Reproductive frost tolerance in field pea (*Pisum sativum* L.)

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By

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Abbreviations

AFLP: Amplified Fragment Length Polymorphism

ANOVA: Analysis of variance

ATFCC: Australian Temperate Field Crops Collection

BC: Backcross

CO_2: Carbon dioxide

CRD: Completely Randomised Design

CTAB: hexa-decyl, tri-methyl, ammonium bromide

DNA: Deoxyribonucleic Acid

dNTP: Deoxyribonucleoside triphosphate

EST: Expression Sequence Tag

FAO: Food and Agriculture Organisation of the United Nations

FS: Flowering Stage

HPLC: High Performance Liquid Chromatography

ICP-AES: Inductively Coupled Plasma Atomic Emission Spectrometry

NPQ: Non-photochemical quenching

OsO_4: Osmium tetraoxide

PBS: Phosphate Buffered Saline

PCR: Polymerase Chain Reaction

PDS: Pod Development Stage

PGER: Pulse Germplasm Enhancement Research

PPFD: Photosynthetic Photon Flux Density

PSII: Photosystem II

qP: Photochemical quenching

QTL: Quantitative Trait Locus

RAPD: Randomly Amplified Polymorphic DNA

RFT: Reproductive frost tolerance

RWC: Relative Water Content

SARDI: South Australian Research and Development Institute

SNP: Single Nucleotide Polymorphism

SSR: Simple Sequence Repeat

STMS: Sequence Tagged Microsatellite Site

STS: Sequence Tagged Site

TEM: Transmission Electron Microscopy

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Abstract

Radiant frost during spring is a significant problem for field pea (*Pisum sativum* L.) grown in Mediterranean environments as plants are at the vulnerable reproductive stage when frost occurs. In such environments, radiant frost events after the commencement of flowering of field pea may lead to severe frost injuries on plants, and can adversely affect the grain yield. Despite the importance of the impact of frost on grain yield, no dedicated study has been conducted on reproductive frost tolerance (RFT) in field pea.

One aim of this research was to develop a simple and reliable screening method to evaluate frost tolerance of eight reproductive organs (from immature buds to mature pods) which are often present at the same time on a single plant. A controlled environment screening method that exposed plants to a defined temperature regime, including a minimum temperature of -4.8 ^{o}C for 4 hr, was developed. A scoring key was devised to record frost symptoms on each reproductive organ, and five categories were defined to evaluate frost damage on seeds. Using this screening method, a diverse collection of germplasm was screened, including 83 accessions sourced from high altitude and frost prone areas in 39 countries. A locally adapted variety, Kaspa, the most widely cultivated field pea variety in southern Australia, was included in the screening. The flowering stage was found to be more susceptible to frost than the pod development stage. Buds and set pods were found to be the most frost-susceptible reproductive organs, and mature pods were the most frost-tolerant reproductive organs. Genetic variation was found among field pea genotypes for frost tolerance at the flowering stage. Eight accessions, ATC 104, ATC 377, ATC 947, ATC 968, ATC 1564, ATC 3489, ATC 3992 and ATC 4204, each from a different country, were identified with more than 20 % frost survival of flowering stage organs. Kaspa was highly susceptible to frost at reproductive stages, with no buds, flowers or pods surviving the frost treatment.

A BC_1F_1 population was derived from frost- tolerant ATC 1564 and frost-sensitive Kaspa, and segregation of the frost survival trait and SSR markers was studied. Little marker polymorphism was observed between the two genotypes, with only 41 (12.3 %) of the 332 primer pairs assayed on DNA samples of the parental lines, exhibiting

polymorphic products in polyacrylamide gel electrophoresis. Unfortunately, most of these markers were not linked with any other loci, and only two linkage groups were developed: one with three markers, and the other with only two. No strong marker-trait associations were observed for frost tolerance.

Responses of reproductive-stage plants to low positive temperature (10/5 ^{o}C day/night, and 150 – 250 µmole m^{-2} s^{-1} PPFD) for 7, 14 and 21 days were studiedas were the effects of these cold treatments on survival of vegetative and reproductive tissues after frost, for frost-tolerant (ATC 968 and ATC 1564) and frost-sensitive (ATC 1040 and Kaspa) genotypes. Under long exposures (21 days), all genotypes exhibited an ability to maintain the photosynthetic rate. All genotypes were found to be adversely affected by chilling at the reproductive stage, however frost-sensitive genotypes were more responsive to low positive temperatures (cold) than frost tolerant genotypes. Evidence of symptoms of chilling injuries was found in the frost-sensitive genotype: distortion in the ultrastructure of chloroplasts was observed in parenchyma cells of stipules in Kaspa. A decrease and/or non-accumulation of soluble sugar in vegetative and reproductive tissues found in all genotypes under cold conditions reflected the inability in reproductive stage plants to acclimate. In contrast to what has previously been observed for pea seedlings, cold treatment of reproductive-stage pea plants did not result in acclimation, did not improve reproductive frost tolerance, and in fact reduced frost tolerance.

In conclusion, a drop in temperature under radiant frost conditions is lethal for reproductive stage pea plants. Reproductive organs are inherently sensitive to frost, and severe frost damage may lead to abortion of buds, flowers and set pods, and significantly reduce the seed weight.

Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution, and to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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Dec, 2012

Statement of authorship

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Shaista Shafiq (Candidate)

Designed experiment, performed experimental work, analysed and interpreted data, and wrote the manuscript.

Jeffery Paull (Co-author, and Principal Supervisor)

Edited the manuscript, and gave advice and suggestions on presenting results and the manuscript. Acted as a corresponding author.

I hereby certify that the statement of contribution is accurate and I give permission for the inclusion of the article in the thesis.

Signed .. .. Date 5/12/12

Diane Mather (Co-author, and Co-Supervisor)

Edited the manuscript, and gave useful criticism on the manuscript and particularly advice on statistical analyses of data.

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Supervised the candidate during the design and execution of the experiments, and provided financial support through South Australian Grain Industry Trust project.

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Chapter 1

General Introduction

Field pea (*Pisum sativum* L.) is an economically important grain legume crop. It is of a fundamental importance for agricultural systems providing sustainable pasture production and cereal rotation capabilities. Field pea seeds are rich in protein; therefore, this crop is extensively used for animal feeding and human consumption (Tian et al. 1999; Mihailović and Mikić 2004; Nunes et al. 2006). However, susceptibility of this crop to frost stress is a major limitation to achieving high grain yield and to enlarging the area of cultivation worldwide.

Frost is a significant problem for field pea at both vegetative and reproductive stages. In temperate environments (e.g. in Europe and North America) frost during winter or early spring can severely damage or kill seedlings (Swensen and Murray 1983; Stoddard et al. 2006). Research has been conducted to study and improve vegetative frost tolerance. Genetic variation has been reported for frost tolerance in field pea seedlings (Bourion et al. 2003) and genetic analysis of vegetative frost tolerance has recently been conducted (Lejeune-Henaut et al. 2008). The ability of field pea to tolerate frost at the vegetative stage decreases with age (Badaruddin and Meyer 2001; Meyer and Badaruddin 2001), and sensitivity to frost has been observed to increase after the commencement of floral initiation (Lejeune-Henaut et al. 1999).

In Mediterranean environments, radiant frost events commonly occur during cold clear nights in spring. At that time of year, field pea plants are likely to be at flowering and podding stages. Exposure to frost at reproductive stages can damage or kill buds, flowers and pods, and can reduce seed weight (Ridge and Pye 1985) leading to a drastic reduction in the final grain yield. Current cultivars of field pea have inadequate tolerance to radiant frost at reproductive stages. Fluctuations in grain yield due to the sensitivity of this crop to radiant frost limit the expansion of pulse industries in many countries, including Australia (Wery 1990; Siddique et al. 1999). Despite the importance of the significant impact of sub-zero temperatures on grain yield, no dedicated study has been conducted on reproductive frost tolerance (RFT) in field pea.

Research on evaluation of RFT has been constrained by the lack of rapid and reliable screening techniques. There is a need to develop screening methods to evaluate RFT in the *P. sativum* genepool. With such methods, it might be possible to identify sources of RFT, to select for RFT in pea breeding and to conduct genetic analysis of RFT, as has been done in cereals (Reinheimer et al. 2004; Chen et al. 2009).

Cold acclimation is a dynamic process in which plants exposed to low but non-freezing temperature acquire tolerance to sub-zero temperatures (Levitt 1980). In field pea, cold acclimation has been studied in seedlings (Bourion et al. 2003) but not at the reproductive stage. Information about whether reproductive-stage plants are capable of acclimating to cold conditions could be useful in evaluating to what extent the sensitivity of reproductive-stage plants is due to the sudden nature of radiant frost events (lack of opportunity for acclimation) and to what extent it is due to inherent sensitivity of reproductive tissues to frost.

The aim of the study was to develop a screening method for evaluating the susceptibility of each reproductive stage against radiant frost, and to use this method to assess RFT in a diverse collection of *P. sativum*. Further, upon the identification of frost tolerant line(s), the segregation of frost survival traits and molecular markers would be studied among progeny of pea genotypes differing in frost tolerance. The cold acclimation process would be studied at the reproductive stage to investigate the natural capacity in field pea to adjust physiological and cellular changes under low positive temperature conditions, and its effect on the reproductive frost tolerance would be determined. For this, physiological response would be studied in vegetative and reproductive tissues. As outlined in Table 1.1, this thesis will review relevant literature (Chapter 2), report on experimental research with methods and results (Chapters 3 – 7), and provide a general discussion (Chapter 8). The final two chapters will describe the main conclusions of the research (Chapter 9) and its contributions to knowledge (Chapter 10).

Table 1.1. Thesis structure

Chapter	Content
Chapter 1	Introduce the thesis topic and objectives.
Chapter 2	Review the literature on radiant frost stress and tolerance in field pea with particular reference to the reproductive stage, methods to assess natural frost tolerance and cold acclimation.
Chapter 3	Develop a screening method to score frost symptoms at each reproductive stage. Screen a diverse collection of germplasm for the identification of accessions with reproductive frost tolerance.
Chapter 4	Develop a backcross population from parents identified in the previous chapter. Investigate the segregation of frost survival traits and molecular markers.
Chapter 5	Study the process of cold acclimation at the reproductive stage in four genotypes selected on the basis of results reported in Chapter 3. Examine physiological changes in leaf tissues.
Chapter 6	Observe ultra-structural changes in leaf parenchyma cells during cold acclimation in two of the genotypes used in the experiments reported in Chapter 4.
Chapter 7	Study physiological changes during cold treatment in two genotypes selected on the basis of results reported in Chapter 3.
Chapter 8	Discuss results of the research reported in the thesis, and potential applications of this research, with suggestions for follow-up studies.
Chapter 9	State the main conclusions of the research.
Chapter 10	State the contributions of the research to knowledge.

Some of the information included in Chapters 2 and 3 has already been published in two peer-reviewed journal articles:

Ahmad M, Shafiq S, Lake L (2010) Radiant frost tolerance in pulse crops-a review. Euphytica 172: 1 – 12

Shafiq S, Mather D, Ahmad M, Paull J (2012) Variation in tolerance to radiant frost at reproductive stages in field pea germplasm. Euphytica 186 (3): 831-845. Doi: 10.1007/s10681-012-0625-0

For the review article (Ahmad et al. 2010), I critically reviewed the available literature on frost stress and tolerance in field pea and faba bean, and wrote sections field pea and faba bean. I also edited and rearranged the other two sections, chickpea and lentil, and added the first two figures in the review paper and wrote the abstract. For the article on variation in RFT (Shafiq et al. 2012), I planned the research, developed a screening including a scoring key to analyse frost symptoms at each stage conducted the experiment, performed statistical analyses, and wrote the manuscript.

My co-author's contributions were as follows:

For the review paper, Lachlan Lake wrote sections on spring radiant frost, and tolerance in chickpeas and lentils. Dr M Ahmad edited the manuscript and submitted it for publication.

In the research paper, my supervisors Dr Jeffrey Paull and Professor Diane Mather gave advice and suggestions on presenting results and contributed towards editing the research manuscript. Professor Diane Mather gave advice on statistical analyses of data. The research was financially supported by South Australian Grain Industry Trust through a project led by Dr M Ahmad at the South Australian Research and Development Institute.

To integrate the information from the review article into this thesis, I updated the literature review and included more information about frost and tolerance in field pea at the reproductive stage. The research paper is included as Chapter 3 of the thesis, with only minor changes in format (e.g. numbering of tables) to provide a consistent and logical format throughout the thesis.

Chapter 2

Literature Review [1]

2.1. Introduction

Field pea (*Pisum sativum* L.) is a major pulse crop. It is economically very important for many agricultural systems, but the relative importance of abiotic stresses affecting its production is poorly understood. Field pea has inadequate tolerance at the reproductive stage to winter stresses, particularly frost. Under Mediterranean environments, radiant frost events are frequent during spring when field pea plants in these areas of cultivation are at the reproductive stage. Exposure to radiant frost can damage the early inflorescence (buds and flowers) and adversely affect pod setting, pod development and seed weight leading to reduction in final grain yield. This chapter reviews the literature on radiant frost stress and tolerance in field pea against radiant frost at the reproductive stage. Here, the methods that could be used to identify reproductive frost tolerance and responsive gene(s) are discussed. Further, the chapter contains information on the cold acclimation process to better understand the response of field pea to low temperatures.

2.2. Field pea, a beneficial grain legume crop

General information and physiology

Field pea is an economically important grain legume, and it is one of the oldest cultivated crops in the world (Zohary and Hopf 1973). Due to its widespread cultivation, field pea was ranked second in the world for pulse production in the last decade, and recent FAO (Food and Agriculture Organisation of the United Nations) statistics show an annual production of 10.2×10^6 t of this crop (FAOSTAT 2010).

[1] This chapter contains some information that is also published in: Ahmad M, Shafiq S, Lake L (2010) Radiant frost tolerance in pulse crops-a review. Euphytica 172: 1-12

Field pea belongs to the Leguminosae family. Field pea plants have advanced root and stem systems. They exhibit varied leaf types, and on this basis are classified into three distinct groups: conventional leafy types, semi-leafless and leafless (Mikić et al. 2011). Conventional leafy types have a pair of stipules, one to three pairs of leaflets, and tendrils. The semi-leafless types are also known as *afila* where the leaflets are replaced by additional tendrils, and leafless types have reduced stipules. Anatomically, stipules are different to normal leaves, however functionally stipules are similar to leaves being representative of a large proportion of the transpiration and photosynthesis area of a pea plant (Lecoeur 2010).

Field pea plants set pods as fruits. At the commencement of the reproductive stage, the reproductive organs (buds, flowers and developing pods) start developing, but vegetative growth also continues. Many reproductive organs can be seen at the same time on the same plant within a few weeks after the commencement of flowering. There is a vast variation among *P. sativum* varieties for characteristics such as number of pods plant^{-1}, number of seeds pod^{-1}, seed weight, flower colour (coloured or white), seed colour (e.g. dun and white), seed coat (smooth, dimpled and wrinkled) and cotyledon colour (yellow and green) (Orland 1917).

Benefits in agriculture systems

Field pea has become an attractive cash crop due to numerous benefits in agricultural systems. Field pea seeds are rich in protein, with grains containing a protein content of 21 to 26 %, therefore, this crop including two varieties in particular, var. *sativum* and var. *arvense* (L.) Poiret, is extensively used for animal feeding (Mihailović and Mikić 2004). The field pea derived proteins are referred as 'new industrial proteins'. Recently, due to an increasing consumption of vegetable protein foods, field pea is now used in the production of intermediary products in human diets, for example, field pea proteins have replaced milk proteins in dairy dessert-like products (Nunes et al. 2006). Further, field pea seeds are a potential source of novel ingredients for food processing (Tian et al. 1999). The seeds are also used to balance the deficiency of an essential amino acid, lysine, in cereal based diets (Tian et al. 1999).

Field pea is of fundamental importance for agricultural systems providing sustainable pasture production and cereal rotation capabilities. Rotational benefits include the ability to increase soil nitrogen levels through biological nitrogen fixation, increased weed control options and as a break crop for cereal diseases. Field pea is also used for roughage, green manure, hay and silage. It uses less soil water than cereal crops and leaves moisture stored for subsequent crops grown in rotation. Therefore, inclusion of field pea in rotation with cereals can increase protein content and yield of the cereal (Rowland et al. 1994).

Autumn sowing for higher grain yield

Field pea is a cool-season crop, and highly responsive to temperature and water supply, particularly at the end of the growth cycle. Any fluctuation in these factors affects the grain yield in this crop (Guilioni et al. 2003; Poggio et al. 2005). Therefore, dry and warm weather at the end of the plant's life cycle is avoided, either through manipulation of time of sowing, selection of varieties with appropriate phenology to enable the life cycle to be completed prior to adverse weather, or a combination of the two. In countries where field pea is sown in spring, such as in northern Europe, the practice to shift sowing time to autumn is in progress (Dumont et al. 2009). Autumn–sowing in such environments will allow longer vegetative growth and increased biomass production and grain yield, while the larger plants produced under these conditions are more suitable for mechanical harvesting (Stoddard et al. 2006). Growth under cool conditions can result in an increase in grain yield from 50 to 100 % (Eujayl et al. 1999; Stoddard et al. 2006). However, the susceptibility of this crop to winter stresses, particularly frost, is a major limitation in European environments to achieve higher yields and to enlarge the area of cultivation of this crop.

2.3. Frost stress and tolerance in field pea

Frost stress at different developmental stages

In order to gain high and stable yield of field pea through autumn sowing under a European environment, such as in northern France, tolerance to winter stresses is very important to enable plants to survive. Survival against winter stresses involves tolerance to many factors such as diseases, frost heaving, water logging and frost. In Britain, this

crop is unsuccessful due to its susceptibility to sub-zero temperatures (Stoddard et al. 2006).

Frost is a major abiotic stress in field pea. A certain degree and duration of frost is lethal to plants. When the temperature drops to sub-zero levels, ice crystals form and lead to intracellular dehydration and damage to tissues. Rupture of the plasma membrane can also occur resulting into cellular death (Uemura et al. 1995; Atici et al. 2003; Jacobsen et al. 2005).

In Northern France, Canada and North America, it is mainly the vegetative stage of plant development that is exposed to sub-zero temperatures. Many researchers have studied frost tolerance during the first few weeks of growth (Hume and Jackson 1981; Yordanov et al. 1996; Ali et al. 1999; Meyer and Badaruddin 2001). It has been found that field pea is sensitive to frost, but there exists some variation for frost tolerance at early vegetative stages (Swensen and Murray 1983; Bourion et al. 2003). Further, winter genotypes are more frost tolerant than spring genotypes at the vegetative stage (Bourion et al. 2003).

The extent of frost damage depends on the developmental stage of the plant and tolerance to frost has an inverse relationship with the plant age (Wery 1990; Fowler et al. 2001). In field pea, a loss in ability to tolerate frost has been observed with advanced growth at the vegetative stage. Four week old seedlings were damaged more from frost stress and their rate of survival was less than two and three week old seedlings (Meyer and Badaruddin 2001). Further, the sensitivity of field pea to frost increased after the commencement of floral initiation (Lejeune-Henaut et al. 1999). In France, efforts are being made to produce varieties that can escape frost stress by delaying floral initiation so that plants do not flower until after freezing periods have passed. Lejeune-Henaut et al. (1999, 2008) found genetic variability in field pea for flowering time, and reported lines that carry *Hr* phenotype. *Hr* is a single dominant gene responsible for the qualitative high response to photoperiod; lines bearing *Hr* phenotype do not initiate flowering until the photoperiod or the day length reaches 13 h 30 min.

The reproductive stage is more sensitive to frost than the vegetative stage. In legumes, flowering and podding are the most sensitive reproductive stages to abiotic stresses, and field pea is highly responsive to frost stress (Siddique et al. 1999). Field pea plants are at the risk of exposure to spring radiant frost at reproductive stages under Mediterranean

environments (Bond et al. 1994; Berger 2007). Radiant frost and the potential damages from radiant frost at reproductive stages in field pea are discussed here.

Spring radiant frost events under Mediterranean environments

During spring, radiant frost events are common under Mediterranean environments. These events occur under clear skies and are caused by a loss of radiant energy from the crop canopy. During the day, the soil and plants absorb energy from the sun, and radiate energy back to the air when they become warmer than the surrounding air. Consequently, the air becomes less dense, rises, and is replaced by cooler air from above. Such convective mixing of warmer and cooler air currents keeps the lower atmosphere warm. But at night, when there is no incoming heat, the soil and plants continue to lose heat through radiation until they are cooler than the surrounding air. The air passes heat to soil and plants and the lower atmosphere cools. If no cloud cover is present to block the outgoing radiation, the soil, plants and the air temperature will continue to decrease significantly (Hocevar and Martsolf 1971).

Temperature and water supply are critical for normal growth and high yield in field pea. Variations in these factors above or below the plant growth optima can adversely affect numbers of seeds pod^{-1} and pods $plant^{-1}$ (Guilioni et al. 2003; Poggio et al. 2005). Under Mediterranean environments, heat and drought conditions are common in late spring and summer, therefore field pea is sown in autumn in such environments (Ridge and Pye 1985; Berger 2007). For example in southern Australia, field pea has been sown in autumn for more than 70 years over the wheat belt (Ridge and Pye 1985). In this region, temperature during autumn – winter does not drop to several degrees below zero, allowing a longer vegetative growth period without any considerable frost damage on plants. In southern Australia, the average temperature ranges recorded during autumn – winter months (April – August) are from 5 ^{o}C to 17 ^{o}C (min – max) (Australian Bureau of Meteorology 2008). Longer photoperiod conditions and elevated temperatures in spring promote the transition from vegetative to reproductive stage in field pea plants. Radiant frost events occur in southern Australia during spring when field pea plants are at reproductive stages (Ahmad et al. 2010). The occurrence, intensity and duration of frost events are unpredictable. Severe damage to plants on exposure to intense frost conditions at the reproductive stage may result in a reduction in grain yield or total crop failure (Ridge and Pye 1985; Siddique et al. 1999).

Tolerance to spring radiant frost at the reproductive stage

In pea, the floral emergence phase is very sensitive (Lejeune-Henaut et al. 1999) and periods of fluctuating temperatures at reproductive stages can lead to a significant loss in grain yield. The literature on radiant frost tolerance at the reproductive stage is scarce in field pea, but frost tolerance at the reproductive stage has been studied in other crops grown in Mediterranean-type environments and variation in tolerance has been identified. Reinheimer et al. (2004) and Chen et al. (2009) studied radiant frost tolerance in barley at the reproductive stage. They found that severe frost damage on plants resulted in frost induced sterility. Similarly in wheat, Frederiks et al. (2004, 2008) reported that radiant frost can result in a 10 % reduction in the long term average yield under the best management practices. Tshewang et al. (2010) also found sensitivity to frost in triticale varieties at the flowering stage, and observed 50 % abortion of florets at -3.8 ^{o}C.

In legumes, limited literature is available on tolerance against chilling (low-positive) temperatures at the reproductive stage. Ohnishi et al. (2010) reported that the flowering stage in soybean (*Glycine max* L.) was the most sensitive stage to low temperatures. In another grain legume species, chickpea (*Cicer aritenum* L.), damage from frost had an adverse effect on pod set and seed development (Siddique et al. 1999). Further, Nayyar et al. (2007) reported that chilling temperatures at the reproductive stage in chickpea resulted in flower abortion, poor pod set and impaired pod filling leading to a drastic reduction in yield.

Field pea is sensitive to radiant frost at the reproductive stage (Siddique et al.1999). However, genetic variation in frost tolerance of field pea has only been evaluated at the vegetative stage (Badaruddin and Meyer 2001; Meyer and Badaruddin 2001; Bourion et al. 2003), despite the importance of the impact of sub-zero temperatures on grain yield. A dedicated study on the reproductive frost tolerance (RFT) in field pea is needed.

2.4. Identification of reproductive frost tolerance (RFT) in field pea

Screening methods to evaluate RFT

The identification of tolerance to radiant frost is important in field pea. It is assumed that the evaluation of RFT is constrained by the lack of rapid and reliable screening techniques. Developing a proper methodology for screening peas at the reproductive stage could be useful in research on frost tolerance and in selection for frost tolerance. With the identification of accessions of field pea with reproductive frost tolerance (RFT), it could be possible to develop frost tolerant cultivars by crossing frost tolerant material with the local adapted varieties. This would increase the grain yield and area of adaptation of this crop in areas of the world where radiant frost during reproductive growth is a significant problem. The key step to evaluating germplasm and identifying frost tolerance is the selection of a reliable and economical screening method.

Previously, Ridge and Pye (1985) observed frost events at the flowering stage under natural conditions in southern Australia while studying the effect of sowing date on grain yield in field pea for three consecutive years. They reported inconsistency in response between seasons due to the variable incidence of frost events and warm spells during spring. In the field, assessment of frost tolerance is a complex process because of natural variability in the intensity and timing of the frost events. Also, a strong interaction of natural environmental conditions with maturity can affect the interpretation of the results. Kahraman et al. (2004) reported that one of the major problems in the characterization of genetic control of frost tolerance is the inconsistency of field and freezing tests. Natural conditions vary during field tests, and several other factors that may be involved are freezing-thawing sequences, soil hardening, humidity, waterlogging and soil pH (Levitt 1980; Blum 1988).

The frost tolerance trait is very complex and influenced by genotype by environment (G x E) interaction (Kahraman et al. 2004). It might be more reliable and repeatable to use controlled conditions to study phenotypic variation among genotypes for frost tolerance. Using growth chambers or cabinets that allow the simulation of frost events under controlled conditions, plants grown to the desired stage could be exposed to specific sub-zero temperatures for specified periods of time and results should be reproducible. Previously, Chen et al. (2009) used artificial controlled conditions to study the effect of radiant frost in barley.

One problem associated with the study of reproductive-phase field pea plants is the presence of various phenological stages or reproductive organs on one plant at the same time. This complicates the scoring of symptoms of frost damage at the reproductive stage. Separate keys may be required for the scoring of symptoms on each type of individual reproductive organ (bud, flower, set pod or developing pod) that is exposed to frost.

Genetic variation for tolerance to radiant frost at the reproductive stage

Reproductive frost tolerance in field pea has not been studied or evaluated before. Before breeding programs can improve radiant frost tolerance at the reproductive stage frost tolerant germplasm should be identified. To evaluate RFT in field pea, germplasm sourced from various pea-cultivating areas of the world from different ecological and geographical conditions, and particularly from frost prone areas or areas that frost tolerance has been reported for other crops, should be screened. Germplasm can be a collection of breeders' lines, commercial varieties, landraces and wild or related species.

2.5. QTL mapping: Importance and methodology

Mapping of RFT gene(s)

Although it has been suggested that molecular techniques can be used to characterize and isolate genes responsible for the development of freezing tolerance (Blum 1988; Galiba et al. 2001), limited research has been carried out on the identification of frost tolerance genes in legumes. Phenotypic variation for vegetative frost tolerance has been studied in the progeny derived from a crosses between tolerant and susceptible parents in lentil (Kahraman et al. 2004) and soybean (Hume and Jackson 1981). In lentil, several QTLs with cumulative effect on winter hardiness have been identified (Kahraman et al. 2004). However, no variation for frost tolerance was observed among soybean genotypes at the first trifoliate leaf stage (Hume and Jackson 1981). In field pea, Liesenfeld et al. (1986) studied the transmittance of winter-hardiness of seedlings in a reciprocal backcross population. They reported that survival under both field and controlled environment conditions increased as the dosage of the winter hardy parent

increased. However, radiant frost tolerance has not been studied in a segregating population in field pea at the reproductive stage.

