R-Sq = 97.66% R-Sq(adj) = 96.51%

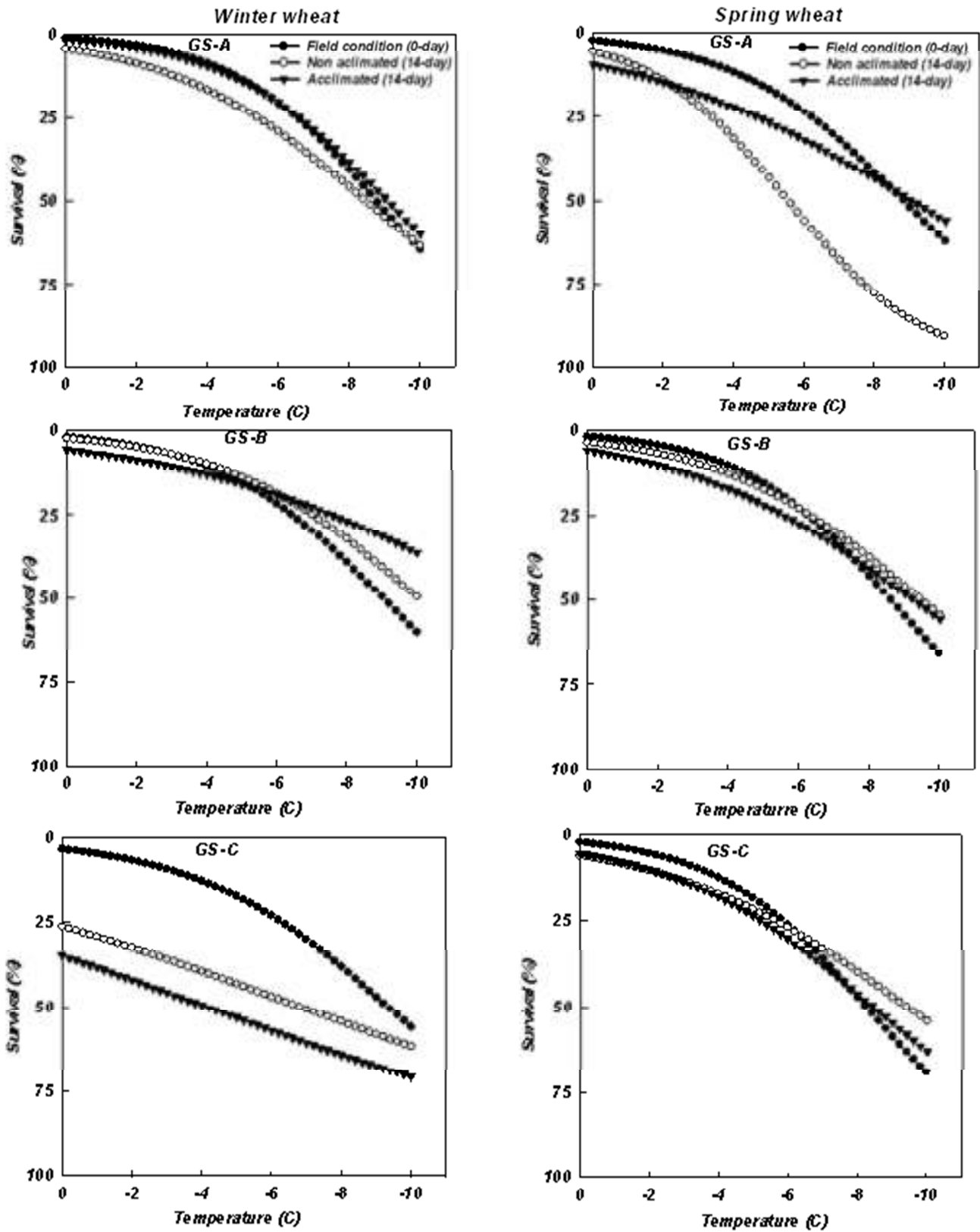
**Appendix (4-A): The logistic differential curves of cultivars Mortality after exposure to freezing temperatures in seedling, leaves and spikes.**



**Appendix (4-B): Logistic differential curves of the survival of leaves after spike emergence following exposure to freezing temperatures in European wheat (left panel) and Iraqi wheat (right Panel).**



Appendix (4-C): Figure 18. Logistic differential curves of the survival of spikes after spike emergence following exposure to freezing temperatures in European wheat (left panel) and Iraqi wheat (right Panel)



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