Microsatellite markers for field pea

Molecular marker-based methods are commonly used to detect, map and characterize loci responsible for quantitative traits in different crops. The availability of markers in field pea is low compared to the other related species such as Medicago (*Medicago trancatula* L.), and often the same mapping population is used in several studies to locate traits, and identify QTLs in field pea (Weeden et al. 1998; Burstin et al. 2001; Loridon et al. 2005; Tar'an et al. 2005). Microsatellite or simple sequence repeat markers (SSR) exhibiting polymorphisms in field pea have been reported (Winter et al. 1999; Burstin et al. 2001). These markers are generally expected to be reliable, independent of environmental interactions, have no stage specific expression of characters and provide a high level of polymorphism (Gutierrez et al. 2005; Choudhury et al. 2007). One constraint in using SSRs is the cost and effort required for their development from genomic or transcript sequences. Markers can also be derived from other genomes and amplified in pea, however such derived primers do not always work successfully (Pandian et al. 2000; Choumane et al. 2004).

QTL mapping of RFT gene(s)

In legumes, QTLs have been mapped for winter hardiness at the vegetative stage in lentils (Eujayl et al. 1999; Kahraman et al. 2004) and faba bean (Arbaoui and Link 2008). In field pea, genetic linkage maps have been developed for various populations (Burstin et al. 2001), and several QTLs associated with frost damage at the vegetative stage have been mapped. Recently, a flowering locus *Hr* has been found to colocalise with a major QTL affecting winter frost tolerance in field pea (Lejeune-Henaut et al. 2008; Dumont et al. 2009). Since frost tolerance before the commencement of flowering is not related to reproductive frost tolerance (Bond et al. 1994), there is no reason to assume that QTLs affecting frost tolerance at the vegetative stage will be effective at reproductive stages. Mapping QTLs for RFT has been reported in barley. Reinheimer et al. (2004) and Chen et al. (2009) identified chromosomal regions associated with RFT, and reported that a locus on chromosome 5H was associated with response to cold stress at both vegetative and reproductive developmental stages in barley. They also

indicated that frost damage scores mapped to a maturity locus and showed a frost-escape effect rather than true tolerance.

2.6. Cold acclimation in field pea

Definition and application

Cold acclimation is a process in which plants that are exposed to low and non-freezing temperature acquire tolerance to sub-zero temperatures (Levitt 1980). This process is also called cold hardening. The cold acclimation process challenges the plant's ability to adjust the growth and cellular metabolism under low positive temperatures (Guy 1990). This process is primarily regulated by temperature; however light intensity, photoperiod, cultural practices and abiotic stresses can also be involved in cold acclimation (Gray et al. 1997; Fowler et al. 2001; Fowler and Limin 2004; Trischuk et al. 2006). Generally, exposure to temperatures below 10 ^{o}C and photoperiod of 12 hr induced acclimation in different plants (Badaruddin and Meyer 2001; Meyer and Badaruddin 2001; Jacobsen et al. 2005). It can be induced by various periods of cold treatment, from days to weeks, depending on the plant species (Sakai and Larcher 1987; Guy 1990; Trischuk et al. 2006). For example, in *Arabidopsis thaliana*, cold acclimation was achieved after plants were exposed to 4 ^{o}C for only 48 hr (Ristic and Ashworth 1993).

Researchers have found that cold-acclimated plants survived frost stress better than non-acclimated plants (Gilmour et al. 1988; Mahfoozi et al. 2001; Jacobsen et al. 2005). Bourion et al. (2003) exposed seedlings of field pea to 10/5 ^{o}C (day/night) under 12 hr photoperiod to induce acclimation, and found an increase in vegetative frost tolerance. It is not known whether prior exposure of pea plants to low positive temperatures would enhance the tolerance of pea plants to reproductive-stage frost.

Physiological responses in leaf tissues during cold acclimation

Cold acclimation is a dynamic process (Guy 1990; Rowland et al. 2005) and during this process, a complex of responses at physiological and cellular levels occurs in plants (Levitt 1980; Niki and Sakai 1981; Sakai and Larcher 1987). During cold acclimation, many physiological changes are observed in leaf tissues that include changes in the protein composition and content, and increase in proline, soluble sugars and starch

content, and accumulation of abscisic acid, polyamines and Glycine betaine (Guy 1990; Guy et al. 1992; Nayyar et al. 2005b; Bakht et al. 2006). During this process, Palonen et al. (2000) observed an increase in small neutral polysaccharides and a decrease in larger pectic polysaccharides in acclimated raspberry cultivars. They reported that hydrolysis of polysaccharide (starch) provided free energy to plant cells in the form of soluble carbohydrates. It has also been observed that accumulation of soluble carbohydrates leads to a decrease in the relative water content and increase in the osmotic potential in leaf tissues (Guinchard et al. 1997; Hekneby et al. 2006; Yap et al. 2008).

An increase in the concentration of soluble carbohydrates plays an important role in survival of freezing temperatures by providing reserve energy for maintenance and plant growth (Sakai and Yoshida 1968). Soluble sugars serve as osmolytes and protect cellular membranes from an alteration in the permeability and damage from cellular dehydration during frost (Steponkus 1984; Nagao et al. 2005). Under low positive temperature treatment, the accumulation of soluble carbohydrates is proportional to the degree of cold acclimation achieved by plant species (Aloni et al. 1996). A high level of soluble carbohydrates such as fructose, glucose, sucrose, raffinose and sorbitol is positively correlated with the level of tolerance to sub-zero temperatures achieved (Palonen et al. 2000).

With the changes in the concentration of sugars and starch grains during acclimation, a decrease in the Rubisco activity and rate of photosynthesis is also observed (Chabot and Chabot 1977b; Feierabend et al. 1992; Savitch et al. 1997). Photosynthesis is an integrative measure of membrane function of chloroplasts, and any changes in starch grains in chloroplasts affect photosynthesis (Musser et al. 1984; He et al. 1994). During cold acclimation, the extent of utilization of absorbed light decreases and photo-inhibition increases due to low temperature and light conditions (Sonoike 1998). This leads to a decrease in the efficiency of photosystem II (Φ_{PSII}). Carmi and Shomer (1979), and Ristic and Ashworth (1993) observed that during cold acclimation photo-assimilates were converted into starch followed by a decrease in rate of photosynthesis in beans and Arabidopsis, respectively. The process of photosynthesis is more resistant against low positive temperatures in acclimated plants, and acclimated field pea seedlings have an ability to maintain the rate of photosynthesis for survival during growth under low temperature conditions (Yordanov et al. 1996).

Changes in leaf ultrastructure during cold acclimation

Frost can cause irreversible damage to plant cells. Frost stress induces the growth of ice crystals that develop mechanical forces and induce pressure on the cell (Nagao et al. 2005). With these changes, the concentration of salts increases and results in cellular dehydration. For frost tolerant plants, the cell structure and intracellular organelles should possess tolerance to these mechanical and osmotic stresses generated by the frost stress. During cold acclimation, the ultrastructure of plants cells undergoes fundamental changes (Geronimo and Herr 1970; Kimball and Salisbury 1973). The morphological changes include fragmentation of vacuoles, thickening of cell walls, invagination of plasma membranes, formation of small vesicles of endoplasmic reticulum and accumulation of phenolic compounds (Ristic and Ashworth 1993; Stefanowska et al. 2002; Helliot et al. 2003). Further, vacuoles shrink and cytoplasmic space increases, and cells become enriched with dicytosomes and endoplasmic cisternae (Ristic and Ashworth 1993; Strand et al. 1999). These cold induced changes may play an important role in mitigation of damage under dehydration conditions during frost (Nagao et al. 2005). For example, reduction in vacuole size reduces the moisture content, leading to depression in the freezing temperature (Hekneby et al. 2006).

Dehydration induced by freezing results in the destabilisation of the membrane and is the primary cause of freezing injury in plants (Yoshida 1984). The osmotic expansion and contraction of protoplasts affect the area of plasma membranes (Steponkus 1984). Steer (1988) observed that all modifications in cell ultrastructure during cold acclimation are associated with the processes related to changes in the composition of the plasma membrane. In cold acclimation, the plasma membrane plays an important role and undergoes biochemical changes (Steer 1988). Changes in the cryostability, lipid and protein composition, and behaviour and fluidity of plasma membranes are observed during cold acclimation (Ristic and Ashworth 1993; Uemura et al. 1995; Nagao et al. 2005).

During cold acclimation, the formation of dark stained small globules has also been observed on the plasma membrane, mitochondrial outer membrane and microvesicle membrane (Ristic and Ashworth 1993). However, very little information is available on the factors that are associated with these changes.

Under low temperature conditions, changes in chloroplasts are also observed (Nagao et al. 2005; Xu et al. 2008). Stefanowska et al. (2002) reported that in chloroplasts, swelling of stroma indicates the accumulation of excess water during thawing on exposure to frost. In field pea, He et al. (1994) found that chloroplasts lost large starch grains and structural integrity, and that their lamella systems were disoriented and intergrana lamella were swollen on exposure to UV-B radiation. In field pea, very little is known about cold acclimation and associated changes in the physiology and cellular ultrastructure changes particularly at the reproductive stage.

2.7. Conclusion and implication to thesis

Field pea is an attractive cash crop providing numerous benefits in agricultural systems. However, sensitivity of this crop to radiant frost is a significant problem at the reproductive stage. Exposure to radiant frost can adversely affect reproductive organs (buds, flowers and developing pod) and lead to a drastic reduction in the final grain yield. Previously, efforts have been made to study genetic variation in field pea for natural vegetative frost tolerance, and to understand the cold acclimation process. However, frost tolerance during the vegetative stage is generally not related to tolerance during the reproductive stage (e.g. for faba bean: Bond et al. 1994) and information on reproductive frost tolerance (RFT) in field pea is scarce.

This thesis addresses the following research gaps to further the understanding of the response and genetic variation of field pea to frost at the reproductive stage.

- There are no reliable or repeatable screening systems for the identification of frost tolerance at each reproductive stage, and no information is available on genetic variability among pea genotypes for RFT (Chapter 3).

- There is no knowledge of genetic control of frost tolerance or segregation of the RFT trait (Chapter 4).

- Physiological and cellular responses of field pea to low temperatures during the reproductive stage are poorly understood and there is no information of the comparative responses of tolerant and sensitive genotypes (Chapters 5 and 6).

- It is not known if field pea undergoes acclimation when exposed to low positive temperatures during the reproductive stage, and if acclimation does occur, does it improve frost tolerance (Chapters 5 and 7).

Chapter 3

Variation in tolerance to radiant frost at reproductive stages in field pea germplasm [2]

Abstract

Radiant frost is a major abiotic stress, particularly at the reproductive stage, in field pea (*Pisum sativum* L.) grown in Mediterranean environments. Here, response to frost was studied for flowering stage (FS) organs (buds, flowers and set pods) and pod development stage (PDS) organs (flat, swollen and mature pods) under controlled conditions, with plants exposed to a minimum temperature of –4.8 ^{o}C for 4 h. This frost treatment adversely affected seed yield through (i) abortion of buds, flowers and set pods, (ii) death of pods and (iii) reduction in seed size. Flowering stage organs were more sensitive to frost than PDS organs. Genetic variation was observed among 83 accessions collected from 34 countries worldwide for survival of FS buds, flowers and set pods. In 60 of 83 accessions, no buds, flowers or set pods survived the frost treatment. Five accessions: ATC 104 (origin: United Kingdom), ATC 377 (Estonia), ATC 968 (Italy), ATC 3992 (Kazakhstan) and ATC 4204 (China), showed the highest frost tolerance of FS organs and lowest numbers of abnormal seeds. The frost tolerant accessions identified in this study may be useful as parents for breeding field pea varieties that will be less likely to suffer yield loss due to radiant frost during the reproductive stage.

[2] This chapter contains information that is also published in: Shafiq S, Mather D, Ahmad M, Paull J (2012) Variation in tolerance to radiant frost at reproductive stages in field pea germplasm. Euphytica 186 (3): 831-845. doi:10.1007/s10681-012-0625-0

3.1. Introduction

Field pea (*Pisum sativum* L.) is an economically important grain legume crop cultivated worldwide. Its agricultural benefits include improved soil nitrogen, better weed management and reduced disease for other crops with which it is grown in rotation. It is proteinaceous in nature (Tian et al. 1999; Tar'an et al. 2004) and is extensively used for animal feed and is increasingly used for the production of food products (Nunes et al. 2006). The growing market for this crop demands adapted varieties with high grain yield and potential to tolerate the stresses prevalent in a range of production environments.

Field pea has three phenological groups: spring, Mediterranean and winter (Stoddard et al. 2006). Across all three groups, inadequate tolerance to low temperature stresses is a significant problem. In many countries where field pea is sown in winter or spring, frost damage occurs at the seedling stage (Badaruddin and Meyer 2001). Severe frost damage can completely kill pea seedlings, requiring re-sowing of the crop (Meyer and Badaruddin 2001). In other environments, including Mediterranean-type environments in southern Australia, severe radiant frost in spring is a hazard during reproductive stages, causing ice formation within plant cells or tissues. Less severe but periodic frost may reduce grain yield (Ridge and Pye 1985). Under wet conditions, physical frost damage on plants can promote infection by *Pseudomonas syringae* pv. *pisi*, the causal agent of bacterial blight (Knott and Belcher 1998).

In field pea, sensitivity to frost stress has been reported to increase after floral initiation commences (Lejeune-Henaut et al. 1999). Lejeune-Henaut et al. (1999, 2008) proposed that reproductive frost damage might be avoided by developing winter varieties with the *Hr* flowering phenotype, in which floral initiation is delayed under short days. In European production environments, *Hr* plants may be able to escape frost stress by delaying flowering until after freezing periods have passed (Lejeune-Henaut et al. 1999). However, the use of the *Hr* phenotype would not protect against frost damage in environments where frost events occur during long-day periods after plants have already flowered. In Mediterranean environments, such frost events occur frequently in spring, when plants are at the reproductive stage. In southern Australia, early flowering is important to avoid terminal drought and heat in late spring and early summer (Siddique et al. 1999; Ahmad et al. 2010). Under these conditions, Ridge and Pye (1985) found

the incidence of radiant frost and/or high temperatures during flowering to be an important determinant of yield in field pea. In spite of the importance of this problem, no dedicated study has been carried out on reproductive frost tolerance in field pea. There is a need to develop screening methods and identify tolerance to frost at the reproductive stage so that breeding for frost tolerant lines can be undertaken.

Frost damage cannot easily be studied under natural conditions, due to unpredictability of the occurrence, duration and frequency of frost events (Ali et al. 1999) and the interaction of various factors including soil moisture, soil type, pathogens, hardening and freezing and thawing sequences (Badaruddin and Meyer 2001). Controlled conditions may provide more reproducible results (Ali et al. 1999) and were selected for use in the work reported here.

A major difficulty associated with studying stress response in field pea during reproductive stages is the simultaneous presence of reproductive organs: buds, flowers and pods, at many stages of development all on the same plant. The level of frost damage may depend on which reproductive organs are present at the time of frost exposure. The impact of frost stress at each reproductive organ has never been quantified, and no methods have been described to assess frost damage at individual reproductive organs. The objectives of the present study were (i) to develop a phenotyping method to assess severity of frost damage at each reproductive organ separately and (ii) to evaluate genetic variation for reproductive frost tolerance in field pea germplasm under controlled conditions.

3.2. Materials and method

Plant material

Field pea germplasm was sourced from a diverse range of environments, particularly high-altitude and frost-prone areas (Table 3.1). A total of 83 accessions from 34 countries was studied, 78 of which were provided by the Australian Temperate Field Crops Collection (ATFCC) and five by the South Australian Research and Development Institute (SARDI).

Table 3.1. Eighty three accessions from a diverse collection of field pea (*Pisum sativum* L.) germplasm screened for reproductive frost tolerance

Accession	Taxon	Origin	Plant type	Flower colour	Seed shape
ATC[^] 18	*Pisum sativum*	Malaysia	Conventional	White	Dimpled
ATC 49	*Pisum sativum*	Colombia	Conventional	Coloured	Dimpled
ATC 104	*Pisum sativum*	United Kingdom	Conventional	Coloured	Dimpled
ATC 377	*Pisum sativum*	Estonia	Conventional	Coloured	Dimpled
ATC 385	*Pisum sativum*	Taiwan	Conventional	Coloured	Wrinkled
ATC 514	*Pisum sativum*	Uganda	Conventional	Coloured	Dimpled
ATC 550	*Pisum sativum var. arvense*	Canada	Conventional	White	Dimpled
ATC 872	*Pisum sativum var. arvense*	China	Conventional	Coloured	Dimpled
ATC 947	*Pisum sativum*	Greece	Conventional	Coloured	Dimpled
ATC 968	*Pisum sativum*	Italy	Conventional	White	Dimpled
ATC 1026	*Pisum sativum*	India	Conventional	White	Round
ATC 1036	*Pisum sativum*	Mexico	Conventional	White	Dimpled
ATC 1039	*Pisum sativum*	Iran	Conventional	Coloured	Dimpled
ATC 1040	*Pisum sativum*	Nepal	Conventional	Coloured	Dimpled
ATC 1211	*Pisum sativum*	Pakistan	Conventional	Coloured	Dimpled
ATC 1263	*Pisum sativum*	Syria	Conventional	White	Dimpled

[^]ATC = Australian Temperate Field Crops Collection accession number

Table 3.1. continued.

Accession	Taxon	Origin	Plant type	Flower colour	Seed shape
ATC[^] 1436	*Pisum sativum*	Kenya	Conventional	White	Round
ATC 1498	*Pisum sativum*	Germany	Conventional	Coloured	Dimpled
ATC 1502	*Pisum sativum*	Turkey	Conventional	Coloured	Wrinkled
ATC 1510	*Pisum sativum*	Turkey	Conventional	Coloured	Round
ATC 1517	*Pisum sativum*	Turkey	Conventional	White	Dimpled
ATC 1541	*Pisum sativum*	Turkey	Conventional	Coloured	Wrinkled
ATC 1564	*Pisum sativum*	Afghanistan	Conventional	White	Round
ATC 1605	*Pisum sativum*	China	Conventional	Coloured	Dimpled
ATC 1759	*Pisum sativum*	Greece	Conventional	Coloured	Dimpled
ATC 1791	*Pisum sativum*	Egypt	Conventional	Coloured	Dimpled
ATC 1862	*Pisum sativum*	Egypt	Conventional	Coloured	Dimpled
ATC 2201	*Pisum sativum*	Egypt	Conventional	White	Round
ATC 2504	*Pisum sativum*	USA	Conventional	Coloured	Wrinkled
ATC 2549	*Pisum sativum*	Netherlands	Conventional	Coloured	Dimpled
ATC 2649	*Pisum sativum*	Sweden	Conventional	White	Dimpled
ATC 2702	*Pisum sativum*	Afghanistan	Conventional	Coloured	Dimpled

[^]ATC = Australian Temperate Field Crops Collection accession number

Table 3.1. continued.

Accession	Taxon	Origin	Plant type	Flower colour	Seed shape
ATC[^] 2710	*Pisum sativum*	Afghanistan	Conventional	Coloured	Dimpled
ATC 3095	*Pisum sativum*	Yugoslavia	Conventional	Coloured	Dimpled
ATC 3198	*Pisum sativum*	Russia	Conventional	Coloured	Dimpled
ATC 3355	*Pisum sativum*	Turkey	Conventional	Coloured	Dimpled
ATC 3362	*Pisum sativum*	Poland	Conventional	Coloured	Dimpled
ATC 3387	*Pisum sativum*	Poland	Conventional	White	Round
ATC 3429	*Pisum sativum*	Turkey	Conventional	Coloured	Wrinkled
ATC 3489	*Pisum sativum*	Poland	Conventional	Coloured	Dimpled
ATC 3754	*Pisum sativum*	Yugoslavia	Conventional	White	Wrinkled
ATC 3755	*Pisum sativum*	Yugoslavia	Conventional	White	Wrinkled
ATC 3975	*Pisum sativum*	Tajikistan	Conventional	Coloured	Round
ATC 3976	*Pisum sativum*	Tajikistan	Conventional	Coloured	Dimpled
ATC 3977	*Pisum sativum*	Tajikistan	Conventional	Coloured	Dimpled
ATC 3979	*Pisum sativum*	Tajikistan	Conventional	Coloured	Wrinkled
ATC 3980	*Pisum sativum*	Tajikistan	Conventional	Coloured	Dimpled
ATC 3984	*Pisum sativum*	China	Conventional	White	Round

[^]ATC = Australian Temperate Field Crops Collection accession number

Table 3.1. continued.

Accession	Taxon	Origin	Plant type	Flower colour	Seed shape
ATC[^] 3987	*Pisum sativum subsp. sativum*	China	Conventional	White	Wrinkled
ATC 3988	*Pisum sativum*	Kazakhstan	Conventional	White	Round
ATC 3989	*Pisum sativum*	Kazakhstan	Conventional	White	Round
ATC 3991	*Pisum sativum*	Kazakhstan	Conventional	Coloured	Dimpled
ATC 3992	*Pisum sativum*	Kazakhstan	Conventional	Coloured	Dimpled
ATC 4035	*Pisum sativum subsp. sativum*	Kazakhstan	Conventional	White	Round
ATC 4197	*Pisum sativum subsp. sativum*	Kyrgyzstan	Conventional	White	Dimpled
ATC 4199	*Pisum sativum subsp. sativum*	Kazakhstan	Conventional	Coloured	Dimpled
ATC 4201	*Pisum sativum subsp. sativum*	China	Conventional	White	Round
ATC 4202	*Pisum sativum subsp. sativum*	China	Conventional	White	Round
ATC 4203	*Pisum sativum subsp. sativum*	China	Conventional	White	Round
ATC 4204	*Pisum sativum subsp. sativum*	China	Conventional	White	Dimpled
ATC 4206	*Pisum sativum subsp. sativum*	China	Conventional	White	Dimpled
ATC 4210	*Pisum sativum subsp. sativum*	China	Conventional	Coloured	Dimpled
ATC 4223	*Pisum sativum subsp. sativum*	China	Conventional	Coloured	Dimpled
ATC 4233	*Pisum sativum subsp. sativum*	China	Conventional	White	Wrinkled

[^]ATC = Australian Temperate Field Crops Collection accession number

Table 3.1. continued.

Accession	Taxon	Origin	Plant type	Flower colour	Seed shape
ATC[^] 4257	*Pisum sativum subsp. sativum*	Uzbekistan	Conventional	Coloured	Dimpled
ATC 4258	*Pisum sativum subsp. sativum*	Uzbekistan	Conventional	White	Round
ATC 4259	*Pisum sativum subsp. sativum*	Uzbekistan	Conventional	White	Round
ATC 4262	*Pisum sativum subsp. sativum*	Uzbekistan	Conventional	Coloured	Dimpled
ATC 4263	*Pisum sativum subsp. sativum*	Uzbekistan	Conventional	Coloured	Dimpled
ATC 4388	*Pisum sativum subsp. sativum*	USA	Conventional	White	Round
ATC 4471	*Pisum sativum subsp. sativum*	Ukraine	Conventional	White	Round
ATC 4472	*Pisum sativum subsp. sativum*	Ukraine	Conventional	White	Round
ATC 4519	*Pisum sativum subsp. sativum*	Armenia	Conventional	White	Round
ATC 4542	*Pisum sativum subsp. sativum*	China	Conventional	White	Round
ATC 4557	*Pisum sativum subsp. sativum*	Tajikistan	Conventional	Coloured	Dimpled
ATC 4906	*Pisum sativum subsp. sativum*	Ukraine	Conventional	White	Round
ATC 5744	*Pisum sativum*	China	Conventional	Coloured	Dimpled
ATC 5745	*Pisum sativum*	China	Conventional	Coloured	Round
Pelikan-1	*Pisum sativum*	Poland	Conventional	Coloured	Wrinkled
Ps-05-01	*Pisum sativum*	USA	Semi leafless	White	Dimpled

[^]ATC = Australian Temperate Field Crops Collection accession number

Table 3.1. continued.

Accession	Taxon	Origin	Plant type	Flower colour	Seed shape
Mukta	*Pisum sativum*	Australia	Semi leafless	White	Dimpled
Nepal	*Pisum sativum*	Nepal	Semi leafless	White	Round
Kaspa	*Pisum sativum*	Australia	Semi leafless	Coloured	Dimpled

Growth conditions and frost treatment

The accessions were grown in four sequential experiments, with each experiment including between 17 and 25 accessions. Kaspa, a local Australian variety with high yield potential but poor frost tolerance at the reproductive stage, was included in all experiments as a control. Plants in all experiments were grown under the same conditions at 18/12 ^{o}C (day/night) and 14 hr photoperiod in a glasshouse. In each experiment, the plants were arranged in two blocks in the same glasshouse; one was a control block (with no frost treatment) and the other was a frost block (from which plants were taken for frost treatment). Each block was laid out as a completely randomised design with two replications. Each seed was sown in a 200 mm pot in new bark mix soil that was inoculated with moist rhizobium culture (*Nodulaid 100*) one week after sowing.

After the plants had started to develop floral buds, flowers and pods, they were exposed to frost in a controlled-environment chamber. An ordinal scale based on the key for stages of development of pea (Knott 1987) was used to classify floral buds, flowers and pods into eight reproductive stages, labelled from 201 to 208 (Fig. 3.1).

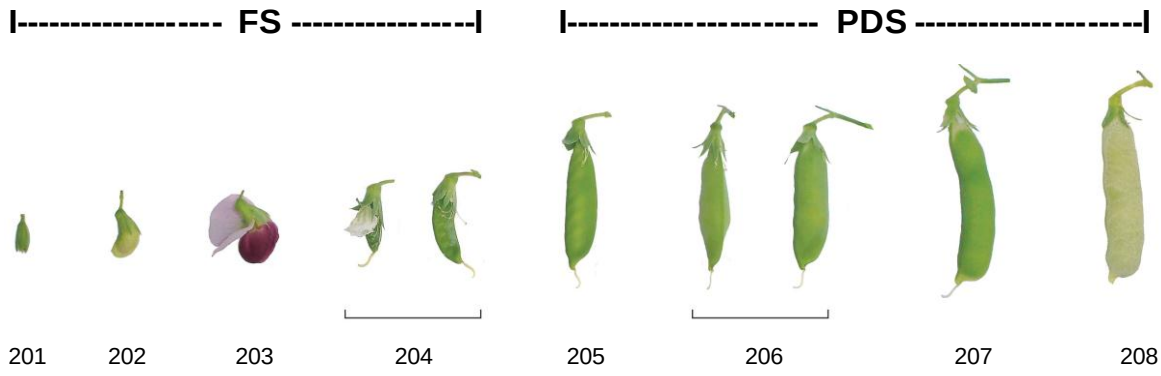


Fig. 3.1. Reproductive organs in of field pea (*Pisum sativum*) at four flowering stages (FS; 201: enclosed bud, 202: visible bud, 203: open flower, 204: pod set) and four pod development stages (PDS; 205: flat pod, 206: pod swell, 207: pod fill, 208: green wrinkled pod)

Each plant was exposed to frost once it exhibited a range of reproductive stages (five, on average). Prior to the frost exposure, each bud, flower or pod was individually tagged to indicate its stage. For example, as shown in Fig 3.2, a visible bud would be labelled "202" and a swollen pod labelled "206". Due to variation in flowering dates among plants within experiments (Appendix 1), plants were screened for frost in four or five groups within each experiment, with a total of 17 groups across experiments. Plants within each group were introduced to the frost chamber, and arranged at random positions, immediately prior to the frost treatment being imposed. The frost chamber was 3 m wide x 4 m long x 2.2 m high, with the capacity for 72 pots that were 75 mm apart. To simulate a radiant frost event, the temperature was controlled within the frost chamber over a 24 hr period (Table 3.2). The minimum temperature (–4.8 ^{o}C) was selected on the basis of actual lowest minimum air temperatures recorded in August in the preceding three years in pea growing areas in southern Australia (Australian Bureau of Meteorology). After frost treatment, plants were returned to the glasshouse and grown to maturity.

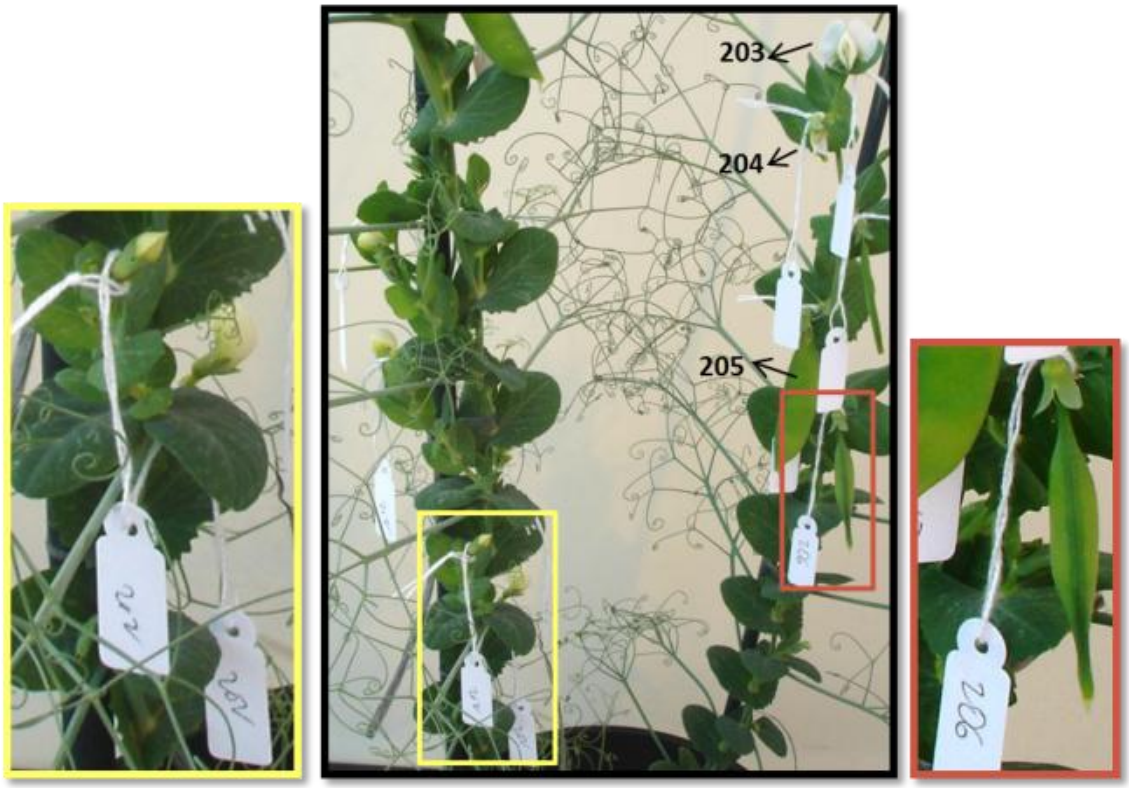


Fig. 3.2. Tags labelled with reproductive stage numbers were placed on buds, flowers and pods before plants were exposed to frost (field pea accession: Ps-05-01)

Table 3.2. Simulated radiant frost conditions in the frost chamber for 24 h

Phase	Temperature	Temperature ramping
Induction	20 ºC → 3.5 ºC	5 °C decrease /hr
	3.5 ºC → –4.8 ºC	1 °C decrease /hr
Frost Exposure	–4.8 ºC	4 hr on hold
Recovery	–4.8 ºC → 3.5 ºC	2 °C increase /hr
	3.5 ºC → 20 ºC	5 °C increase /hr

Collection of data on symptoms and seed traits

Symptom data were collected 72 hr after frost exposure. Frost symptoms were recorded for each bud, flower and pod by using visual scores as shown in Table 3.3. Using these scores, percentage survival was calculated for each stage and cumulatively across frost screened flowering stages (FS) (201 to 204) and pod development stages (PDS) (205 to 208).

In the third week after the frost treatment, accessions were classified as having regrowth potential if new shoots had grown from the basal nodes of the frosted stem. At maturity, all pods, irrespective of their position on the plant, were harvested from control plants and from the frosted stems of frost- treated plants (but not from any new shoots that had regrown after frost treatment). Pods and seeds were counted and the mean numbers of seeds per pod were calculated. A subsample of the harvested seeds was randomly selected and weighed to estimate the 100-seed weight of frosted and control plants separately. Further, seeds from frosted plants were compared to those from control plants on the basis of seed size, seed colour and seed-coat texture, and classified as normal (categories 1 and 2 in Fig. 3.3) or abnormal (dark and shrivelled, categories 3, 4 and 5 in Fig. 3.3). The percentage of abnormal seeds was calculated. For 10 accessions (ATC 104, ATC 377, ATC 550, ATC 1039, ATC 1040, ATC 1498, ATC 4259 ATC 4262, ATC 4263 and ATC 4542) the seeds in each of five categories (Fig. 3.3) were counted and weighed.

Table 3.3. A scoring key for frost symptoms at each reproductive organ in field pea (*Pisum sativum*)

Reproductive stage	Organs	Label	Score	Description
Flowering stage (FS)	Enclosed bud	201	1	No visible symptoms of frost damage
			2	Immature inflorescence is dead
	Visible bud	202	1	No visible symptoms of frost damage
			2	Immature inflorescence is dead
	Open flower	203	1	No visible symptoms of frost damage
			2	Immature inflorescence is dead
	Pod set	204	1	No visible symptoms of frost damage
			2	Pod is dead
Pod development stage (PDS)				
	Flat pod	205	1	No visible symptoms of frost damage
			2	Frozen cracks/lesions visible
			3	Flat pod is dead
	Pod swell	206	1	No visible symptoms of frost damage
			2	Frozen cracks/lesions visible but seeds appear normal
			3	Frozen cracks/lesions visible and seeds appear dead
			4	Pod is dead

Table 3.3. continued.

Reproductive stage	Organs	Label	Score	Description
Pod development stage (PDS)	Pod fill	207	1	No visible symptoms of frost damage
			2	Frozen cracks/lesions visible but seeds appear normal
			3	Frozen cracks/lesions visible, seeds smaller and damaged but may be viable
			4	Pod is dead
	Mature pod	208	1	No visible symptoms of frost damage
			2	Frozen cracks/lesions visible but seeds appear normal
			3	Frozen cracks/lesions visible, seeds smaller and damaged but may be viable
			4	Pod is dead

Fig. 3.3. Five categories of frosted seeds (1-5) in field pea based on their seed weight, colour and seed coat texture: (C) control seeds without frost stress (1) after frost seeds normal and similar to control (2) slightly discoloured and reduced in size compared to control (3) dark seeds but seed size comparable to control (4) dark and shrunken seeds (5) dark and highly shrivelled seeds. These seeds are from accession ATC 1039 (control and after frost) that showed all five categories of frosted seeds

Data analysis

Polynomial regression analysis was performed to analyze the variation for frost survival among eight organs. The quadratic model was used as follows;

$$y_i = \alpha + \beta_1 x_i + \beta_2 x_i^2 + R_i$$

Where y is a response variable, α is the y intercept when x is zero, β is the regression coefficient, x is an explanatory variable, R represents the residual variation, which was assumed to be normally distributed with mean 0 and constant variance σ^2 and i represents the stage from 1 (label: 201) to 8 (208). The regression equation for predicting percentage survival at each organ was as follows;

$$(Survival\%)_i = \alpha + \beta_1 i + \beta_2 i^2 + R_i$$

For further analysis of the data containing zero, scoring data were subjected to the empirical logistic transformation (McCullagh and Nelder 1989) using the following formula:

$$y = \ln\left[\frac{p + 1/2n}{(1-p) + 1/2n}\right]$$

where p is the proportion ($0 < p < 1$) of organs (from 201 to 204 at FS, and from 205 to 208 at PDS) that survived frost and n is the total number of organs ($n \geq 0$) subjected to frost stress. Transformed values were calculated for FS organs and PDS organs. Predicted values for frost survival of organs at FS and PDS was calculated via a linear mixed model (REML) using GenStat (GenStat Statistical Software) for each of the 83 accessions. Accessions were taken as fixed effects and experiments and frost-screened groups as random effects. Predicted values were transformed back to the percentage scale using the following formula;

$$\hat{p} = 100 \times \left[\frac{\left(-1/24 + e^y\right) \times \left(1 + 1/24\right)}{\left(1 + e^y\right)}\right]$$

In this formula, $\hat{p}$ is the predicted percentage frost survival where 0 % is no survival and 100 % is absolute survival with no frost symptoms, and y is the predicted value on the transformed scale.

3.3. Results

Frost tolerance at flowering and pod development stages

After exposure to frost treatment at −4.8 ºC, plants showed characteristic frost symptoms including bleaching of leaves, bud and flower abortion, lesions on pod walls, flattening of pods and blackening of seeds (Fig 3.4). Flowering stage (FS) was more susceptible than pod development stage (PDS) (Fig 3.5). Frost survival increased from 3% at organ 201 (enclosed buds) up to 32 % at organ 208 (mature pods). Open flowers (organ 203) were somewhat more tolerant than visible buds (202) and young set pods (204). The regression analysis showed a highly significant effect of organs on frost survival (Table 3.4). A regression equation for predicting frost tolerance at each organ was obtained as follows:

$$(Survival\%)_i = 8.15 - 3.28i + 0.741i^2 + R_i$$

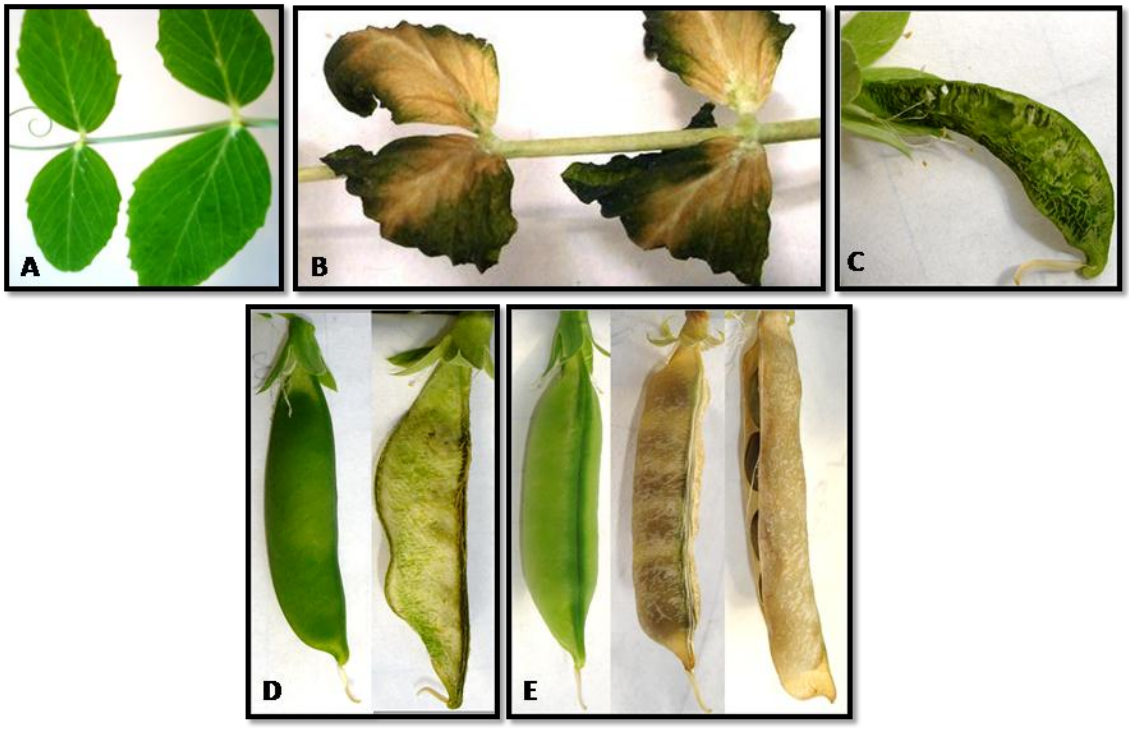


Fig. 3.4. Characteristics of frost symptoms observed under controlled conditions in field pea. **A**: leaves at reproductive stage before frost, **B**: bleaching of leaves after frost, **C**: a set pod killed by frost exposure at stage 204 (score 2; dead), **D**: a swollen pod (stage 206) before (left) and after (right) frost treatment, (score 4; dead), **E**: a filled pod (stage 207) before (left) and after (middle and right) frost treatment (score 3; blackening of seeds)

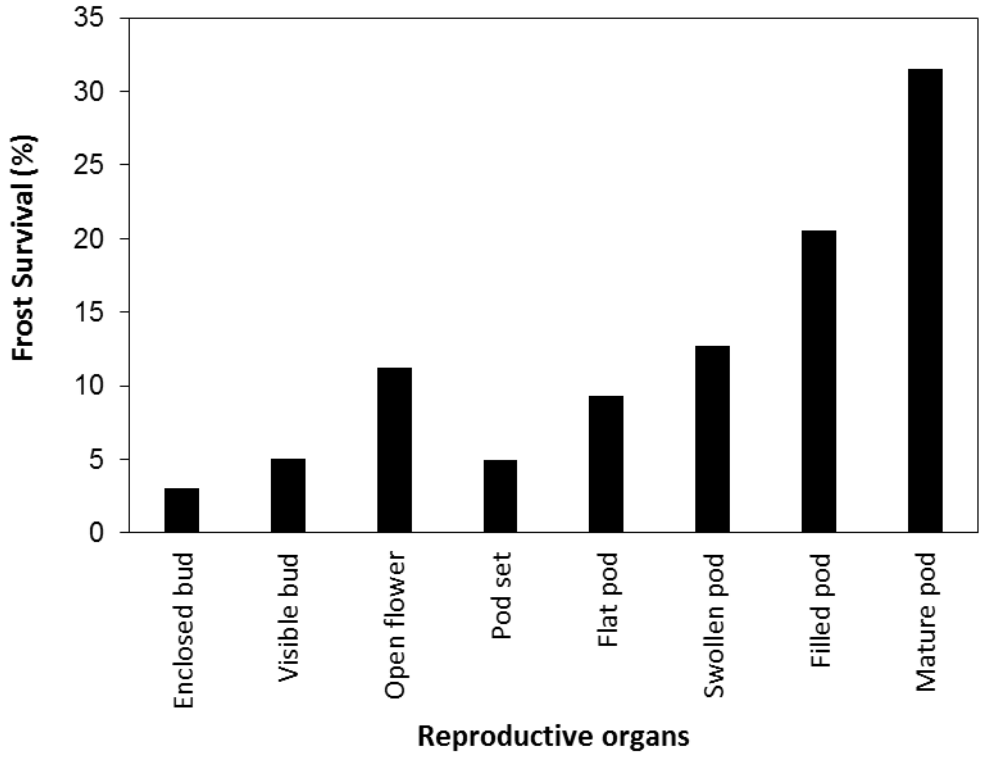


Fig. 3.5. Survival of reproductive organs after exposure to frost at stages from enclosed bud to mature pod across 83 field pea (*Pisum sativum*) accessions. FS: flowering stage; PDS: pod development stage

Table 3.4. Polynomial (quadratic) regression analysis showing variation in response to frost stress over eight reproductive organs from immature bud to mature pod in field pea (*Pisum sativum* L.)

Source	Degree of freedom	Sum of squares	Mean square	F	P
Regression	2	576.85	288.43	23.02**	0.003
Residual	5	62.66	12.53		
Total	7	639.51	91.36		

Estimated standard error: 3.54

Variation for reproductive frost tolerance in field pea

a. Analysis of frost symptoms

Partitioning of the variation in response to frost stress by REML revealed significant variation among accessions and groups of accessions at FS but not at PDS (Table 3.5).

In 14 of the 83 accessions (ATC 18, ATC 1211, ATC 1502, ATC 1517, ATC 2504, ATC 3095, ATC 3362, ATC 3429, ATC 3754, ATC 3980, ATC 3989, ATC 4197, Ps-05-01 and Nepal) no buds, flowers or pods survived the frost stress (Fig 3.6). In another 46 accessions, there was some survival of pods at PDS but none at FS. In only 21 accessions, some of the FS organs survived the frost stress (Fig 3.6). For two accessions (ATC 2201 and Pelikan-1) there was no opportunity to assess frost survival for FS organs because all reproductive nodes were at PDS when screened for frost.

Table 3.5. Percentages of the observed variation in survival of buds, flowers and pods against frost stress attributable to differences among 83 field pea accessions and 17 groups exposed to frost during reproductive growth

Source of variation	**Percentage of Variation**	
	Flowering Stages (buds, flowers and set pods)	**Pod Development Stages (flat, swollen, filled and mature pods)**
Random effects		
Group	75.0 %	8.1 %
Residual	25.0 %	91.9 %
Fixed effects		
Accessions (*F* value)	2.58*	0.81^{NS}
	(*P*= 0.011)	(*P*= 0.739)

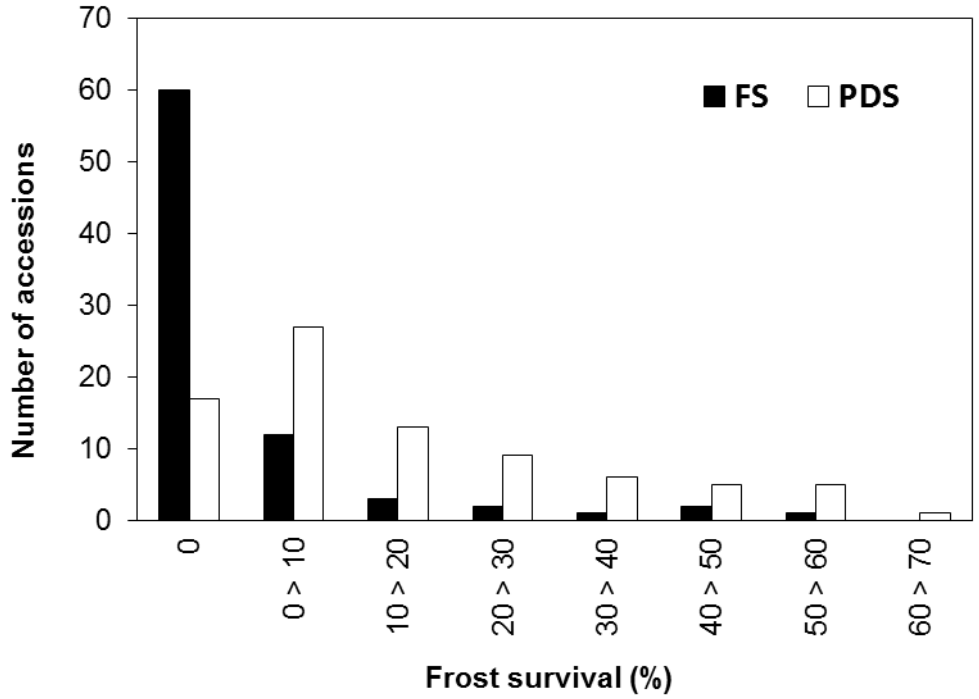


Fig. 3.6. The frequency distribution of 83 field pea (*Pisum sativum*) accessions for frost survival of flowering stage (FS) organs (enclosed and visible buds, flower and pod set) and pod development stage (PDS) organs (flat, swollen, filled and mature pod).

In Table 3.6, predicted frost survival values are presented only for accessions that survived at the critical FS. Accessions showed frost survival between 1.5 % and 45.9 %. Seven accessions had predicted frost survival values above 25 %: ATC 104, ATC 377, ATC 947, ATC 1564, ATC 3489, ATC 3992 and ATC 4204 (Table 3.6).

b. Analysis of yield components

In the control block with no frost treatment, Kaspa, a semi-leafless Australian variety with strong stems, had the highest number of seeds per pod (7.2) (Table 3.6) while Pelikan-1, ATC 1541 and ATC 5745 exhibited the highest 100-seed weights: 51.9 g, 40.5 g and 39.1 g, respectively (Table 3.6).

Forty-five accessions, including Kaspa, showed regrowth after frost and developed shoots from the basal nodes of the frosted stem (Table 3.6). These included some of the most frost-sensitive accessions, but also some of the most frost-tolerant accessions

Table 3.6. Observed yield components and regrowth after frost stress and predicted frost survival of flowering stage (FS) buds, flowers and set pods for 83 accessions of field pea

Accession	Number of seeds/pod (Control)	100 seed weight (g)		Abnormal seeds (%)	[^]Regrowth after frost	[#]Predicted % frost survival (and standard error) at FS
		Control	Frosted			
ATC 18	5	37.5	4.1	100	Yes	–
ATC 49	4.9	15.3	7.1	89	No	–
ATC 104	4	6.7	5.1	42.9	Yes	45.9 (0.8)
ATC 377	3.7	29.7	27.9	39.1	Yes	35.0 (1.0)
ATC 385	6	21.1	1.5	100	No	–
ATC 514	3.4	22.9	9.2	100	No	–
ATC 550	3.1	28.6	20.9	51.6	Yes	–
ATC 872	4.1	11.2	8.4	100	No	–
ATC 947	3.8	29.9	14.2	79.7	Yes	25.7 (0.9)
ATC 968	3.8	32.6	18.4	64.1	Yes	23.4 (0.7)
ATC 1026	4.7	10.4	5.9	54.5	No	–
ATC 1036	3.7	23.5	3.7	100	Yes	–
ATC 1039	4.3	22.7	8.5	40.9	Yes	–
ATC 1040	5.2	11.1	6.6	56.5	No	–
ATC 1211	3.3	9.6	8.2	16.7	Yes	–

[^]Yes indicates growth from basal nodes of frosted stem and No indicates no such regrowth. [#]– indicates no frost survival of FS organs.

Table 3.6. continued.

Accession	Number of seeds/pod (Control)	100 seed weight (g)		Abnormal seeds (%)	^Regrowth after frost	#Predicted % frost survival (and standard error) at FS
		Control	Frosted			
ATC 1263	1.7	24.5	0.4	100	No	–
ATC 1436	4.2	15.4	0.4	100	No	–
ATC 1498	4.8	24.1	1.6	100	Yes	–
ATC 1502	4.7	35.2	5	100	Yes	–
ATC 1510	3.9	5.8	3	100	No	–
ATC 1517	2	17.8	4.6	100	No	–
ATC 1541	4.5	40.5	10.2	100	No	–
ATC 1564	4.1	21.2	5.8	85.6	Yes	28.3 (0.7)
ATC 1605	4.9	27.6	7.4	96.9	Yes	–
ATC 1759	6.5	11.6	2.8	96.5	No	1.5 (0.8)
ATC 1791	5.4	25.3	5.8	100	Yes	–
ATC 1862	6.7	22.1	8.5	81.2	Yes	6.6 (0.6)
ATC 2201	4.3	20.8	16.9	52.8	No	X
ATC 2504	5.9	38.7	6.8	100	No	–
ATC 2549	5	8.6	5.3	100	No	–

^Yes indicates growth from basal nodes of frosted stem and No indicates no such regrowth. #– indicates no frost survival of FS organs. #x indicates that ATC 2201 had pod development stage organs only at the time of frost exposure.

Table 3.6. continued.

Accession	Number of seeds/pod (Control)	100 seed weight (g)		Abnormal seeds (%)	[^]Regrowth after frost	[#]Predicted % frost survival (and standard error) at FS
		Control	Frosted			
ATC 2649	4.9	16.3	6	95.5	Yes	–
ATC 2702	5.9	8.7	4.5	88.9	Yes	9.8 (0.7)
ATC 2710	4.3	9.5	5.2	81.7	No	–
ATC 3095	5	16.8	0.1	100	Yes	–
ATC 3198	5.1	18.2	4.2	96	No	9.4 (0.8)
ATC 3355	3.5	10	6.9	100	No	–
ATC 3362	5	9.7	0.2	100	No	–
ATC 3387	4.2	35.2	3.9	99.7	Yes	–
ATC 3429	4.9	31.1	11.1	100	No	–
ATC 3489	4	34.4	3.4	100	Yes	32.5 (0.7)
ATC 3754	5.7	25.5	1.2	100	Yes	–
ATC 3755	5.3	25.6	4.6	97.1	No	–
ATC 3975	5.6	14	2.7	100	No	–
ATC 3976	4.1	22	11.7	73.5	Yes	13.2 (1.0)
ATC 3977	5.2	34.4	9.9	100	Yes	–

[^]Yes indicates growth from basal nodes of frosted stem and No indicates no such regrowth. [#]– indicates no frost survival of FS organs.

Table 3.6. continued.

Accession	Number of seeds/pod (Control)	100 seed weight (g)		Abnormal seeds (%)	[^]Regrowth after frost	[#]Predicted % frost survival (and standard error) at FS
		Control	Frosted			
ATC 3979	5.4	20.5	9.2	93.9	No	5.0 (1.0)
ATC 3980	3.7	20.1	1.4	100	No	–
ATC 3984	4.4	21.1	9.7	89.5	Yes	3.4 (0.7)
ATC 3987	5.6	18.6	1.9	100	Yes	–
ATC 3988	4.8	12.2	1.7	98.2	No	–
ATC 3989	3.9	20.2	0.5	100	No	–
ATC 3991	5.7	17.7	0.6	100	No	–
ATC 3992	3.7	19.8	8.5	45.5	Yes	29.6 (0.9)
ATC 4035	3.7	25.8	8.7	84.8	No	–
ATC 4197	4.5	37	12.6	93.2	No	–
ATC 4199	6	24.3	3.8	100	Yes	–
ATC 4201	4	22.3	5.2	100	Yes	4.5 (0.6)
ATC 4202	4.5	21.7	2.2	100	Yes	–
ATC 4203	5.3	25.6	5.1	100	Yes	–
ATC 4204	3.9	13.8	10.3	42.8	Yes	34.5 (0.7)

[^]Yes indicates growth from basal nodes of frosted stem and No indicates no such regrowth. [#]– indicates no frost survival of FS organs.

Table 3.6. continued.

Accession	Number of seeds/pod (Control)	100 seed weight (g)		Abnormal seeds (%)	[^]Regrowth after frost	[#]Predicted % frost survival (and standard error) at FS
		Control	Frosted			
ATC 4206	3.7	22.3	9.7	88.3	No	–
ATC 4210	5.9	24.5	1.4	100	Yes	–
ATC 4223	5.1	16.6	2.3	96.6	Yes	5.9 (0.6)
ATC 4233	4.8	23.5	9.5	95.3	Yes	–
ATC 4257	3.8	14.6	7.3	94	Yes	–
ATC 4258	4	12.9	1.4	100	No	–
ATC 4259	3.7	23.2	1.9	98.1	No	–
ATC 4262	3.8	15.6	0.7	100	Yes	0 (1.0)
ATC 4263	4.7	9.6	2.1	90	No	–
ATC 4388	4.1	35.8	6.3	98.9	Yes	–
ATC 4471	4.5	15.4	5.9	84.5	No	–
ATC 4472	4	15.4	3.6	86.9	No	10.8 (0.9)
ATC 4519	4.2	25.6	5.6	100	Yes	–
ATC 4542	5.1	20.6	13.6	75.8	No	–
ATC 4557	4.3	18.8	4.8	95.7	Yes	–

[^]Yes indicates growth from basal nodes of frosted stem and No indicates no such regrowth. [#]– indicates no frost survival of FS organs.

Table 3.6. continued.

Accession	Number of seeds/pod (Control)	100 seed weight (g)		Abnormal seeds (%)	[^]Regrowth after frost	[#]Predicted % frost survival (and standard error) at FS
		Control	Frosted			
ATC 4906	4.4	18	11.7	65.2	No	9.3 (0.8)
ATC 5744	3.1	20.9	7.2	92	Yes	–
ATC 5745	5.4	39.1	10	96.9	Yes	4.1 (0.7)
Pelikan-1	2.8	51.9	15.5	99	Yes	X
Ps-05-01	4.3	19.5	3.6	80	No	–
Mukta	6.1	26.1	6.4	100	Yes	–
Nepal	4.2	31.6	2.6	100	Yes	–
Kaspa	7.2	26	7.5	100	Yes	–

[^]Yes indicates growth from basal nodes of frosted stem and No indicates no such regrowth. [#]– indicates no frost survival of FS organs. [#]x indicates that ATC 2201 and Pelikan-1 had pod development stage organs only at the time of frost exposure.

Across the 83 accessions, on average a 70 % loss in seed weight was observed after the frost treatment (Table 3.6). Most of the frosted seeds were black, irregular-shaped, small and shrunken. They tended to stick to the inner pod walls and were difficult to harvest. Across all accessions, seeds were highly affected by the frost treatment. None of the accessions had 100 % normal seeds. In 38 accessions, including Kaspa, all seeds from frost-treated plants were classed as abnormal, with 100-seed weights ranging from 0.1 to 11.1 g (Table 3.6). In another 32 accessions, over 75 % of the seeds from frost-treated plants were classified as abnormal, with 100-seed weights ranging from 1.7 to 15.5 g. In seven accessions, between 50 and 75 % of the seeds from frost-treated plants were classified as abnormal, having 100-seed weights ranging from 5.9 to 20.9 g. In the six remaining accessions, including the three accessions with the best frost survival (ATC 377, ATC 3992 and ATC 4204) less than 50 % of the seeds from frost-treated plants were classified as abnormal, with the 100-seed weight ranging from 5.1 to 27.9 g (Table 3.6). ATC 1211 had the lowest proportion of abnormal seeds (16.7 %), despite poor frost survival at FS; this accession had a high proportion of mature pods at the time of frost exposure.

After the frost treatment, the mean seed weights of accessions for which all seeds were classified as abnormal ranged from 1 mg to 111 mg. As illustrated in Table 3.7 for 10 of these accessions, the overall seed weight depended on both the mean weight of normal seeds (category 1) for the accession and on the distribution of seeds among categories, with mean seed weight declining from category 1 to category 5. The most highly frost affected seeds (abnormal seeds in categories 4 and 5) contributed very little to total seed weight (yield).

Table 3.7. A representative set of ten field pea (*Pisum sativum*) accessions showing the proportion and mean weight of frosted seeds (%) in five categories based on seed size, colour and seed coat texture

Accessions	Proportion of frosted seeds in each category (%)					Mean weight of frosted seeds in each category (mg)				
	[#]1	2	3	4	5	1	2	3	4	5
ATC 104	50.0	7.1	14.1	9.9	18.9	63	42	48	26	7
ATC 377	57.3	3.6	10.0	1.8	27.3	296	255	172	93	13
ATC 550	27.4	21.0	11.6	5.3	34.7	284	232	112	46	7
ATC 1039	6.8	52.3	12.5	13.6	14.8	225	175	99	71	16
ATC 1040	1.6	41.9	27.9	8.5	20.1	72	75	62	42	5
ATC 1498	-	-	-	55.0	45.0	-	-	-	23	5
ATC 4259	1.9	-	-	38.5	59.6	225	-	-	18	7
ATC 4262	-	-	-	-	100	-	-	-	-	7
ATC 4263	10.0	-	4.3	7.1	78.6	70	-	48	40	10
ATC 4542	-	24.2	30.3	12.2	33.3	-	200	140	80	20

[#]Seed categories: 1- frosted seeds normal and comparable to control, 2- slightly discoloured and reduced in size compared to control, 3- dark seeds but seed size comparable to control, 4- dark and shrunken seeds, 5- dark and highly shrivelled seeds

3.4. Discussion

Frost tolerance at flowering and pod development stages

The present study examined responses of buds, flowers and pods at flowering and pod development stages (FS and PDS respectively) to frost stress under controlled conditions. Individual plants were exposed to frost when they had reproductive organs at both FS and PDS simultaneously. After the frost treatment, most of the buds, flowers and set pods in FS were aborted, while pods in PDS were retained on the plant and had mild to severe symptoms including lesions on pod walls, pod flattening and completely killed pods. Similar abscission of floral buds, flowers and pods has been observed in field pea after severe heat stress (Guilioni et al. 1997). Similar findings on cold sensitivity of FS organs have been reported for related species, with both chickpea (Nayyar 2005) and soybean (Ohnishi et al. 2010) found to be more sensitive to chilling at flowering than during pod development. The abscission of sensitive FS organs may be due to a decrease in level of polyamine, i.e. putrescine, on exposure to low temperature conditions (Nayyar 2005).

The analysis of individual reproductive organs showed that buds (enclosed and visible) and pod set were the most sensitive reproductive organs to frost stress (frost survival ≤ 5 %) in *P. sativum*. Similar findings have been reported for soybean by Ohnishi et al. (2010), who found that the period from four to three days before anthesis was the most sensitive to low temperature stress. In cowpea, Ahmed and Hall (1993) found similar suppression under high temperature stress in the development of floral buds. The present results show that flowers have somewhat higher frost tolerance than buds and set pods. For the retention of a reproductive organ, accumulation of photosynthetic assimilates such as sugars and starch in the tissues of bud, flower or a pod may be important to inhibit abscission during temperature stress (Aloni et al. 1996; Nayyar et al. 2005a). Perhaps these photosynthates are higher in flowers and contributed to greater survival against frost stress than floral buds and young set pods.

Based on the substantial abortion of set pods after exposure to frost, it seems that the pod set stage may be crucial in determining reproductive frost tolerance. In related species, poor pod set and pod abortion under chilling temperature conditions have been attributed to abnormality of pollen grains (Ohnishi et al. 2010) and reduced pollen tube growth down the style (Clarke and Siddique 2004), respectively. The present results

show that frost tolerance increased as pods matured and a mature pod is the most tolerant of the reproductive organs. This may be due to the relatively low moisture content of mature pods (Sasaki et al. 1998).

Variation for reproductive frost tolerance in field pea genotypes

Genetic variation for frost tolerance is present in field pea at FS organs. Sixty accessions (72.3 %) showed no frost tolerance of any FS organ. Five accessions (ATC 104, ATC 377, ATC 968, ATC 3992 and ATC 4204) showed the highest frost tolerance at FS and the least number of abnormal seeds. These accessions are originally from the United Kingdom, Estonia, Italy, Kazakhstan and China, respectively. The growing conditions for field pea in these countries are characterized by low temperatures during the early stages of plant growth. Perhaps these accessions have gene(s) that promote plant survival and growth at low temperatures and also contribute to some tolerance at the reproductive stage. It would be useful to assess additional accessions from these countries and/or environments to seek out additional tolerant lines that could be used as parents in pea breeding. Further, information on the genetic diversity of genotypes to be selected for frost tolerance studies, would be useful for later selection in breeding. Across the 83 genotypes, no interaction was observed between reproductive timing (days from flowering to the frost exposure) and frost tolerance (Appendix 2).

The frost treatment induced a considerable loss in yield across 83 field pea accessions. Pods that were already at PDS at the time of exposure to frost were more likely to survive than buds, flowers and set pods at FS, but many of the surviving pods were flattened and contained shrunken and blackened seeds. Severe frost symptoms at PDS resulted in complete pod abortion. These results are similar to previous findings showing that extreme temperatures during the reproductive stage altered the number of pods, number of seeds/pod and seed weight in field pea (Poggio et al. 2005). Similar effects on seed yield in chickpea have also been observed after exposure to chilling temperature (Nayyar et al. 2007).

Across all accessions, developing seeds within maturing pods were significantly affected by the frost treatment. This is in agreement with a previous study that showed the sensitivity of the seed filling stage to temperature stress (Srinivasan et al. 1998). During seed filling, embryos have high moisture contents and low solute concentrations. As seeds mature, their water contents decrease and their protein concentrations increase

(Longvah and Deosthale 1998). With less free water available, the freezing point temperature is depressed and seeds do not freeze (Woltz et al. 2005). Thus exposure of pods containing relatively mature seeds to frost will not reduce seed weight (and thus grain yield) as much as frost exposure of pods containing less developed seeds. In conclusion, the grain yield can be adversely affected by frost at the reproductive stage through (i) abortion of buds, flowers and set pods (ii) death of pods and (iii) loss in seed weight (shrinking and blackening of seeds).

Exposure to freezing temperatures such as −6 ^{o}C at the vegetative developmental stage has been reported to decrease regrowth ability in field pea (Meyer and Badaruddin 2001). The present results showed regrowth in about half of the screened accessions after the frost treatment at −4.8 ^{o}C. These results indicate that in these genotypes, the growing points between the seed and the soil surface were not damaged. Here, it was observed that there was no interaction between regrowth after frost and the reproductive timing (days from flowering to frost exposure) (Appendix 3). Regrowth ability may help compensate for frost damage that occurs early in plant development. However, after frost events at the reproductive stage, there is not likely to be enough time for regrowth to contribute much towards the final yield.

Semi-leafless genotypes of field pea have strong stems that provide reduced crop lodging and facilitate mechanical harvest (Siddique et al. 1999). However, the afila gene (*af*) conferring the semi-leafless type has been reported to be associated, with reduced frost tolerance (Cousin et al. 1993). The present study included four semi-leafless accessions. For two of these (Nepal and Ps-05-01) no buds, flowers or pods survived the frost treatment. For the other two (Kaspa and Mukta), none of the FS organs: buds, flowers or set pods survived the frost treatment. The five frost tolerant accessions identified in this study were all conventional types. In future investigations, underlying mechanisms (physiological parameters or changes in cellular ultrastructures) involved in response to frost stress could be explored and compared between semi-leafless and conventional genotypes to determine if there is an intrinsic difference between the two plant types or if the difference observed in this experiment was a consequence of the tolerance of the particular genotypes tested.

Minimum temperature and duration of exposure for frost treatment

In general, the field pea accessions were susceptible to the frost treatment that was applied at reproductive stages in this experiment. The frost treatment of 4 hr duration at a minimum temperature of −4.8 ^{o}C resulted in abortion of buds, flowers and set pods, severe symptoms on pods and substantial loss in seed weight in more than 50 % of accessions. The frost treatment used here was selected on the basis of meteorological data in the target field pea production region. Under controlled conditions, this treatment may have been too harsh to identify minor differences in tolerance among the majority of accessions. This explanation is consistent with previously reported results in which extreme heat stress resulted in the abscission of buds, flowers and pods (Guilioni et al. 1997). The magnitude of the stress and the crop developmental stage are two important factors that account for the grain yield in response to stress and after-stress recovery. Research on field pea seedlings has shown only 32% survival at −4 ^{o}C for 4 hrs duration (Badaruddin and Meyer 2001; Meyer and Badaruddin 2001). Given that the reproductive stage is more sensitive to frost than the vegetative stage, the −4.8 ^{o}C treatment used in the present study may be too low and the duration of 4 hr too long. For frost screening experiments, the best temperature or duration is the one that permits differentiation between tolerant and susceptible accessions. The use of quite harsh conditions highlights the frost tolerance of the five accessions that exhibited the best survival in this experiment.

Identification of accessions with tolerance to radiant frost should facilitate breeding frost-tolerant varieties of field pea. This will likely require crossing of frost-tolerant introductions with locally adapted genetic material, followed by field evaluation of progeny under frost-prone conditions. To our knowledge, this study is the first report on frost tolerance of field pea reproductive organs at different reproductive stages. The present results describe the sensitivity of each stage to radiant frost. In future, a study at the reproductive stage on cold acclimation, the process that may increase frost tolerance, could help to understand the ability in field pea to acquire physiological changes associated with cold acclimation to induce/enhance frost tolerance, and defence mechanisms against frost stress at the reproductive stage.

Chapter 4

Segregation of frost survival traits and molecular markers among backcross progeny of pea genotypes differing in sensitivity to frost at the reproductive stage

4.1. Introduction

Low temperature is a major environmental constraint to achieving high yield in field pea, worldwide. Tolerance against low temperatures, particularly frost, is important at vegetative and reproductive stages under European and Mediterranean environments, respectively (Ridge and Pye 1985; Lejeune-Henaut et al. 1999). Genetic variation has been reported for vegetative-stage frost tolerance (Swensen and Murray 1983; Bourion et al. 2003), and there has been some genetic analysis of differences for frost tolerance, and six chromosomal regions affecting winter frost tolerance were identified (Lejeune-Henaut et al. 1999, 2008). On the other hand, literature on frost tolerance at the reproductive stage is limited. Results of a recent study (Chapter 3 of this thesis; Shafiq et al. (2012) demonstrated variation for frost tolerance at the reproductive stage among pea genotypes, but the genetic control of reproductive frost tolerance (RFT) has not been explored.

Several genetic maps have been developed and/or used for diversity assessment and to locate QTL in field pea (Weeden et al. 1998; Burstin et al. 2001; Loridon et al. 2005; Tar'an et al. 2005). For such genetic studies, various molecular markers have been used, including RAPD, AFLP, SSR, STS and STMS markers (Burstin et al. 2001; Ford et al. 2002; Tar'an et al. 2004). Microsatellite polymorphism has been studied in field pea (Burstin et al. 2001), and it was found that database-derived SSR markers were highly variable and degree of polymorphism among genotypes was high.

Genetic analysis of frost tolerance is a prerequisite for the development of lines that are tolerant to frost at reproductive stages. Here, segregation of the frost tolerance trait and PCR-based molecular markers are studied among a backcross population of pea derived

from Kaspa and ATC 1564 genotypes, differing in response to frost at the reproductive stage.

4.2. Material and Methods

Plant material

The genotypes Kaspa and ATC 1564 were selected for use as parents based on their differential responses to frost particularly at the flowering stage, according to the observations reported in Chapter 3 of this thesis and by Shafiq et al. (2012). Kaspa is a variety grown in southern Australia, and is sensitive to frost at reproductive stages. In contrast, ATC 1564 originated from Afghanistan and has comparatively higher tolerance at reproductive stages. Kaspa and ATC 1564 also differ in morphological traits, such as foliage formation type (*afila* and conventional leafy type, respectively), flower colour (pink and white, respectively) and seed coat colour (dun: reddish brown and white, respectively). These traits can provide useful classical makers for anchoring the genetic map to published pea linkage maps.

The F_1 plants derived from a single cross between Kaspa x ATC 1564, were grown in the glasshouse under 18/12 ^oC (day/night) and 14 hr photoperiod conditions. The F_1 plants were all conventional leafy type with purple flower colour. At the reproductive stage, a small set of F_1 hybrids constituting 10 plants was exposed to frost under controlled conditions. All frost-treated F_1 plants were observed to be susceptible at the reproductive stage. The reproductive-stage frost tolerance was then assumed to be a recessive trait, and (remaining) F_1 individuals were crossed with the tolerant accession ATC 1564, and a backcross (BC) population of 119 individuals was developed.

Growth conditions

Parental lines and BC_1F_1 plants were grown in the same glasshouse under 18/12 ^oC (day/night) and 14 hr photoperiod conditions, in three random sets (constituting 40, 40 and 39 plants set^{-1}) with four-days interval in sowing time between each set. Two plants of each parental lines were included in each set of BC_1F_1s. Each seed was sown in a 200 mm pot in new bark mix soil. The soil around seeds was inoculated with moist rhizobium culture (*Nodulaid 100*) one week after sowing. Plants were watered regularly

and supported with wooden stakes. After six weeks from sowing, leaf tissues were sampled from each BC_1F_1 individual and the two parents for DNA extraction.

DNA extraction and molecular markers analysis

DNA was extracted from young leaf tissue (200 – 400 mg) from the parental plants and BC_1F_1 plants following CTAB (hexa-decyl, tri-methyl, ammonium bromide) miniprep method as described by Doyle and Doyle (1987). All DNA preparations were scored for equivalent quantity using gel analysis and Bio-photometer 6131 (Eppendorf AG, Germany).

A total of 332 SSR primer pairs were screened via PCR and gel electrophoresis to detect polymorphisms between the two parents Kaspa and ATC 1564. Of the 332 primer pairs, 58 and 44 had been designed based on sequences derived from pea (Burstin et al. 2001, and Ford et al. 2002) and chickpea (Winter et al. 1999). These chickpea-derived primers had previously been used in pea (Choumane et al. 2004). For the remaining 230 primers, SSR-containing sequences were obtained from Medicago genomic databases (Oklahoma State University plant data base; http://www.plantgdb.org/MtGDB/index.php) and NCBI's dbEST (http://www.ncbi.nlm.nih.gov/sites/enterz?db=nucest&cmd=search&term=medicago%20truncatulla%20EST%20database) by Mr. Jamus Stonor and Dr Abdolreza under Pulse Germplasm Enhancement Research (PGER) program in South Australian Research and Development Institute (SARDI), using a simple sequence repeat identification tool (SSRIT, in http://www.gramene.org/db/searches/ssrtool). Primers were then designed to flank the repeats using Primer 3 software (http://frodo.wi.mit.edu/primer3/) (Rozen and Skaletsky 1999) by the above two researchers and myself. Some of these sequences were derived from ESTs and some from genomic sequence.

The protocols for SSR assays were performed following the established procedures described by Burstin et al. (2001) and Choumane et al. (2004). Primer pairs that gave reproducible and clearly resolvable amplification products were selected to be assayed on the BC population.

Polymerase chain reactions (PCR) were performed with Eppendorf thermocycler (Mastercycler® ep, Eppendorf AG, Germany). PCR for SSRs were performed in 25 µl

reactions volumes. The reaction mixture contained 20 – 75 ng of field pea genomic DNA, 1 U of *Taq* polymerase (Platinum® Taq), 100 µM of each dNTP (Roche®), 0.2 – 40 µM SSR primers and 1X PCR reaction buffer (50 µM KCl, 10 µM Tris-HCl pH 8.3, 2.5 µM $MgCl_2$ and 0.1 % v/v Triton X-100). The PCR regime consisted of an initial denaturation step (96 ^oC for 2 min), followed by 35 cycles, each consisting of a 96^oC denaturing step (30 s), a 55 ^oC annealing step (30 s) and a 72 ^oC elongation step (50 s). A final extension period of 72 ^oC for 5 min was included. For a few primers (EST SSRs), PCR were performed using the same protocol except that the reaction volume was 20 µl, and 20 ng of genomic DNA and 20 – 40 µM primers were used.

The PCR products were denatured at 94 ^oC for 5 min and immediately placed on ice. Six µl of each PCR product was separated on 0.4 mm thick 6 % (w/v) polyacrylamide gels in 1X TBE running buffer using a vertical gel electrophoresis system (Scientific, USA). Products were resolved at 500 V and a constant power 50 W for 90 min, however products for a few primer pairs (EST-SSR) were better resolved at 400 V for 50 min. Bands were visualized by silver staining and gels were scored visually and recorded using a Gel Doc system (Bio-Rad, Australia). Only strong visible polymorphic bands were scored. To demonstrate, a gel with PCR products of parents and progeny is shown in Fig 4.1. For each primer pair assayed, the PCR products exhibiting single or multiple polymorphic bands were recorded as a single score of 0 or 1 if it showed identical pattern as recurrent parent (ATC 1564) or hybrid (heterozygous), respectively.

Phenotypic evaluation of frost damage

Three weeks after the appearance of the first flower on each plant, 119 BC_1F_1s and six plants of each parent were exposed to frost under controlled conditions in six sequential batches. Each batch contained 19 – 26 plants and there were three to four days interval between exposures of each batch to frost conditions. Plants were randomly placed in the artificial frost chamber and treated at a minimum temperature of – 4.8 ^oC for 4 hr, using the temperature treatment regime detailed in Table 3.2 in Chapter 3 of this thesis.

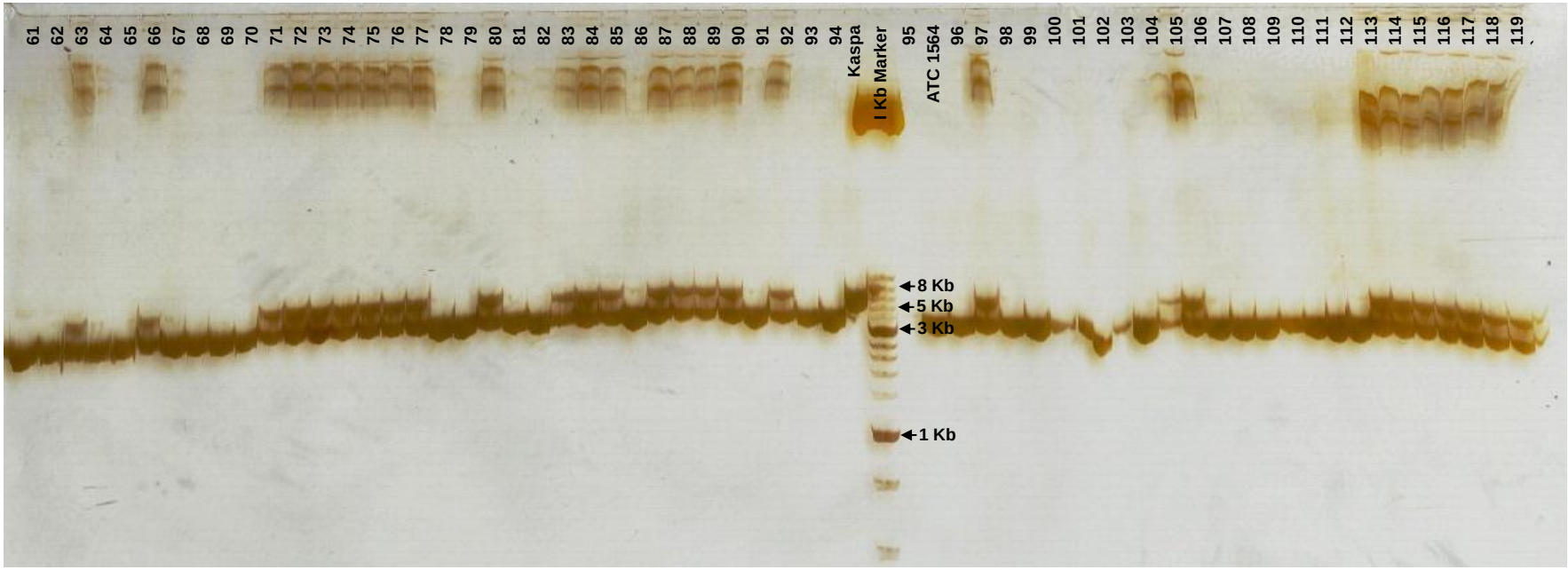


Fig. 4.1. Amplified products of Medicago-derived primer PGER-E53, on polyacrylamide gel in BC population (individuals 61-119 shown) and two parents Kaspa and ATC 1564. Both parents exhibited one product (6 Kb in Kaspa, and 4Kb in ATC 1564). In this gel, 28 of the BC_1F_1 DNA samples exhibited both products and were scored as heterozygotes, while 31 of the BC_1F_1 DNA samples exhibited only the ATC 1564 product (4Kb) and were scored as recurrent-parent homozygotes.

Prior to the frost treatment, each bud, flower and pod on each plant was labelled using tags, according to its reproductive stage as described by Knott (1987): immature bud as 201, mature bud: 202, open flower: 203, set pod: 204, flat pod: 205, swollen pod: 206, filled pod: 207 and mature pod: 208 (as described in Chapter 3 of this thesis; Shafiq et al. 2012). After frost treatment, all frost-treated plants were placed in the glasshouse at 18/12 ^{o}C (day/night) until pods were dry at which time they were harvested.

Seventy two hr after frost exposure, frost symptoms were recorded on all buds, flowers and pods. The assessment of symptoms was based on visual observations and a scoring key was used as described in Chapter 3 of this thesis and in Shafiq et al. (2012). For buds and flowers, score 1 was used for alive and 2 for dead. For developing pods to mature pods, score 1 was used for no frost damage, 2 for frost lesions on pod with seeds slightly discoloured and reduced in size, 3 for frost lesions on pod with dark and shriveled seeds and 4 for dead pods. The percentage for the survival of each reproductive stage was determined. Further, survival of immature and mature buds, flowers and set pods were cumulatively presented as survival at flowering stage (FS) and for flat, swollen, filled and mature pods as survival at pod development stage (PDS). On maturity, plants were harvested and seed weight of all frosted backcross plants and parents were determined.

Data analysis

A Chi-square test was used to observe the deviation of molecular markers in backcross population of 119 individuals from the expected 1:1 ratio using MapDisto (Lorieux 2012). Markers were assigned to linkage groups using 'find groups' command, and association of markers with the frost survival tested using single-marker F-tests implemented in the 'QTL/ANOVA' function of MapDisto (Lorieux 2012).

4.3. Results

Phenotypic evaluation of frost damage

As expected, all BC_1F_1 plants were of conventional leaf type like their recurrent parent ATC 1564. Sixty-three (52.9 %) of the backcross plants had white flowers and 56 (47.1 %) had purple flowers.

On plants of ATC 1564, only 2.3 % of the flowering-stage organs survived the frost treatment. For Kaspa, this value was only 0.6 % (Fig. 4.2A). On 79 (66.4 %) of the backcross plants, no flowering-stage organs survived the frost. Among the remaining 40 plants, the survival of flowering-stage organs ranged from 1 % to 100 % (Fig. 4.2A).

On plants of ATC 1564, only 4.2 % of the pod development-stage organs survived the frost treatment. For Kaspa, this value was 4.7 % (Fig. 4.2B). On 73 (61.3 %) of the backcross plants, no pod development-stage organs survived the frost, and this value included 63 of the 79 plants in which none of flowering-stage organs survived. Among the remaining 46 plants, the survival of pod development-stage organs ranged from 1 % to 100 % (Fig. 4.2B).

On 33 (27.7 %) and 34 (28.6 %) of the backcross plants, the frost survival of reproductive organs was above that of both parents at the flowering stage and pod development stage, respectively.

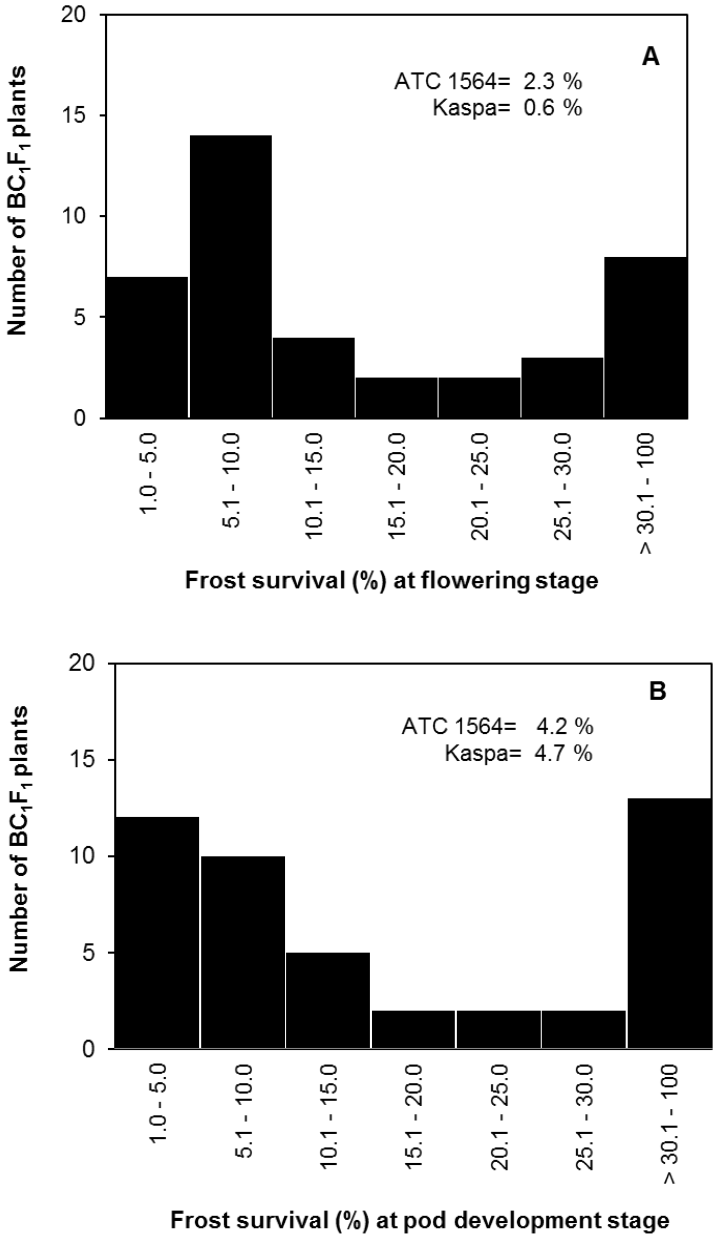


Fig 4.2. The frequency distribution of plants on which there was more than 0 % survival against frost stress in ATC 1564 x Kaspa backcross population at (**A**) flowering stage (buds, flowers and set pods) and (**B**) pod development stage (flat, swollen, filled and mature pods) after the frost treatment.

The 100 seed weight of ATC 1564 plants was observed to be 3.9 g and 21.0 g with and without frost treatments, respectively (Fig. 4.3). The 100 seed weight of Kaspa plants was observed to be 26 g and 5.9 g with and without frost treatments, respectively (Fig. 4.3). For most (69%) of the backcross plants, the 100 seed weight was between 2 – 6 g (Fig 4.3).

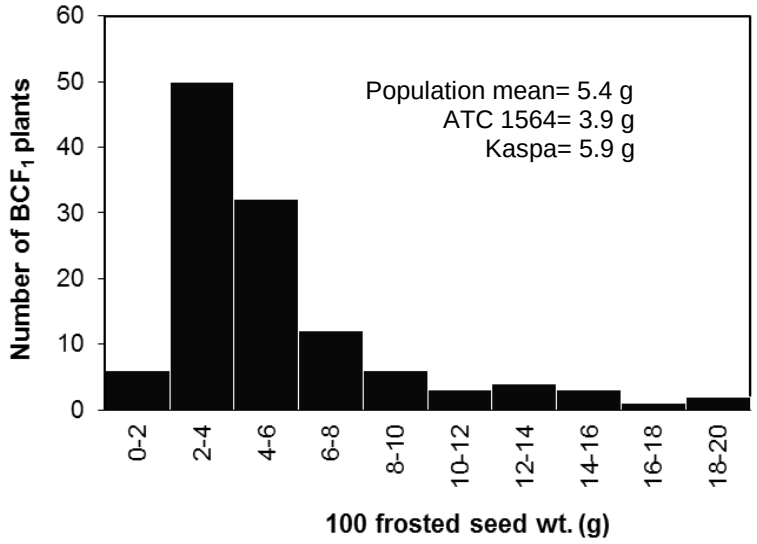


Fig 4.3. The frequency distribution of backcross plants derived from cross between Kaspa and ATC 1564 for weight of 100 seeds after the frost treatment.

Molecular marker analysis

The degree of polymorphism was very low between the parental lines for the markers used in this study (Table 4.1). Only five (8.6 %) of the pea-derived primers exhibited polymorphism between the parents (Table 4.1). The sequences of these primers from which these markers were designed: PSGSR1, PSU58830, PSU51918, PS11824 and PSAJ3318, are as described by Burstin et al. (2001). Similarly, only five (11.4 %) of the chickpea-derived primers exhibited polymorphism between parents (Table 4.1), and sequences of these primers from which these markers were designed: TA3, TA34, TA43, TA45 and TA76, are as described by Winter et al. (1999). Further, only 31 (13.5

%) of the *Medicago*-derived markers exhibited polymorphism between parents (Table 4.1), and sequences of these markers are listed in Table 4.2. Three markers, PGER-S13, PGER-S159 and PGER-E34, were highly distorted; therefore, these markers were not considered in further analyses (Table 4.3).

Table 4.1. Degree of polymorphism for simple sequence repeats (SSR) and sequence tagged microsatellite site (STMS) markers assayed on genomic DNA of two *Pisum sativum* L. genotypes Kaspa and ATC 1564

Primer	Genome derived	Number of markers assayed	Number of polymorphic markers detected
SSR	Pea (*P. sativum*)	43	5
STMS	Pea (*P. sativum*)	15	0
STMS	Chickpea (*Cicer arietinum* L.)	44	5
SSR	Medicago (*Medicago truncatula*)	230	31

Table 4.2. Sequences for *Medicago*-derived SSRs (microsatellite) primer pairs (18-23 bp) assayed on DNA samples (of parent plants) of two *Pisum sativum* L. genotypes ATC 1564 and Kaspa, and exhibited polymorphism.

Primer pairs[1]	Forward primer	Reverse primer	Tm (ºC)
PGER-S5	CGAATAAATCAAATGCGTAAACA	GGTTCGTCGTAACAGGTGGT	56
PGER-S13	TCAAAATCAAAATCGGCGAC	TGTGTGCATCTAAGACCAAAAA	53
PGER-S27	CCGAAGCTTGTTATCCAAGG	TGAATGAAAGAGGAAGAAGTGAA	56
PGER-S28	CCCTTAACCAAGCAAGCAAA	GAAAAGGGGAGGAGATCAATG	55
PGER-S48	TGGCTTTCTTACGATCCACA	GCGTAGTGTGATCAATTGGC	54
PGER-S60	GGAAAAGGGAGGGTGTGAAT	GGCTGTTTTCTTCACCCACA	55
PGER-S88	CGCCGTCATGCTTGATACTA	GCAAAACGCCCCCTAAAT	52
PGER-S114	TTGGCATACAAAGGCACAAG	TTCGTCCAAGGCGGTATATT	53
PGER-S139	CAAGGCTCGCTTTTTCTTCA	ATGAGCGTAGGCGATAGTGG	55
PGER-S140	GCCATCAAAGTTAAAATGGCTT	ATTTAGGCACCCCGAGGTTA	55
PGER-S152	TCTTGCTCTCTGGTGGTGAA	TTCTCTCCATACCTCGCTCAA	56
PGER-S159	TCCACCACAAATCACAGGAA	TCCTAATCCAATACACCCCC	54
PGER-S161	CAGTCCATTTGCTCACGTTG	CAGGATGTGCGATAAATGGA	54
PGER-S162	CCTGGTGTCAACGGATCCTA	TTTCACTGTCACCTTCAGAGGA	58
PGER-S178	TACCGTAGCTCCCTTTTCCA	TTTGTGTGCCCATGAATGAC	54

[1]PGER-S and PGER-E were abbreviated for Pulse Germplasm Enhancement Research SSR and EST sequences, respectively.

Table 4.2. continued.

Primer pairs[1]	Forward primer	Reverse primer	Tm (°C)
PGER-S180	TTCATCCTCACACAAACCCA	TTCATGGGGTCTGTTTCTGA	53
PGER-S200	AGGAGTGGCAGTTGAACCAC	TTGCCAATTTGAATCGAACA	53
PGER-E5	TGACGTGGACGATTTCATGT	ACAAACTCTTGGGGCAGAGA	51
PGER-E18	TGGGAACTCCCTTTTCACAC	ATGGCAGTGCATCGAACATA	51
PGER-E21	TGCAACAGCCAACTGGTATC	TTGACTTTACAGCGCAGGTG	52
PGER-E23	GCATCTCTGCTGGTGATGAA	ATGTCGCCCTGTCAACCTAC	53
PGER-E31	TGATCGGTGATTCCTTGTGA	TGGTCAACAGTTTGGGGATT	50
PGER-E34	TTAATTGGGGTTGGACCAGA	ACCACTACCTCCACGACCAG	53
PGER-E46	ATGAAGCATATGGGCTCACC	AAGGGGCATTAAGCCAAACT	51
PGER-E48	ACATTTGTGCGGACTGTGTC	ATTGATGCTCTGCCAGGAAC	52
PGER-E50	GCTATGGGGCTTAGTGACCA	AACAAGTGGGCGGAATACAG	53
PGER-E53	CGACCCATTTCCTCACAACT	TAGCAAGAAAAAGCCCCAGA	51
PGER-E62	TGTTGTTGCCGAGTGTTGTT	TCCGCCTGTAAGCTGAAACT	51
PGER-E67	GACCCCCTTGTTCATTTTCA	AGCAGCGATGAGTTTGTGTG	51
PGER-E115	TCCTTCTCATTCCAACCTTGTT	CCAGCCAAGATAAGCGAAAG	52
PGER-E131	GTGGGAAGATGTTTGGAGGA	GCCTCTTGGAGAGGTGTCAG	54

[1]PGER-S and PGER-E were abbreviated for Pulse Germplasm Enhancement Research SSR and EST sequences, respectively.

Table 4.3. Chi-square test for the deviation of observed molecular marker genotypes of 119 backcross plants from the expected 1:1 ratio, and association of markers with the frost survival at flowering stage (FS).

Marker	**Observed frequency[a]**	χ^2	***F***
PGER-E50	46:73	6.13*	0.00
PGER-E62	50:69	3.03	4.15*
PGER-E115	55:64	0.68	2.74
PGER-E5	62:57	0.21	0.07
PGER-E131	63:56	0.41	2.96
PGER-E23	58:61	0.08	0.69
PGER-E31	57:62	0.21	0.43
TA34	73:46	6.13*	1.02
TA45	61:58	0.08	0.17
TA76	59:60	0.01	0.71
PGER-E18	70:49	3.71	3.44
PGER-S48	62:57	0.21	1.47
PGER-E53	56:63	0.41	0.04
PSGSR1	48:71	4.45*	0.00
PSAJ3318	50:69	3.03	1.52
PSU51918	68:51	2.43	1.96
PS11824	55:64	0.68	1.62
PSU58830	64:55	0.68	0.65
PGER-S27	62:57	0.21	0.01
PGER-S28	45:74	7.07**	0.36
PGER-S13	78:41	11.50***	0.23

[a]Homozygous (as parent ATC 1564): Heterozygous (ATC 1564 and Kaspa alleles), * $P<0.05$, ** $P<0.01$, *** $P<0.005$.

Table 4.3. continued.

Marker	Observed frequency[a]	χ^2	*F*
PGER-S60	72:47	5.25*	0.00
PGER-S114	60:59	0.01	0.57
PGER-S139	67:52	1.89	4.25*
PGER-S140	50:69	3.03	0.01
PGER-S88	70:49	3.71	0.09
PGER-S152	44:75	8.08**	0.24
PGER-S162	61:58	0.08	0.15
PGER-S161	60:59	0.01	0.22
PGER-S200	74:45	7.07**	0.04
PGER-S159	89:30	29.25***	0.13
PGER-S180	54:65	1.02	0.01
PGER-S178	45:74	7.07**	0.68
PGER-S5	77:42	10.29**	0.11
PGER-E67	73:46	6.13*	3.96*
PGER-E21	62:57	0.21	0.95
PGER-E46	77:42	10.29**	1.47
PGER-E48	68:51	2.43	0.66
TA43	74:45	7.07**	0.12
TA3	52:67	1.89	3.64
PGER-E34	35:84	20.18***	4.11*
Flower Colour	63:56	0.41	0.01

[a]Homozygous(as parent ATC 1564): Heterozygous (ATC 1564 and Kaspa alleles), * $P<0.05$, ** $P<0.01$, *** $P<0.005$.

Marker-trait association

Thirty nine markers including one morphological marker (flower colour), were assigned to linkage groups to develop a map using find groups command in MapDisto (Lorieux 2012) at LOD min= 3.0 and r max= 0.3. Thirty five loci were unlinked, and two linkage groups were developed with only two and three markers on each group (Fig. 4.4).

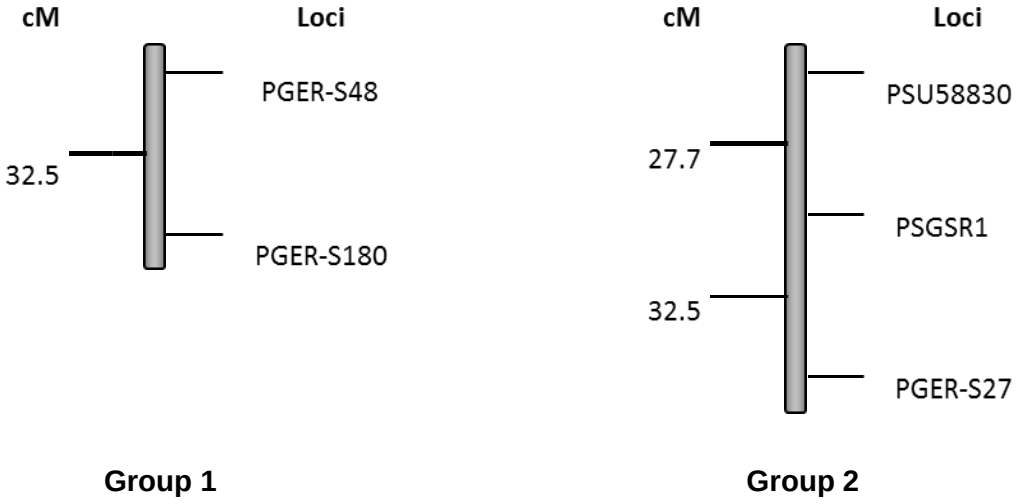


Fig. 4.4. Five loci were linked on two linkage groups at LOD min= 3.0, and r max= 0.3 using MapDisto (Lorieux 2012).

Two markers: PGER-E62 and PGER-S139, found to be associated ($P<0.05$) with frost survival at FS, and none was found associated with frost survival at PDS (Appendices 2 and 3). The additive effects of PGER-E62 and PGER-S139 were -5.94 (R^2= 3.4 %) and 5.99 (R^2= 3.5 %), respectively. Plants that were homozygous for the ATC 1564 allele of PGER-E62 exhibited slightly better frost tolerance than those that were heterozygous for the ATC 1564 and Kaspa alleles of that marker. Among 50 homozygotes, 19 (38%) exhibited at least some survival of FS organs, compared to only 21 of 69 (30.4%) for the ATC 1564/Kaspa heterozygotes. Among the 19 homozygotes survival rate of FS organs ranged from 2 % to 100 % (Fig. 4.5A). For the 21 heterozygotes, this rate was from 2 % to 65 % (Fig. 4.5B).

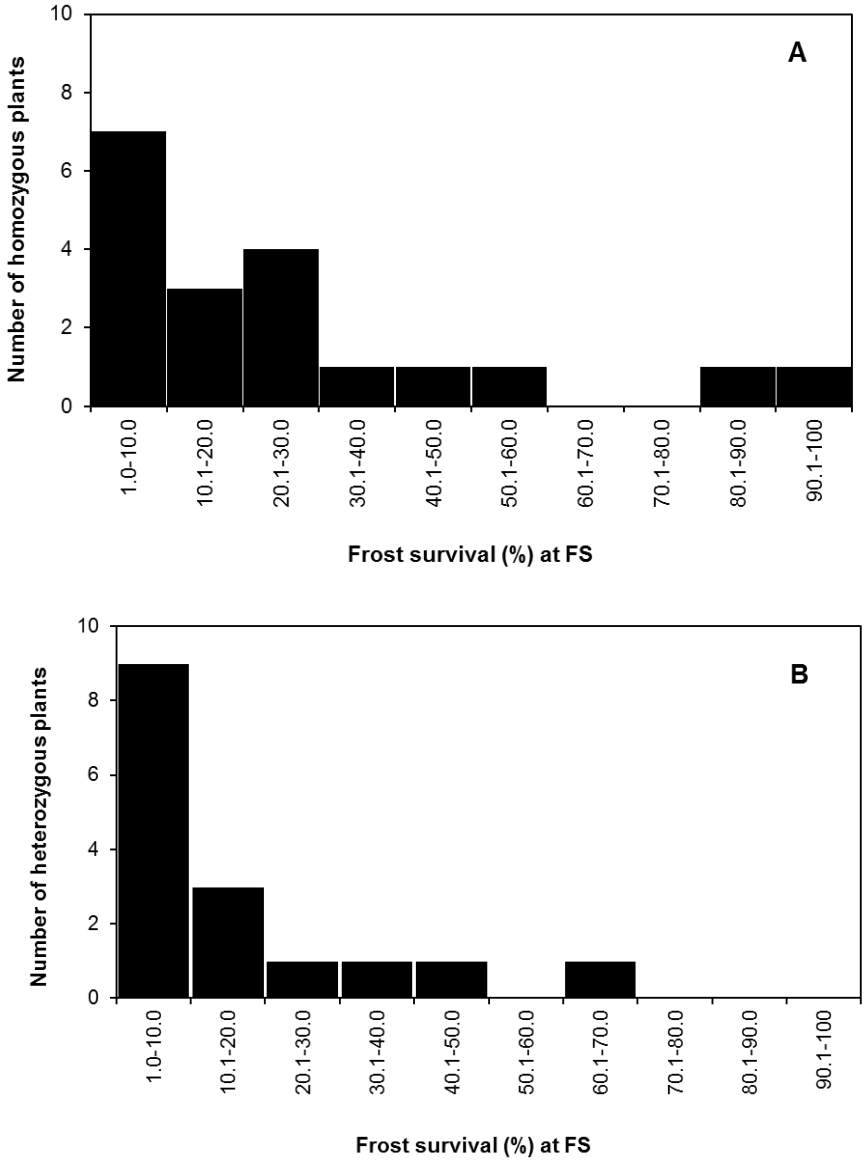


Fig. 4.5. The frequency distribution of backcross plants exhibited survival of flowering stage organs above 0 % against frost for (**A**) homozygous (for ATC 1564 allele) and (**B**) heterozygous (for Kaspa/ATC 1564 alleles) classes of marker PGER-E62. In addition to the plants included in these frequency distributions, there were 31 homozygous plants and 48 heterozygous plants on which no flowering stage organs survived the frost treatment.

For marker PGER-S139, the Kaspa/ATC 1564 heterozygotes exhibited slightly better frost survival than the ATC 1564 homozygotes (Fig 4.6). For marker PGER-S139, the numbers of plants with some survival of FS organs were 18 of 67 (26.9%) of

heterozygotes and 22 of 52 (42.3%) of homozygotes. Among the 18 homozygotes, the survival rate of FS organs ranged from 2 % to 53 % (Fig. 4.6A). For the 22 heterozygotes, this range was from 3 % to 100 % (Fig. 4.6B).

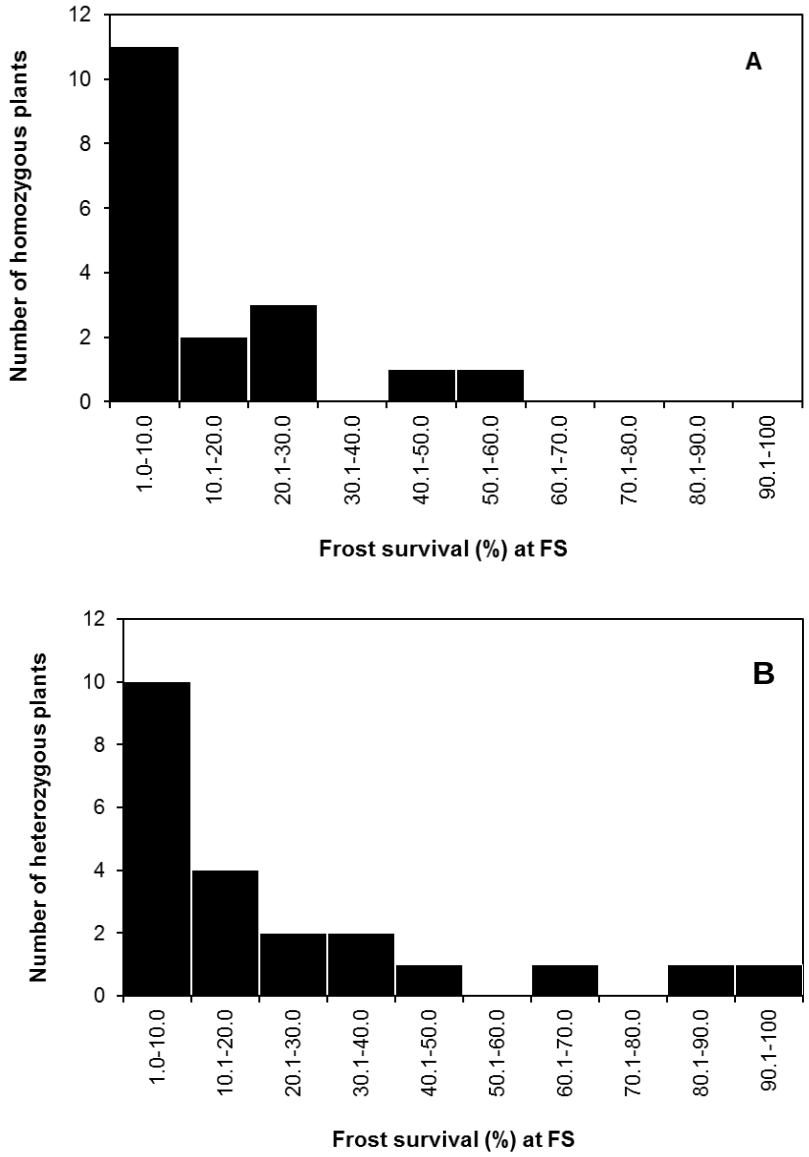


Fig. 4.6. The frequency distribution of backcross plants exhibited survival of flowering stage organs above 0 % against frost for (**A**) homozygous (for ATC 1564 allele) and (**B**) heterozygous (for Kaspa/ATC 1564 alleles) classes of marker PGER-S139. In addition to the plants included in these frequency distributions, there were 49 homozygous plants and 30 heterozygous plants on which no flowering stage organs survived the frost treatment.

4.4. Discussion

In this experiment, Kaspa and ATC 1564 were selected as susceptible and relatively tolerant parents, respectively, on the basis of findings reported in the previous chapter (Table 3.6 in Chapter 3 of this thesis). As expected, Kaspa did not survive frost at the flowering stage (FS) while ATC 1564 exhibited some survival. The survival rate for ATC 1564 was lower than in the experiment reported in the previous chapter (Table 3.6 in Chapter 3 of this thesis). Greater replication may be needed to obtain repeatable results in frost screening under controlled conditions. Another possibility is that ATC 1564 is somewhat heterogeneous and that the plants tested here were not as frost tolerant as those tested in the earlier experiment. In retrospect, ATC 1564 was probably not the best choice as a frost-tolerant parent. Based on all of the evaluations conducted in this thesis research, a better choice might have been ATC 968, which has shown high frost tolerance repeatedly in experiments reported in this thesis (Chapters 3, 5 and 7).

The BC population was, in general, susceptible to frost, and in 63 (53 %) of the backcross plants none of the FS- and PDS-reproductive organs survived frost. However, on 33 to 34 (27 to 29 %) of the backcross plants, the survival of FS- and PDS-reproductive organs was higher than frost tolerant parent ATC 1564.

Of the total 332 markers assayed, only 41 (13 %) of the markers exhibited polymorphic products between the parents. In field pea, restricted polymorphism and low heterozygosity can be expected due to self-pollination.

It was observed that there is high cross-species transferability in leguminous species. The *Medicago*-derived primers exhibited polymorphism between pea parents. This is similar to the findings of Mishra et al. (2012) who reported the amplification of 68 % of *Medicago* SSR primer pairs in pea.

Due to the limited number of polymorphic markers found for parents used here, it was not feasible to develop a genome-wide map, and unfortunately 85 % of the total markers were not found to be linked with any other loci. One marker, PSU51918, (sequences sourced from Burstin et al. 2001) that was polymorphic between parental lines in this study, was previously mapped on pea linkage group I (Loridon et al. 2005) , but was not found to be associated with frost survival here. On the other hand, marker PSU81288 which was also mapped on linkage group I (Loridon et al. 2005), and was reported to be

closely linked to a QTL for winter frost damage at the vegetative stage (Lejeune-Henaut et al. 2008), did not exhibit polymorphism between parents used in the present study.

The significant ($P<0.05$) association of the marker PGER-E62 with frost survival at FS, and negative additive effect indicated as expected that the favourable effect of reproductive frost tolerance came from the frost tolerant parent ATC 1564. In contrast, for the other marker (PGER-S139) with significant ($P<0.05$) association with frost survival at FS, the additive effect was positive, indicating that some tolerance was contributed from the frost-sensitive parent Kaspa.

This study is the first attempt to investigate segregation of frost survival at the reproductive stage in a backcross population in pea. Due to limited polymorphism between the two parents, the marker data could not be further analysed for mapping and QTL detection. In future, development of RILs (recombinant inbred lines) would be advantageous allowing the frost screening (phenotyping) to be replicated and/or repeated which was not possible in the backcross population used here. Since this work has been carried out, new genomic resources have been developed for pea (Bordat et al. 2011; Smýkal et al. 2012). In future, SNP (single nucleotide polymorphism) markers could be used to improve the chances of detecting sufficient allelic variation for genetic mapping (Deulvot et al. 2010).

Chapter 5

Responses of field pea plants to cold treatment at the reproductive stage

5.1. Introduction

Exposure to temperatures below 0 ^{o}C can lead to frost damage in plants. This can involve cellular freezing or ice nucleation in plant tissues that may result in cellular death (Guy 1990). Therefore, inadequate tolerance to sub-zero temperatures and resulting frost damage can significantly affect plant growth and health and can lead to plant death.

Exposure to low but non-freezing temperatures may induce and/or improve subsequent tolerance to frost through a process called cold acclimation (Levitt 1980). Cold acclimation has been reported to trigger physiological changes in leaf tissues including alteration in the photosynthesis rate (Gray et al. 1997), increase in protein content (Guy 1990), accumulation of soluble sugars and starch (Sasaki et al. 1998; Bourion et al. 2003), increase in calcium influx (Monroy and Dhindsa 1995), reduction in water content and depression of freezing temperature (Collins and Rhodes 1995; Hekneby et al. 2006; Swensen and Murray 1983). The drop in water content limits the availability of free water, preventing ice formation within cells and enhancing frost tolerance (Guy 1990), while over-expression of a gene encoding Ca-dependent protein kinase confers tolerance against low temperatures (Saijo et al. 2000). Some of these cold-induced cellular and physiological changes have been reported to be associated with the acquisition of frost tolerance (Guy 1990; Ristic and Ashworth 1993; Guinchard et al. 1997; Sasaki et al. 1998; Jacobsen et al. 2005; Rashed Mohassel et al. 2009).

Field pea is sensitive to frost at reproductive stages (Chapter 3 of this thesis; Shafiq et al. 2012), and exposure to below 0 ^{o}C during flowering leads to reduced yield (Ridge and Pye 1985). The process of cold acclimation has been observed at the vegetative stage in field pea and cold-acclimated pea seedlings survived sub-zero temperatures better than non-acclimated seedlings (Swensen and Murray 1983; Badaruddin and Meyer 2001; Meyer and Badaruddin 2001; Bourion et al. 2003). However, the response

to cold treatment at the reproductive stage has not been reported in field pea, and it is not known whether cold treatment at the reproductive stage will induce cold acclimation and influence reproductive frost tolerance (RFT) of field pea.

Previously, the physiological changes related to the process of acclimation were studied in leaf tissues only. In the present study, field pea plants were exposed to cold (low positive) temperatures at the reproductive stage. Physiological changes were investigated in leaf tissues to determine whether similar acclimation responses occurred in plants at the reproductive stage to those reported for vegetative stage plants. Further, development of reproductive organs was studied to determine whether the cold treatment affected frost tolerance.

5.2. Materials and methods

Plant material

Four accessions (ATC 968, ATC 1040, ATC 1564 and Kaspa) were selected for use in this study on the basis of previously observed contrasting responses to frost at the critical flowering stage (Chapter 3 of this thesis; Shafiq et al. 2012) (Table 5.1).

Growth conditions and cold treatment

Sowing times for the four accessions were staggered to synchronize flowering on the basis of previous observations (recorded during the study presented in Chapter 3 of this thesis; Appendix 1), so that plants would be at the same stage of development at the time of cold treatment. ATC 968 and Kaspa were sown on the same day and ATC 1564 and ATC 1040 were sown 16 and 20 days later, respectively, all in the same glasshouse. All plants were grown at 18/12 ^{o}C (day/night) and 14 hr photoperiod. Each seed was sown in an individual 200 mm diameter pot containing new bark mix soil. Plants were arranged in the glasshouse in a completely randomised design (CRD) with eight plants per genotype. One week after sowing, the soil around each seed was inoculated with pea rhizobium culture *Nodulaid 100* (Bio-care Tech Pty Ltd. NSW, Australia). Plants were watered regularly and supported with wooden stakes. About two weeks after the appearance of the first flower on all plants, three plants of each genotype were transferred to a cold chamber and all other (non-cold treated) plants were kept in the glasshouse (Fig 5.1).

Table 5.1. Origin, leaf type, flower colour, seed coat and flowering time of four field pea (*Pisum sativum* L.) genotypes

Accessions	Origin	Leaf type	Flower colour	Seed coat type	Flowering time (days)[a]	Frost survival of buds and flowers[b]
^ATC 968	Italy	Conventional	White	Non-pigmented	68	23.4
ATC 1040	Nepal	Conventional	Purple	Pigmented	48	0.0
ATC 1564	Afghanistan	Conventional	White	Non-pigmented	52	28.3
Kaspa	Australia	Semi leafless	Light pink	Pigmented	67	0.0

^ATC: Australian Temperate Field Crops Collection accession number; **[a]**flowering times were determined during the study presented in Chapter 3 of this thesis (Appendix 1); **[b]**derived from Chapter 3 of this thesis and Shafiq et al. (2012)

Temperature Treatments

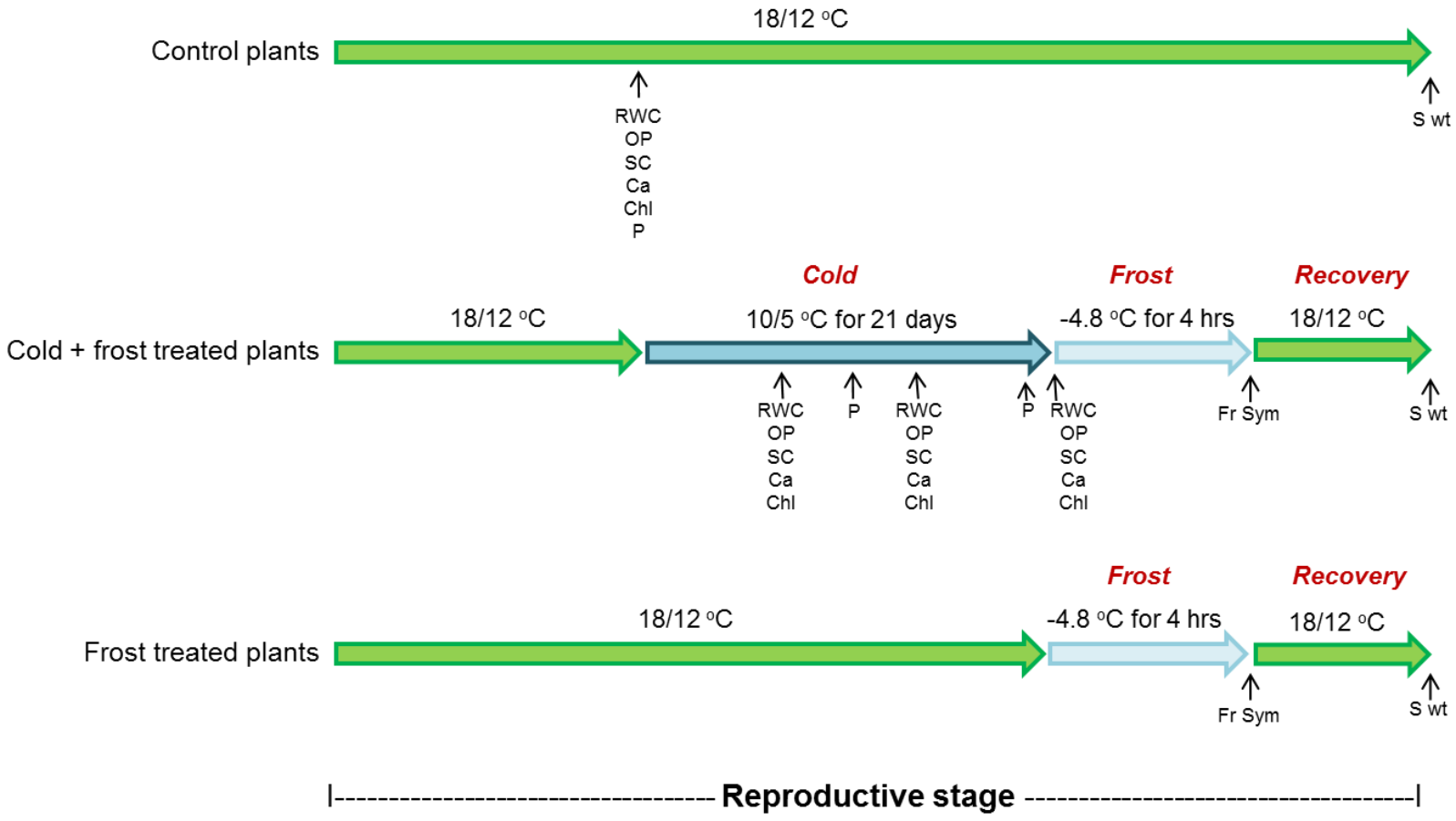


Fig 5.1. Schematic diagram of temperature treatments of plants in the experiment; Three plants of each genotype were assigned to each treatment. From non-cold-treated (control) plants, measurements were taken at 0 day cold treatment for the following: RWC, relative water content; OP, osmotic potential; SC, sugar concentration; Ca, calcium concentration; Chl, chlorophyll content; P, rate of photosynthesis. From cold treated plants, all measurements for physiological analyses were made after 7, 14 and 21 days of cold treatment, and rate of photosynthesis was determined after 10 and 20 days of cold treatment. Frost symptoms (Fr Sym) were determined from cold and non-cold treated plants 72 hr after frost exposure. Seed weight (S wt) was determined from control plants and all frosted plants at pod maturity.

In the cold chamber, plants were arranged in a CRD. Plants were treated at 10/5 ºC (day/night) under 12 h photoperiod and 150 µmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD) generated by high-pressure sodium lighting system (Vialox® 400 Watt NAV-T, OSRAM) for 21 days. Every two days, the plants in the cold room were rearranged into a new CRD layout.

Frost exposure

After 21 days of cold treatment, all cold-treated plants and two non-cold-treated plants of each accession were exposed to frost in a chamber (www.agrf.org.au) under controlled conditions. Prior to frost treatment, each bud, flower or pod was individually labelled to indicate its stage of development (Knott 1987) using the following ordinal scale: immature bud: 201, mature bud: 202, open flower: 203, set pod: 204, flat pod: 205, swollen pod: 206, filled pod: 207 and mature pod: 208. To simulate a frost event in the chamber, the temperature was controlled over a 24 hr period and dropped by 5 ºC per hr from 10 ºC to 3.5 ºC and by 1 ºC per hr from 3.5 ºC to - 4.8 ºC. The temperature was held at - 4.8 ºC for 4 hr and then increased by 2 °C per hr to 3.5 °C and by 5 °C per hr to 20 °C. After frost treatment, all frosted plants were placed in the glasshouse at 18/12 ºC (day/night) and maintained along with non-cold-treated control plants of each accession until pods were dry at which time they were harvested.

Symptom data were collected 72 hr after the frost exposure, using scores as described in Table 3.3 in Chapter 3 of this thesis. All seeds from pods that had been exposed to frost or that had developed from buds or flowers that were exposed to frost were harvested when dry, counted and weighed. Seeds from control plants were harvested at maturity, counted and weighed. The weight of 100 seeds was calculated for frosted and control plants for each genotype.

Physiological Analyses

Samples were taken at the beginning of the cold treatment and after 7, 14 and 21 days of the cold treatment from non-cold-treated (control) and cold-treated plants, respectively, for the following physiological analyses: relative water content, osmotic potential, concentration of soluble sugars and calcium and chlorophyll content. The rate of photosynthesis was determined after 10 and 20 days of cold treatment (Fig 5.1).

Rate of photosynthesis

The rate of photosynthesis was recorded on intact leaflets (of ATC 968, ATC 1040 and ATC 1564) and stipules (Kaspa) three times: at the beginning of the cold treatment, in the middle and a day before the end of the cold treatment i.e. 0, 10 and 20 days after the beginning of the cold treatment, respectively. Youngest fully emerged leaves on the main stem were selected in all plants and the rate of photosynthesis was measured using a portable photosynthesis system LICOR-6400® (LI-COR Inc., USA) under the light and temperature conditions (Mosaleeyanon et al. 2005) in the glasshouse and cold room. All observations for rate of photosynthesis were taken at the same time of the day to avoid diurnal variation.

Five of the youngest fully emerged leaflets or stipules on the main stem of each plant were excised and a single leaflet/stipule per plant was randomly allocated for each of the following analyses.

a. Relative water content

Relative water content (RWC) was determined for leaflets and stipules as described by Sánchez et al. (2004). The fresh weight (*FW*) of the sample was recorded, and then the sample was allowed to float on distilled water in the dark, overnight. Turgid weight (*TW*) of the hydrated sample was determined the following day. The hydrated sample was then oven-dried at 80 ^{o}C for 48 hr. The dry weight (*DW*) was recorded and *RWC* was determined using the following formula (Sánchez et al. 2004):

$$RWC = \frac{(FW - DW)}{(TW - DW)} \times 100$$

b. Osmotic potential

Osmotic potential was measured for leaflets and stipules following Turner et al. (2007). Excised leaf tissues were instantly frozen in liquid nitrogen and kept in a freezer at -20 ^{o}C until required. Each sample was allowed to thaw, placed in a 2 ml microcentrifuge tube and pushed to the bottom of the tube with a 5 ml syringe. The tubes were centrifuged (with syringes) at 4000 rpm and 4 ^{o}C for 20 min. From the extract, 10 µl of the cell sap was placed on a disc of blotting paper and inserted in the chamber of a

vapour pressure micro-osmometer (Vapro® 5500, Wescor, UT, USA). Readings were noted and multiplied by 2.469 x 10^{-3} for conversion into mPa units (Borg 1989).

c. Soluble carbohydrates

The soluble carbohydrates sucrose, glucose and fructose were analysed by reverse phase high-performance liquid chromatography (HPLC) as described by Naidu (1998). The sap of the tissue (leaflets and stipules) was obtained in the same manner as described above for osmotic potential and extracted with 10 volumes of methanol:chloroform:water (60:25:15). D-sorbitol (8.4 µmol) was added as an internal standard to each sample and centrifuged at 10,000 g for 10 min at 4 ^{o}C. The supernatant was dried and redissolved in 200 µl of nanopure water before the assay. Samples were analysed using a DX-500 HPLC system (Dionex) with a SIL-10AD autoinjector (Shimadzu) and an LC 1210 UV/VIS detector (ICI Instruments). Separation was performed on a 300 x 6.5 mm SugarPak column (Waters) with a 7.5 x 4.6 mm BC-100 calcium guard cartridge (Benson) maintained at 90^{o}C in a column oven (Model 2155, Pharmacia LKB) with Ca-EDTA (50 mg L^{-1}) mobile phase. Data were analysed with Peaknet Chromatography Workstation version 4.3 (Dionex). Concentrations of all three sugars were summed and presented as total sugars in the tissue.

d. Chlorophyll content

Chlorophyll content for leaflets and stipules was determined as described by Arnon (1949). Leaf sections of 1x1 cm were cut and weighed. The leaf sections were ground in ice cold acetone (80 %) and the concentrations of chlorophyll *a* and *b* in fresh extract were determined using a spectrophotometer (Pharmacia LKB Biochrom Ltd, England). The absorbance of pigments was recorded at λ_{645} and λ_{663} and total contents were calculated using the following formula (Arnon 1949);

$$C = 20.2\ D_{645} + 8.02\ D_{663}$$

e. Concentration of Calcium

The concentration of calcium (Ca) was determined for leaflets and stipules. Excised leaves were dried at 80 ^{o}C for 48 hr. Due to the limitation of the weight of individual leaflets, three dried samples from each genotype were combined and ground together to a finely homogenised powder and weighed. A minimum of 300 mg of the dried powder was digested with a mixture of nitric acid and hydrogen peroxide (Zhu et al. 2000). The digested material was filtered, diluted and analysed by Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES). During analysis for each treatment (0, 7, 14 and 21 days cold treatment), two randomly selected samples were repeated to observe any variation in measured values.

Statistical analysis

For the results of physiological analyses made prior to frost treatment, two-way analysis of variance (ANOVA) was performed to test the statistical significance of genotypes and treatments (0, 7, 14 or 21 days of cold treatment) using GenStat (GenStat software, 11th Edition). Similarly, two-way ANOVA was used for the survival of buds, flowers and pods and for seed weight after frost exposure, however with only two treatment levels i.e. cold (21 days) and no-cold.

For concentration of Ca, regression analysis was performed over time (0, 7, 14 and 21 days of cold treatment) for each genotype individually, using the following model;

$$[Ca]_i = \alpha + \beta * x_i + R_i$$

where $[Ca]_i$ is the concentration of Ca at the *i*th sampling time, x_i is the number of days of cold treatment (0, 7, 14 or 21 days) prior to the *i*th sampling time, α is the *y* intercept when x is zero, β is the regression coefficient, and R represents the residual variation, which was assumed to be normally distributed with mean 0 and variance σ^2.

5.3. Results

Reproductive frost tolerance after 21 days of cold treatment

There was significant variation among the four accessions and between the two treatments (cold and no-cold) for the survival of buds, flowers and pods when plants were exposed to frost (Table 5.2). With or without the prior cold treatment, ATC 968 exhibited the best survival and Kaspa the worst (Table 5.3). The cold treatment reduced the tolerance of reproductive tissues (buds, flowers and pods) against the frost stress (Table 5.3).

Table 5.2. Analysis of variance for the frost survival of buds, flowers and pods, and seed weight of four accessions of field pea (*Pisum sativum* L.) exposed to frost with and without prior cold treatment

Sources of variation	**d.f**	**F value**	***P***
Frost survival			
Genotypes	3	3.81*	0.043
Treatment (cold - no cold)	1	10.12*	0.009
Genotypes x Treatment	3	1.59	0.248
Residual	11		
^***100 seed weight***			
Genotype	1	0.06	0.812
Treatment (cold – no cold)	1	5.92	0.059
Genotype x Treatment	1	0.10	0.763
Residual	5		

*indicates that the value is significant at $P < 0.05$. ^The 100 seed weight was analysed for only two accessions (ATC 968 and ATC 1564), as the other two accessions (ATC 1040 and Kaspa) did not produce any seeds after exposure to both cold and frost.

Table 5.3. Mean frost survival of buds, flowers and pods, and 100 seed weight of control and frost exposed plants with and without cold (low positive temperature) pre-treatment at the reproductive stage for four accessions of field pea (*Pisum sativum* L.)

Genotype	Frost survival of buds, flowers and pods (%)		100 seed weight (g)		
	with no-cold	with cold	control (no-cold, no-frost)	Frost with no-cold	Frost with cold
ATC 968	27.5	10.0	32.6	5.3	2.0
ATC 1040	18.1	0.0	11.1	0.6	-
ATC 1564	12.5	4.4	21.2	4.5	2.0
Kaspa	0.8	0.0	26.0	3.9	-

- indicates that the plants of ATC 1040 and Kaspa that were exposed to cold for 21 days did not produce seeds following exposure to frost

For all genotypes, seeds harvested from plants that had been exposed to frost were very small (Table 5.3), and this effect was intensified by cold treatment prior to frost exposure, with two accessions (ATC 1040 and Kaspa) setting no seeds at all, and the other two having 100-seed weights of only about 2 g (Table 5.2 and 5.3).

Physiological changes in leaf tissues during cold treatment

The cold treatment significantly reduced the concentration of soluble sugars, and increased the chlorophyll content in leaf tissues (Table 5.4, Fig 5.2). The rate of photosynthesis first dropped under cold conditions between 0 and 10 days, but rose again by 20 days (Table 5.4, Fig. 5.2).

Variation among genotypes was significant for osmotic potential, concentration of soluble sugars, the chlorophyll content and the rate of photosynthesis during cold treatment (Table 5.4). The frost-sensitive variety Kaspa was characterised by relatively low osmotic potential and soluble sugar concentration, and high photosynthetic rate during the cold treatment (Fig 5.2). Further, ATC 1564 responded differently from the other three accessions for the chlorophyll content (Fig. 5.2). There was a little variation among genotypes in the concentration of Ca under control conditions (0 days), and Ca appeared to be increased in ATC 1040 compared to other accessions after 7 days of low temperature treatment (Fig 5.2). However, the cold treatment had no significant effect on the concentration of Ca. The regression equations for predicting the concentration of Ca at time periods (days) of cold treatment, were obtained for each accession as follows:

ATC 968: $[Ca] = 611 + 45.1x$

ATC 1564: $[Ca] = 385 + 12.9x$

ATC 1040: $[Ca] = 1163 + 13.3x$

Kaspa: $[Ca] = 1072 + 26.7x$

where x is the number of days of cold treatment.

Table 5.4. Analysis of variance for relative water content, osmotic potential, concentration of soluble sugars (fructose, glucose and sucrose) and chlorophyll content in four accessions of field pea (*Pisum sativum* L.) subjected to 0, 7, 14 or 21 days of cold treatment

Sources of variation	d.f	F value	*P*
Relative water content			
Genotypes	3	1.72	0.204
Treatments	3	1.56	0.238
Genotypes x Treatment	9	1.45	0.248
Residual	16		
Osmotic Potential			
Genotypes	3	7.05*	<0.001
Treatments	3	1.41	0.259
Genotypes x Treatment	9	0.58	0.801
Residual	32		
Soluble sugar concentrations			
Genotypes	3	4.16*	0.019
Treatments	3	7.80*	0.001
Genotypes x Treatment	9	1.34	0.280
Residual	20		
Chlorophyll content			
Genotypes	3	8.92*	<0.001
Treatments	3	31.10*	<0.001
Genotypes x Treatment	9	3.23*	0.014
Residual	20		
Rate of photosynthesis			
Genotypes	3	5.44*	0.003
Treatments	2	8.11*	0.001
Genotypes x Treatment	6	0.88	0.522
Residual	36		

*indicates that the value is significant at $P < 0.05$.

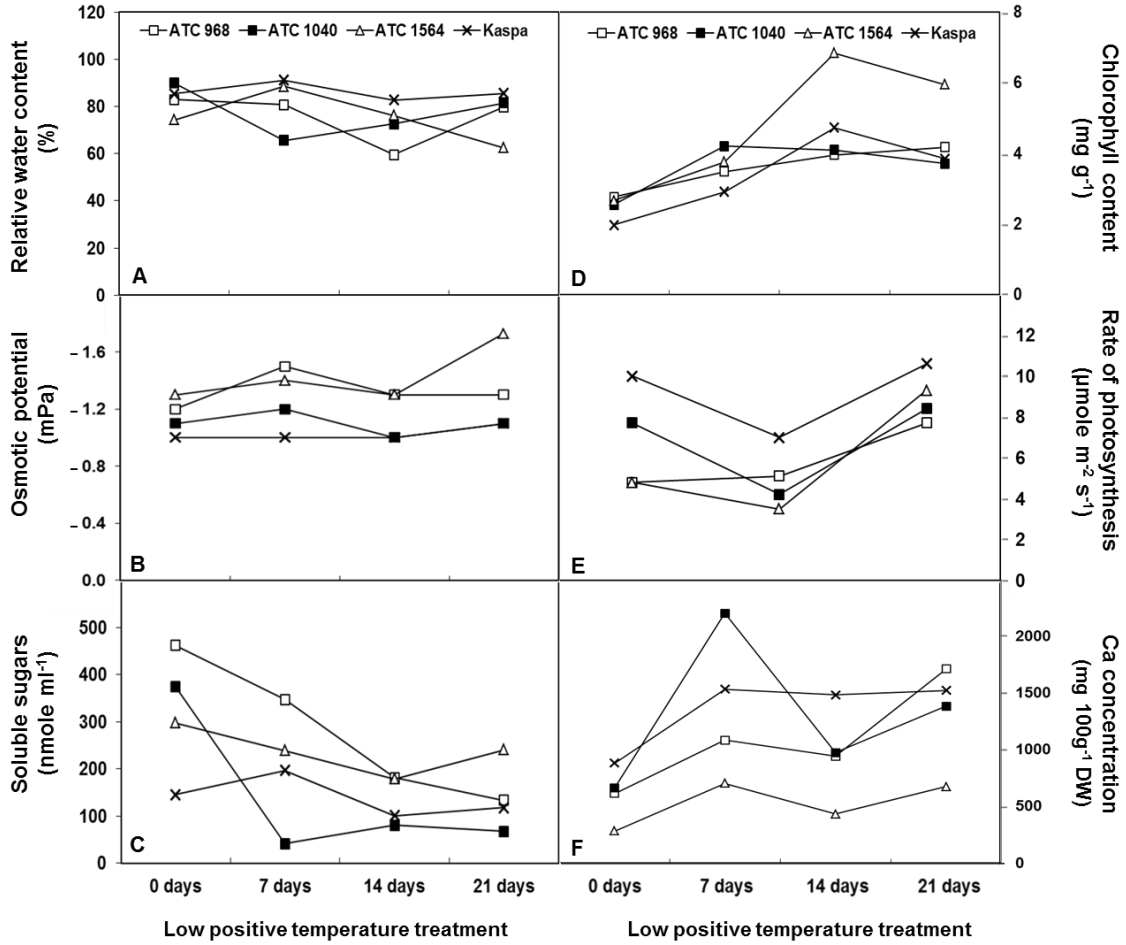


Fig 5.2. Response (mean values) of four field pea genotypes to 7, 14 and 21 days of low positive temperature exposure (10/5 ^{o}C D/N) at the reproductive stage. **A**: relative water content; **B**: osmotic potential; **C**: concentration of soluble sugars (glucose, fructose and sucrose); **D**: chlorophyll content; **E**: rate of photosynthesis (after 10 and 20 days of cold treatment); **F**: concentration of calcium

5.4. Discussion

The reproductive-stage frost tolerance in field pea was significantly reduced with the cold pre-treatment (of 21 days). This was reflected by the lower survival of reproductive organs (buds, flowers and pods) and seed weight in cold-treated plants on exposure to frost, than non-cold-treated plants. This is in contrast to reports on vegetative-stage frost tolerance of pea (Badaruddin and Meyer 2001; Bourion et al. 2003) and chickpea (Bakht et al. 2006), which were improved after cold acclimation. Here, it appears that the reproductive-stage plants were not acclimated after the end of the cold treatment. The plants may have lost their ability to respond to low positive temperatures. This would be consistent with the observations of Meyer and Badaruddin (2001) that older pea seedlings were less responsive to acclimation and exhibited reduced frost tolerance than younger acclimated seedlings.

The results indicate that frost-sensitive genotypes may be more chilling-sensitive than frost-tolerant genotypes. Cold treatment enhanced vulnerability to frost in all genotypes but its effect was more severe for frost-sensitive Kaspa and for ATC 1040 than the more frost tolerant ATC 968. After exposure to cold temperatures, no reproductive organs (buds, flowers or pods) of Kaspa or ATC 1040 survived frost. In the present study, with or without prior cold treatment, ATC 968 had the highest survival of reproductive organs against frost. In the present study, the combined survival of all reproductive organs (flowering stage and pod development stage organs) against frost are presented. These results confirm the findings of Chapter 3 of this thesis where ATC 968 and ATC 1564 showed high frost survival at the flowering stage (with no pre-cold treatment). The loss in seed weight in this study was about the same in all genotypes after exposure to frost (without pre-cold treatment), but it should be noted that in Chapter 3 the tolerant genotypes had more viable seeds than the susceptible genotypes.

The decrease in the concentration of soluble sugars (fructose, glucose and sucrose) in leaves that was observed during cold treatment may have been a factor in increasing frost sensitivity. Sugars are known to protect the plasma membrane in cells from freezing when plants are exposed to frost (Sakai and Yoshida 1968; Steponkus 1984). The accumulation of soluble sugars has previously been reported to be positively associated with frost tolerance in seedlings of cabbage (Sasaki et al. 1996; 1998) and pea (Bourion et al. 2003). Here, a decline in soluble sugar concentration was associated

with frost sensitivity in reproductive-stage pea plants. Further, the accessions that were the most sensitive to frost after cold treatment (ATC 1040 and Kaspa) were also the ones with the lowest concentrations of soluble sugars after cold treatment. It should be noted, however, that sugar concentration was measured here on leaf tissues, and not directly on the reproductive organs for which frost survival was recorded. Data on the concentration of sugars in reproductive organs could provide information on how assimilates are partitioned in response to low temperature; this will be addressed in Chapter 7 of this thesis.

In contrast to what has been observed during cold acclimation of pea seedlings (Bourion et al. 2003), the cold treatment imposed here on reproductive-stage pea plants did not affect relative water content of leaf tissues. Further, no change in the Ca-concentration is in contrast to the findings of Monroy and Dhindsa (1995) where an increase in the concentration of Ca was observed during cold acclimation. No alteration in relative water content, and non-accumulation of soluble sugars in leaf tissues, may have contributed towards the lack of change in leaf osmotic potential observed here during the cold treatment. Compared to frost-sensitive accessions (ATC 1040 and Kaspa), lower osmotic potential was observed during the cold treatment in relatively frost-tolerant accessions, ATC 1564 and ATC 968.

The decline in rate of photosynthesis after the first 10 days of cold treatment and then its upward adjustment after 20 days of cold treatment may reflect the ability in pea plants to adjust the rate of photosynthesis under cold conditions at the reproductive stage, as previously reported for pea seedlings (Georgieva and Lichtenthaler 2006). The upward adjustment in rate of photosynthesis observed here indicates that the photosystem in pea plants was not damaged under low light and positive temperature conditions, and the changes (decline) in rate of photosynthesis observed earlier were reversible as described by Antolín et al. (2005). However, plants might have compensated for the decline in rate of photosynthesis by increasing the chlorophyll content which is observed in the present study during the cold treatment. Among accessions, Kaspa had the highest rate of photosynthesis, and ATC 1564 exhibited the highest concentration of chlorophyll at the end of the cold treatment.

This is the first study carried out to investigate whether cold acclimation could improve frost tolerance of field pea at the reproductive stage. The results indicate that, rather

than becoming acclimated to cold, reproductive-stage pea plants suffered chilling injury and became more sensitive to frost. Although measurements taken during cold treatment provided some insights into physiological responses, there were some limitations in the way the experiment was designed and conducted. At the beginning of the cold treatment (day 0) measurements were taken only on the control plants and not on the cold-treated plants. At subsequent sampling times (7, 14 and 21 days) measurements were taken only on the cold-treatment plants. This meant that any comparisons to the controls (7 vs. 0, 14 vs. 0 and 21 vs. 0 days) were between plants of different ages. Given the limited amount of controlled environment space that was available, it was not feasible to assign different plants to each sampling time. This meant that comparisons among durations of cold treatment (7, 14 and 21 days) were potentially confounded by the fact that repeated measurements were taken on the same plants. In retrospect, it would have been better to have taken measurements on all plants at day 0 and at each subsequent sampling time.

Given the limitations in the experimental design, it was not obvious how best to analyse the data. Had all plants been assessed at each sampling time, then the comparisons of treated plants with their respective controls against sampling times could have been considered. For simplicity, a factorial analysis was used, even though the experiment was not conducted as a true four x four factorial, with different experimental units (plants) for each of the 16 treatment combinations. The limitations of this experiment were addressed in a later experiment (presented in Chapter 7 of this thesis) designed to investigate cold-induced changes in vegetative and reproductive tissues of two accessions.

In conclusion, the response of field pea at the reproductive stage to low positive temperatures is different than previously reported at the vegetative stage. The 21-day cold treatment did not result in the acclimation of plants as reflected through the non-accumulation of sugars and decrease in the reproductive frost tolerance. Pea plants showed an ability to maintain active photosynthesis under low positive temperature conditions. With the decrease in the concentration of soluble sugars during cold treatment, and reduction in the survival of buds, flowers and pods, and seed weight after a subsequent frost exposure, it is concluded that cold treatments applied here induced chilling stress that enhanced frost sensitivity, rather than providing acclimation and enhancing frost tolerance.

Chapter 6

Cold-induced changes in leaf ultrastructure and starch concentration at the reproductive stage in field pea

6.1. Introduction

Cold acclimation of plant tissues is accompanied by fundamental changes in cellular ultrastructure, and these cold induced changes in cells may enable plants to resist low temperatures (Levitt 1980). For example, alteration in the lipid composition of the plasma membrane has been observed during cold acclimation (Yoshida 1984; Uemura et al. 1995). Such compositional changes in the plasma membrane may help maintain membrane fluidity and prevent frost-induced membrane rupture (Steponkus 1984; Nagao et al. 2005). Various other modifications that have been observed during acclimation in plant cells, include thickening of cell walls, invagination of plasma membranes, deposition of phenolics and appearance of various microvesicles (Ristic and Ashworth 1993; Strand et al. 1999; Stefanowska et al. 2002; Helliot et al. 2003).

Among cell organelles, chloroplasts have been found to be highly responsive to low temperatures (Kimball and Salisbury 1973). Chloroplasts are larger than other cell organelles (Levitt 1980), so changes in chloroplast structure are relatively easy to observe with microscopy. Accumulation of starch grains has been observed in chloroplasts of *Arabidopsis thaliana* (L.) Heynh. during cold acclimation (Ristic and Ashworth 1993). Further, alteration in plastids in apple (*Malus domestica* Borkh.) has also been reported (Kuroda and Sagisaka 2001).

There are no reports on cold-induced modifications in the cellular ultrastructure at the reproductive stage in field pea. Variation has been observed in physiological responses to low positive temperatures among frost-sensitive and frost-tolerant genotypes of field pea at the reproductive stage (Chapter 5 of this thesis). In the present study, parenchyma cell ultrastructure and starch concentration in leaf tissues were investigated in two genotypes of field pea during 21 days of cold treatment at the reproductive stage.

6.2. Materials and methods

Plant material

Leaf tissue samples of two field pea genotypes, Kaspa and ATC 1564, were used in the present study. Kaspa is a dwarf, semi-leafless *afila* type with pink flowers, strong stems and determinate growth habit. ATC 1564 is a conventional leaf type with white flowers and prostrate growth pattern. Under controlled conditions, ATC 1564 was relatively frost tolerant during flowering (Chapter 3 of this thesis; Shafiq et al., 2012), and was less susceptible to low positive temperatures (cold) at the reproductive stage (Chapter 5 of this thesis) than Kaspa.

The leaf tissues used to generate the results presented in this chapter were sampled from plants of Kaspa and ATC 1564 grown in the experiment described in Chapter 5 of this thesis.

Stipules from Kaspa and leaflets from ATC 1564 were harvested from three plants per genotype, for the analyses of cellular ultrastructures and starch from non-cold-treated control plants and after 7, 14 and 21 days of cold treatment. At each sampling time, the three youngest fully emerged leaflets (or stipules) were harvested from the main stem of each plant.

Transmission Electron Microscopy

Thin sections of 1 mm^2 were cut from stipules and leaflets with a sharp razor blade, avoiding the midribs, and processed for transmission electron microscopy (TEM) using a technique modified from Ristic and Cass (1992) and Knight et al. (2001) as described below.

Stipule and leaflet sections (1 mm^2) were fixed with 4 % glutaraldehyde and 4 % paraformaldehyde, in phosphate buffered saline pH 7.2 (PBS) at 4 ^oC. Vials containing the sections in fixative were kept at 4 ^oC for a few days to allow thorough penetration of fixative into leaf tissue. Samples were then post-fixed with 2 % aqueous osmium tetraoxide (OsO_4) for 1 h at room temperature, followed by dehydration through 70 %, 90 % and 100 % ethanol. Samples were placed in a 1:1 mixture (v/v) of Procure/Araldite embedding resin and 100 % ethanol overnight and then into pure resin the following day. Samples were left in a second change of resin overnight and

embedded on the third day, then were polymerised at 70 ^{o}C for 24 h. Thin sections (70 nm) were cut using a diamond knife on a Reichert Ultracut E ultramicrotome. A minimum of one stipule (or leaf) from each of three plants, at 0, 7, 14 and 21 days of cold treatment, was sectioned for each genotype. Sections were stained with 4 % aqueous uranyl acetate and Reynold's lead citrate for 15 min each. Sections were viewed in a Philips CM100 TEM at 80 kV.

Starch Analysis

Two stipule or leaflet samples from each genotype were freeze dried and ground finely. Starch concentration was determined following the Megazyme Total Starch Assay Kit (AA/AMG 11/01) procedure. Dried samples (15 mg) were washed and centrifuged with 70 % cold ethanol at 1000 rpm for 10 min. The supernatant was discarded and washed repeatedly with 70 % ethanol. The pellet was finally centrifuged with 100 % cold ethanol at 1000 rpm for 5 min followed by the procedure described in the Megazyme kit.

Starch samples were treated with thermostable α-amylase in a boiling bath for 6 min. After the addition of acetate buffer, amyloglucosidase was added and incubated for 30 min at 50 ^{o}C. Each sample was diluted to 10 ml and centrifuged. Glucose content was analysed using the glucose determination reagent, GOPOD (Megazyme kit) containing glucose oxidase, peroxidase and 4-aminoantipyrine. For quantification, glucose standard solution was analysed and absorption of coloured solution was read at 510 nm. Total starch content was calculated according to the formula provided in the kit procedure.

$$Starch(\%) = \Delta E \times \frac{F}{W} \times 90$$

Where ΔE is the absorbance read against the reagent blank, F is a conversion factor from absorbance to µg, W is the weight of leaf sample and multiplication by 90 is for the following adjustments: the dilution factor, conversion of free glucose to anhydro-glucose and final conversion of starch contents into percentage. The significance of differences in starch concentration between cold-treated plants and non-treated control plants was tested at a significance level of 0.001 using t-tests with GenStat 13 Statistical software (VSN International, UK).

6.3. Results

Cell ultrastructure

Parenchyma cells from Kaspa and ATC 1564 control plants (not exposed to cold temperatures) had large centrally located vacuoles and very thin layers of cytoplasm (Fig. 6.1A and B). Chloroplasts had starch grains, small plastoglobuli (Fig. 6.1C and D) and compact thylakoid membranes (Fig. 6.1E and F). The plasma membranes were smoothly aligned with cell walls (Fig. 6.2C and D).

After 7 days of cold treatment, there were noticeable modifications in parenchyma cell ultrastructure in both genotypes, relative to their respective controls (Figs. 6.3 and 6.4). In Kaspa, large centrally located vacuoles were observed with few chloroplasts that often appeared to have only one or two starch grains (Fig. 6.3A). However, in ATC 1564, cells contained a large (shrunken) vacuoles, small (irregular shaped) vacuoles, and clusters of chloroplasts (Fig. 6.3B). Further, starch appeared to have accumulated in chloroplasts, in contrast to non-cold-treated control plants (Fig. 6.3B). In Kaspa, chloroplasts had dilated thylakoid membranes (Fig. 6.3C) whereas in ATC 1564, thylakoid membranes were still compact but less visible than in non-treated control plants and Kaspa (Fig. 6.3D). Kaspa had more small vesicles present in the cytoplasm and more than in control plants (Fig. 6.4A). InATC 1564, small vesicles were observed in the vicinity of plasma membranes (Figs. 6.3F and 6.4B). In Kaspa, engulfment of vesicle into vacuole, through the process of endocytosis, was observed (Fig. 6.4C).

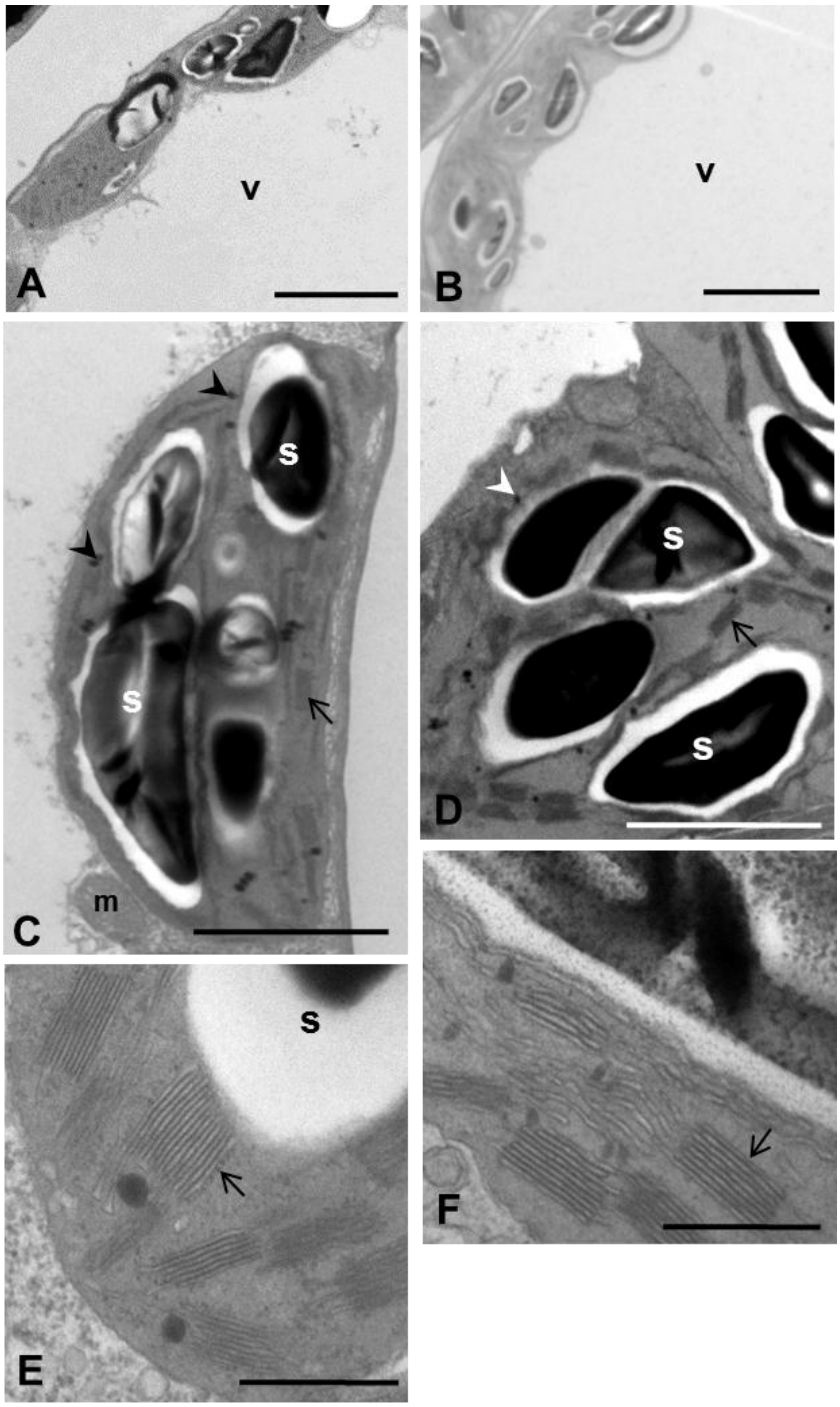


Fig 6.1. Electron micrographs of leaf parenchyma cells of reproductive stage control (non-cold-treated) plants of the *Pisum sativum* L. genotype Kaspa (stipules; left panel) and ATC 1564 (leaflets; right panel). General view of a cell containing a large regular central vacuole in **A** and **B**; Close up of a chloroplast with starch grains, grana (e.g. arrow) and small plastoglobuli (arrowheads) in **C** and **D**; Close up of grana showing compact thylakoids (arrow) in **E** and **F**. Abbreviations: m, mitochondrion; s, starch grain; v, vacuole. Bars: A and B, 5 µm; C and D, 2 µm; E and F, 0.5 µm.

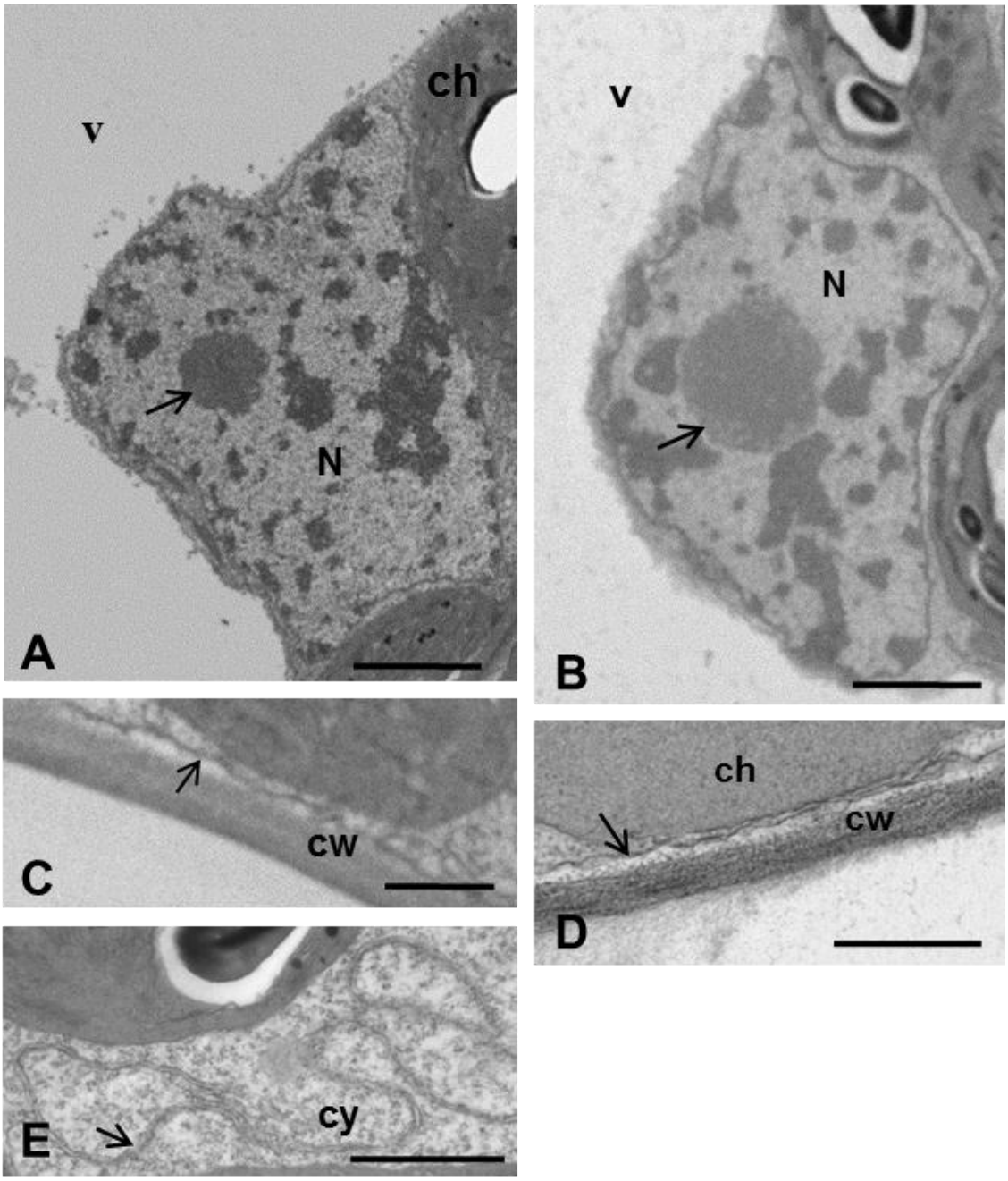


Fig 6.2. Electron micrographs of leaf parenchyma cells of reproductive stage control (non-cold-treated) plants of the *Pisum sativum* L. genotype Kaspa (stipules; left panel) and ATC 1564 (leaflets; right panel). A typical nucleus with nucleolus (arrow) in **A** and **B**; Plasma membrane (arrow) aligned with cell wall in **C** and **D**; Long endoplasmic reticulum cistern (arrow) in cytoplasm in **E**. Note small ribosomes present in the cytoplasm and attached to the endoplasmic reticulum. Abbreviations: ch, chloroplast; cw, cell wall; cy, cytoplasm; n, nucleus; v, vacuole. Bars: A and B, 2 µm; C and D, 1 µm; E, 1 µm.

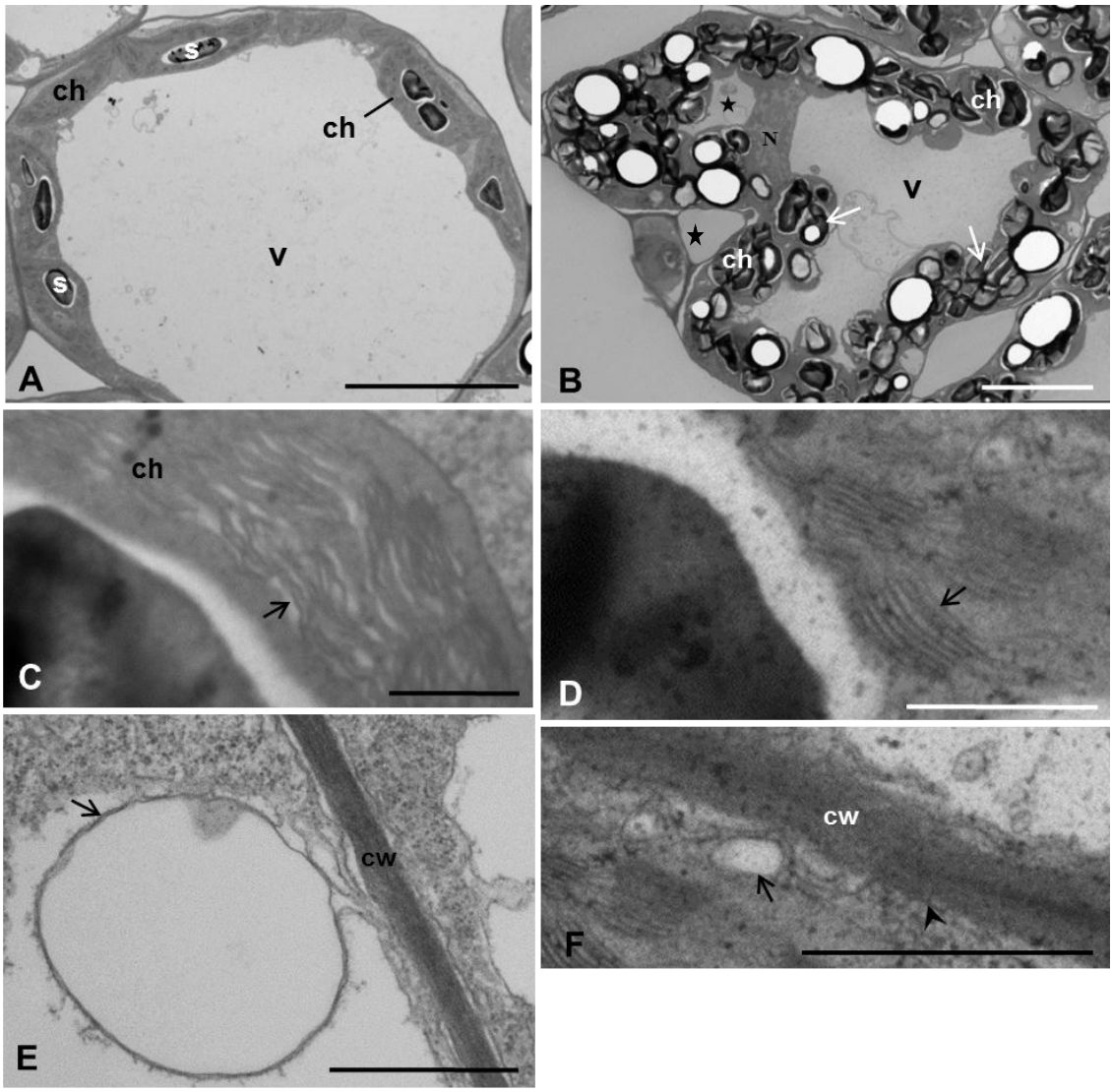


Fig 6.3. Electron micrographs of leaf parenchyma cells of reproductive stage plants of *Pisum sativum* L. genotype Kaspa (stipules; left panel) and ATC 1564 (leaflets; right panel), after 7 days of cold treatment. General view of a cell, showing chloroplasts with a few small starch grains and a large central vacuole in **A**; numerous chloroplasts with large starch grains (arrows), large shrunken vacuole, and small irregular vacuoles (stars) in **B**; Close up of thylakoids (arrow) in chloroplasts. Note the dilation in the lacunae between membranes in **C**; Compact thylakoid membranes in **D**; Close to the cell wall, a vesicle (arrow) attached to the plasma membrane in **E**; A small vesicle (arrow) in the vicinity of the plasma membrane (arrowhead) in **F**. Abbreviations: ch, chloroplast; cw, cell wall; n, nucleus; s, starch grain; v, vacuole. Bars: A and B, 10 µm; C and D, 0.5 µm; E and F, 1 µm.

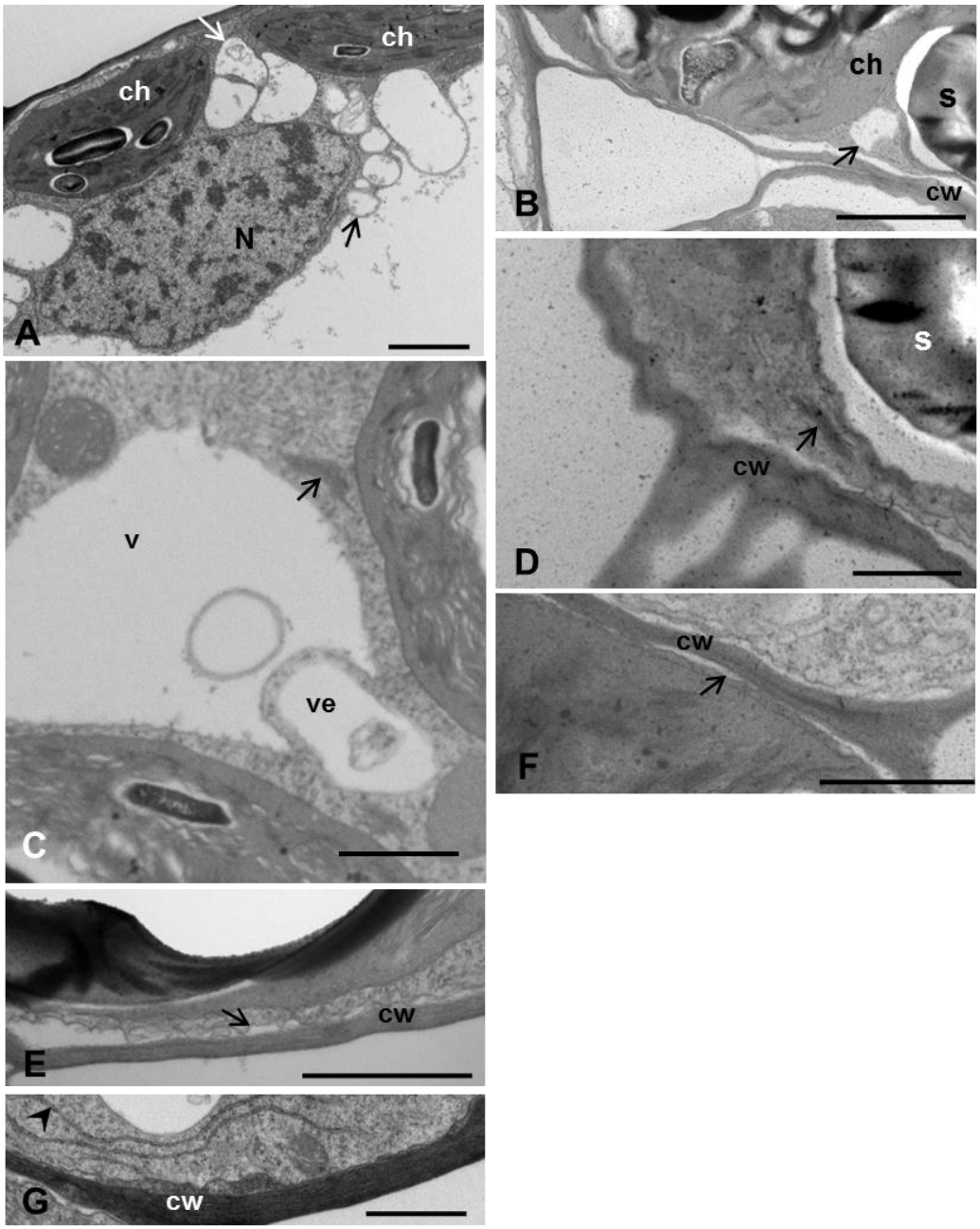


Fig 6.4. Electron micrographs of leaf parenchyma cells of reproductive stage plants of *Pisum sativum* L. genotype Kaspa (stipules; left panel) and ATC 1564 (leaflets; right panel), after 7 days of cold treatment. Numerous vesicles (arrows) near a nucleus and between chloroplasts (ch) in **A**; and a less frequently observed vesicle (arrow) between a chloroplast and cell wall in **B**; Dictyosome (arrow) in the cytoplasm in **C** and **D**. Note the vesicle is being engulfed by the vacuole from cytoplasm (endocytosis process) in **C**; The plasma membrane (arrow) in **E** and **F**; Endoplasmic reticulum cisterna (arrowhead) in the cytoplasm in the vicinity of the plasma membrane in **G**. Abbreviations: ch, chloroplast; cw, cell wall; n, nucleus; s, starch grain; v, vacuole; ve, vesicle. Bars: A and B, 2 µm; C and D, 1 µm; E and F, 1 µm; G, 2 µm.

In tissues sampled after 14 days of cold treatment, more pronounced modifications were observed in the cellular ultrastructure of both genotypes (Figs. 6.5 – 6.7). In Kaspa, large centrally located vacuoles were present as before (Fig. 6.5A). However in ATC 1564, cells contained shrunken vacuoles and numerous chloroplasts with starch grains similar to those observed after 7 days of cold treatment (Fig. 6.5B). In Kaspa, more vesicles were observed in the cytoplasm than ATC 1564 (Fig 6.5C and D). The protrusion of vesicles into the vacuole was noted in ATC 1564 (endocytosis) (Fig 6.5D). In contrast to this process, fusion of a vesicle into the plasma membrane (exocytosis) was observed in Kaspa (Fig 6.5E). Phenolic deposits were observed on vesicle membranes, mitochondrial outer membranes and plasma membranes (Figs. 6.5F and 6.6B). These phenolic deposits were noted on mitochondrial outer membranes and on tonoplast membranes more frequently in Kaspa (Fig 6.6A) than in ATC 1564. In Kaspa, prominent changes in the structure of chloroplasts were observed, with low electron density portions in the swollen stroma (Fig. 6.6C), and the formation within the stroma of "vesicles" with a similar electron density to the cytoplasm (Figs. 6.6C and 6.7A). The thylakoid membrane system was sometimes displaced to one half of the chloroplast (Fig. 6.7C). In ATC 1564, no such structural changes in chloroplasts were observed and thylakoid membranes were compact (Fig. 6.6D), however cells appeared to be shrunken and cell walls often appeared irregular (Fig. 6.7B).

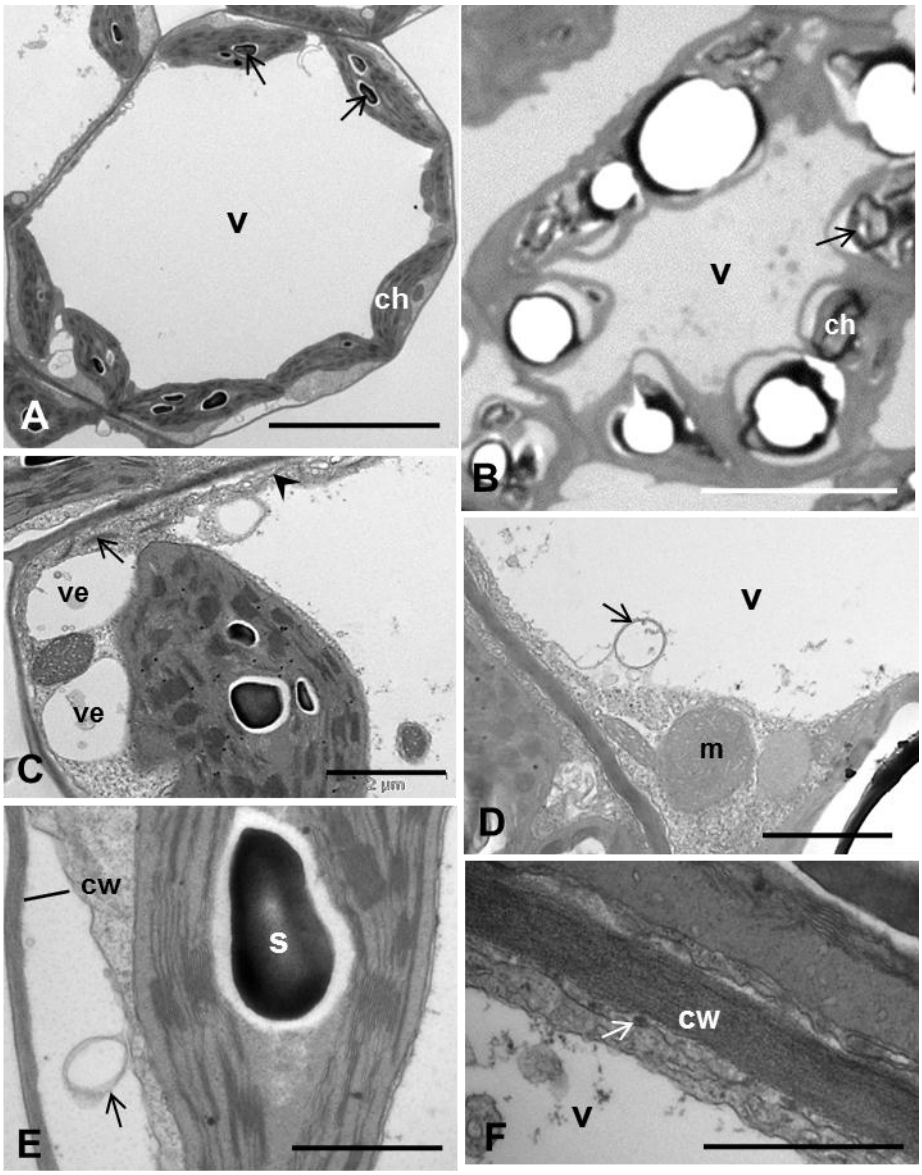


Fig. 6.5 Electron micrographs of leaf parenchyma cells of reproductive stage plants of *Pisum sativum* L. genotype Kaspa (stipules; left panel) and ATC 1564 (leaflets; right panel), after 14 days of cold treatment. General view of a cell containing a large vacuole and chloroplasts with very few starch grains (arrows) in **A**; and with large starch grains (e.g. arrow) in **B**; In the cytoplasm, dictyosomes (e.g. arrow), endoplasmic reticulum (arrowhead) and vesicles in **C**; and protrusion of a vesicle (arrow) into a vacuole (endocytosis) in **D**; A fusion (arrow) of a membrane-bound vesicle into the plasma membrane (exocytosis) in **E**; and dictyosome (arrow) in the cytoplasm and membraglobulus (small phenolic deposits) (arrow) on the plasma membrane in **F**. Abbreviations: ch, chloroplast; cw, cell wall; m, mitochondrion; s, starch grain; v, vacuole, ve, vesicle. Bars: A and B, 10 µm; C and D, 2 µm; E and F, 1 µm.

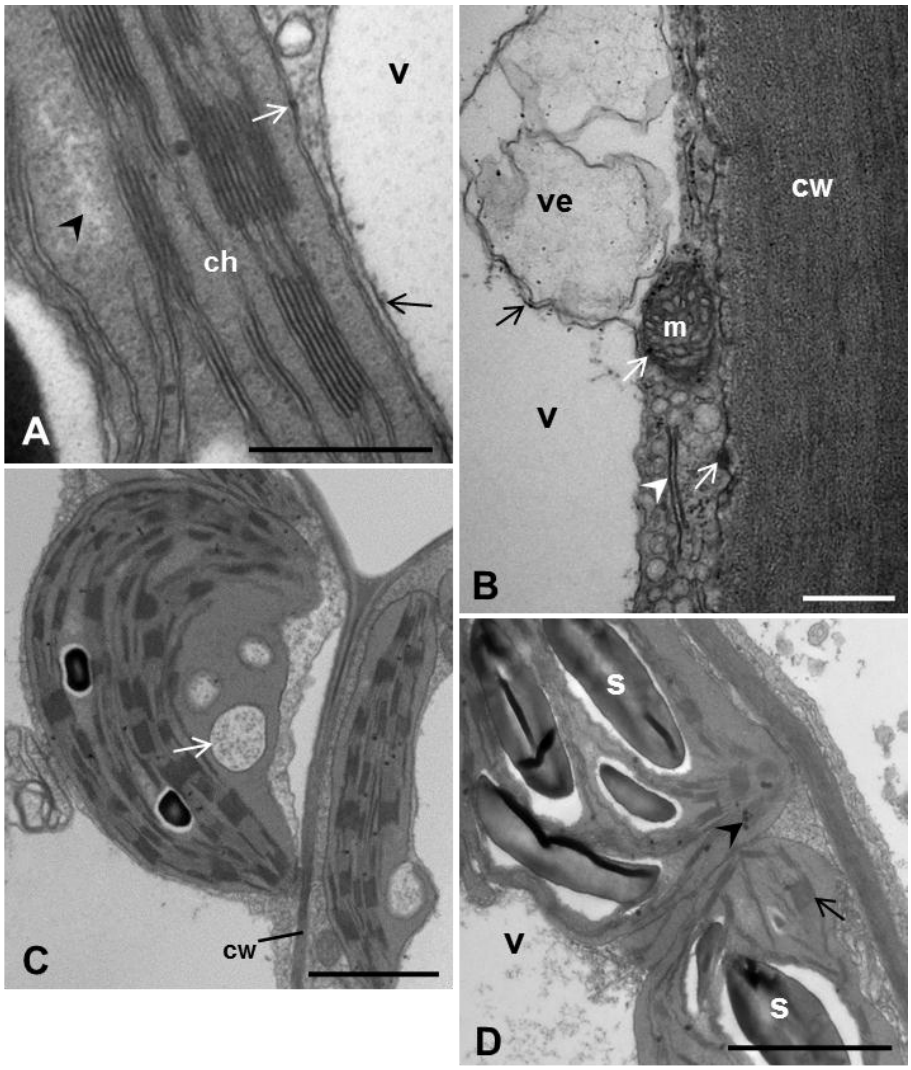


Fig. 6.6 Electron micrographs of leaf parenchyma cells of reproductive stage plants of *Pisum sativum* L. genotype Kaspa (stipules; left panel) and ATC 1564 (leaflets; right panel), after 14 days of cold treatment. Phenolic deposits (arrows) on the outer layer of the chloroplast and note the less electron dense part in the stroma (arrowhead) in **A**; Protrusion of a vesicle into the vacuole, and dictyosome (arrowhead) close to the cell wall and note the phenolic deposits (arrows) on the vesicle, and mitochondrial and plasma membranes in **B**; A "vesicle" (arrow) in the chloroplast, and note the structural changes in the chloroplast, including sickle shape and swelling of the stroma to one side in **C**; and chloroplasts containing grana with compact thylakoid membranes (e.g. arrow), starch grains, and small plastoglobuli (arrowhead) in **D**. Abbreviations: ch, chloroplast; cw, cell wall; m, mitochondrion; s, starch grain; v, vacuole, ve, vesicle. Bars: A and B, 0.5 µm; C and D, 2 µm.

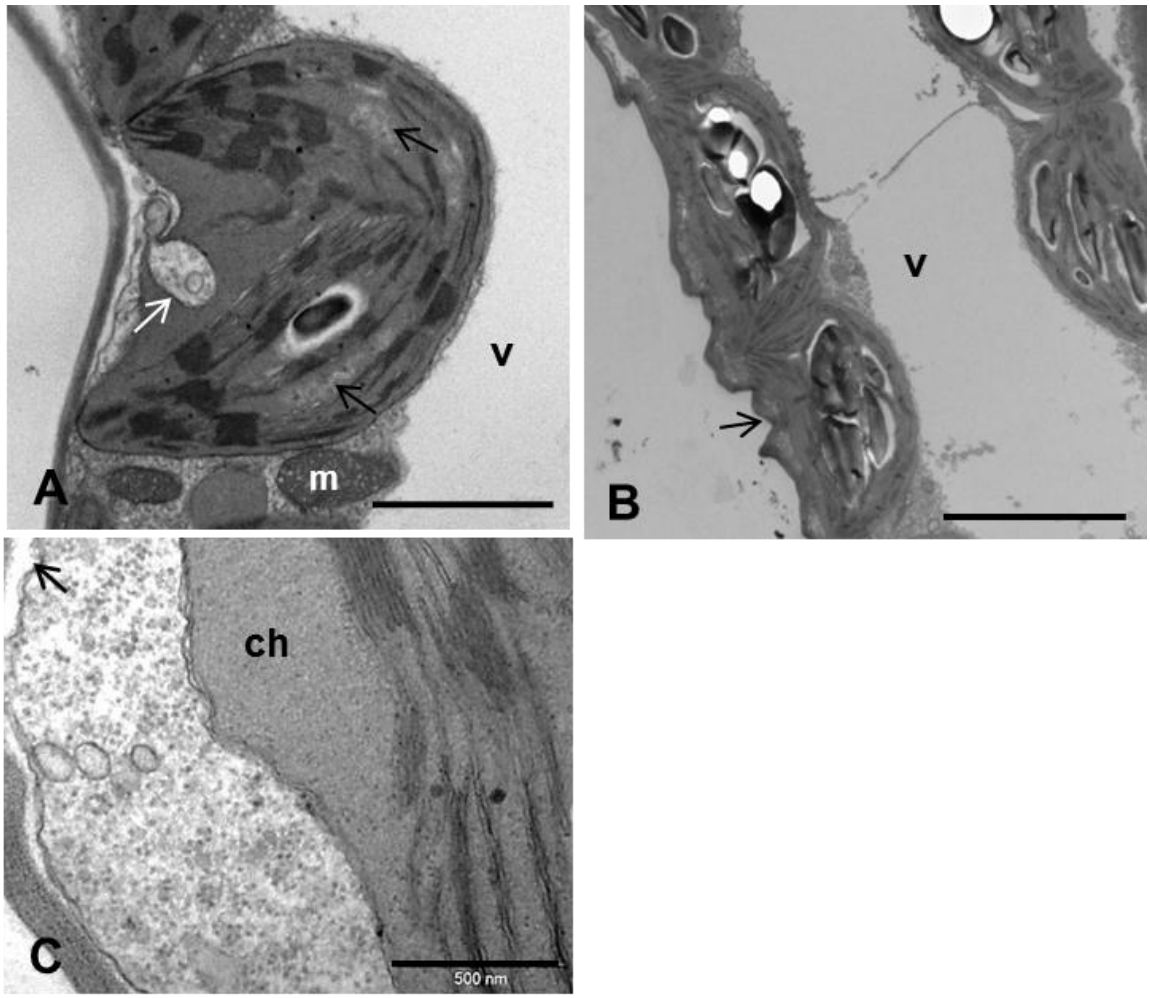


Fig. 6.7 Electron micrographs of leaf parenchyma cells of reproductive stage plants of *Pisum sativum* L. genotype Kaspa (stipules; left panel) and ATC 1564 (leaflets; right panel), after 14 days of cold treatment. Chloroplast containing a "vesicle" (white arrow), and parts of the stroma with low electron density (black arrows) in **A**; Contraction in the cell wall (arrow) in **B**; Note in the chloroplast, thylakoids are pushed to one side due to swelling of the stroma, and the plasma membrane (arrow) along the cell wall in **C**;. Abbreviations: ch, chloroplast; m, mitochondrion; v, vacuole. Bars: A, 2 µm; B, 5 µm; C, 0.5 µm.

In tissues sampled after 21 days of cold treatment, further modifications were observed in the cellular ultrastructure of both genotypes (Figs. 6.8 and 6.9). In Kaspa, large centrally located vacuoles were observed and there appeared to be more starch grains in chloroplasts than after 14 days of cold treatment (Fig. 6.8A). In ATC 1564, cells had shrunken vacuoles, and chloroplasts with smaller starch grains than after 14 days of cold treatment were observed (Fig. 6.8B). Phenolic deposits were observed more frequently in the cytoplasm of Kaspa (Figs. 6.8C and 6.9B) than ATC 1564 (Fig. 6.8D). In both genotypes, vesicles were noted in the vicinity of the plasma membrane (Figs. 6.8E and F). Invagination of the plasma membrane was observed in Kaspa (Fig 6.8E). Vesicles were observed in vacuoles (endocytosis) of both genotypes (Figs 6.9A and B). In Kaspa, dilated thylakoid membranes were noted (Fig. 6.9C), but other modifications in the structure of chloroplasts (vesicle formation, lower electron density and swelling of stroma) that had been seen after 14 days of cold treatment were less pronounced. In ATC 1564, no modification in thylakoid membranes was noted. In Kaspa, small phenol deposits were observed on the tonoplast layer (Fig. 6.9E).

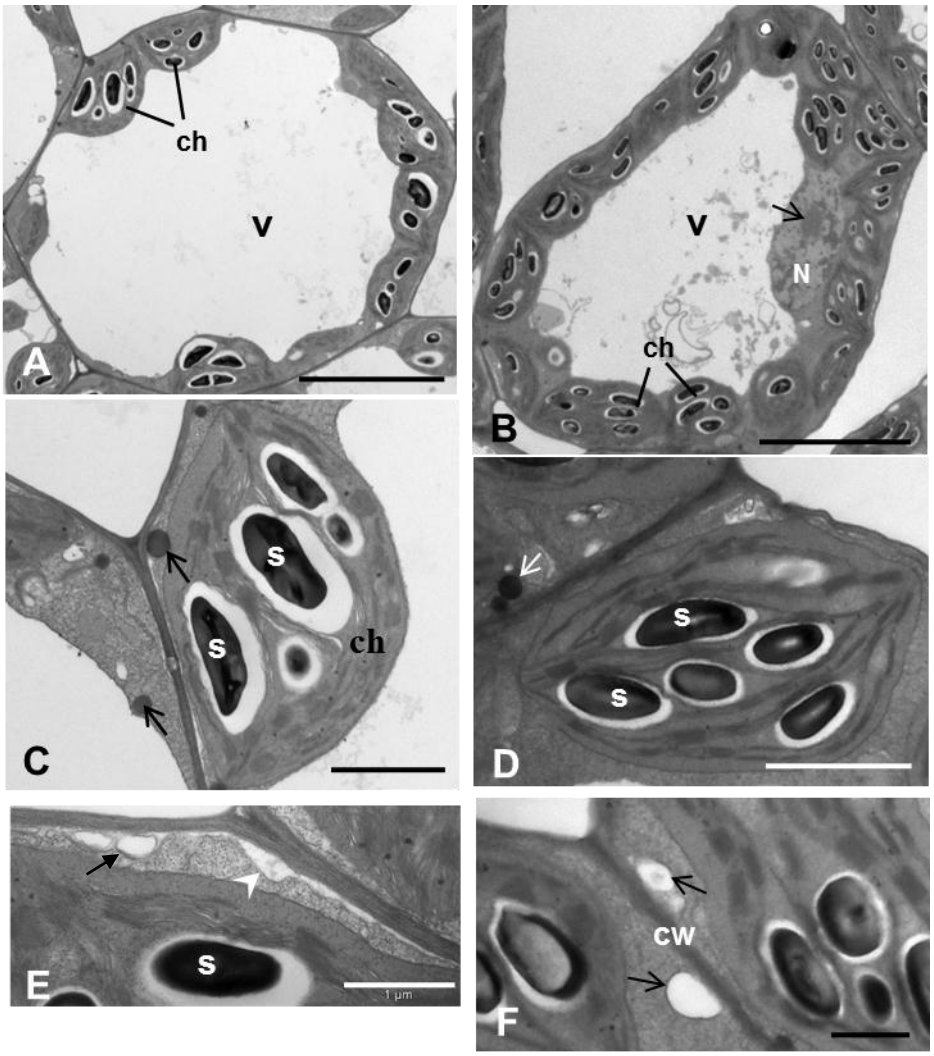


Fig. 6.8 Electron micrographs of leaf parenchyma cells of reproductive stage plants of *Pisum sativum* L. genotype Kaspa (stipules; left panel) and ATC 1564 (leaflets; right panel), after 21 days of cold treatment. General view of a cell with a large vacuole and chloroplasts with starch grains in **A**; and numerous chloroplasts, and a nucleus with nucleolus (arrow) in **B**; Chloroplast, and phenolic aggregations (arrows) in the cytoplasm in **C**, and **D**; Vesicles (arrows) present in the vicinity of the plasma membrane in **E** and **F**, note the invagination of the plasma membrane (arrowhead) in **E**. Abbreviations: ch, chloroplast; cw, cell wall; n, nucleus; s, starch grain; v, vacuole. Bars: A and B, 10 µm; C and D, 2 µm; E and F, 1 µm.

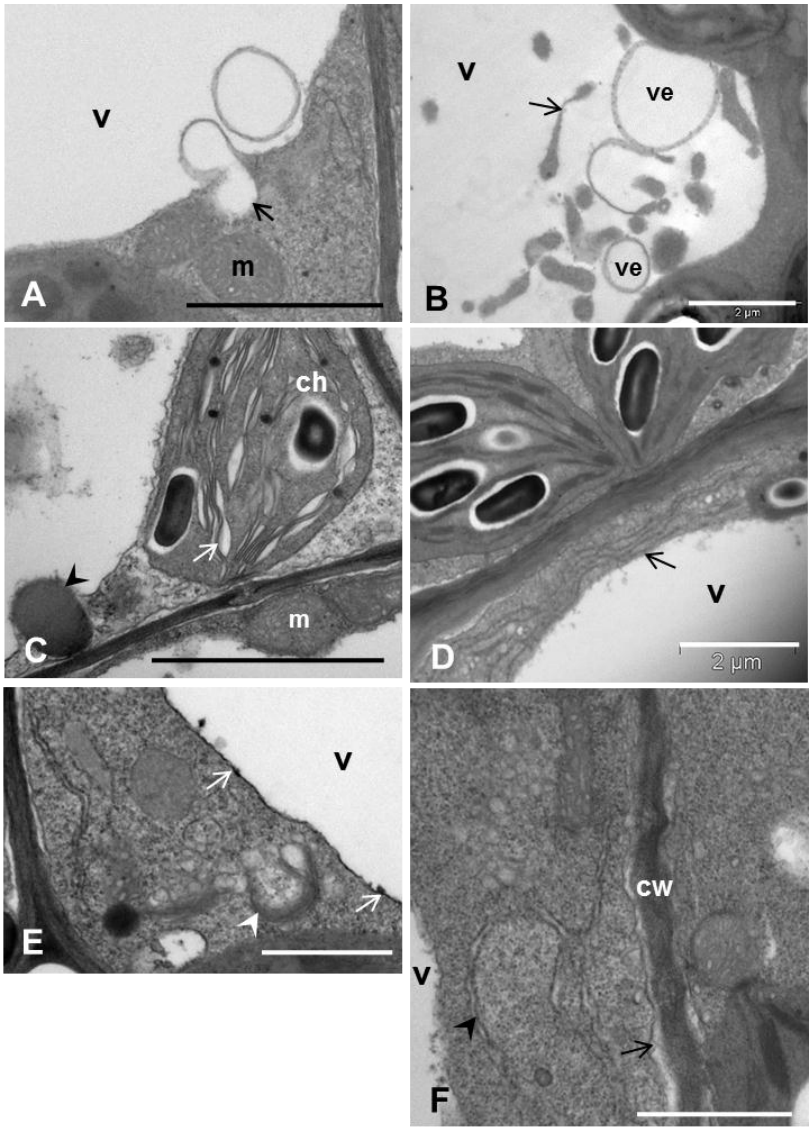


Fig. 6.9 Electron micrographs of leaf parenchyma cells of reproductive stage plants of *Pisum sativum* L. genotype Kaspa (stipules; left panel) and ATC 1564 (leaflets; right panel), after 21 days of cold treatment. Protrusion of a vesicle (arrow) into a vacuole (endocytosis) in **A**; Presence of vesicles and paramural bodies (e.g. arrow) in a vacuole (endocytosis) in **B**; Chloroplast with dilated thylakoid membranes (e.g. arrow), and phenolic aggregation (arrowhead) in the cytoplasm in **C**; Long endoplasmic reticulum cisternae (e.g. arrow) in the cytoplasm in **D**; Dictyosome (arrowhead) in the cytoplasm, and small phenolic deposits (arrows) on the tonoplast in **E**; Long endoplasmic reticulum cisternae (e.g. arrowhead) in the cytoplasm and in the vicinity of the plasma membrane (arrow) in **F**. Abbreviations: ch, chloroplast; cw, cell wall; m, mitochondrion; v, vacuole; ve, vesicle. Bars: A and B, 2 µm; C and D, 2 µm; E and F, 1 µm.

Starch concentration

Contrasting responses in the concentration of starch were observed after cold treatment in the two genotypes (Fig. 6.10). In Kaspa, stipules sampled after 7, 14 and 21 days of cold treatment had significantly lower starch concentration than those from non-treated control plants. In ATC 1564, individual comparisons at 7, 14 and 21 days were not statistically significant, but the overall mean starch concentration across all cold-treated samples was significantly higher than the mean for control plants.

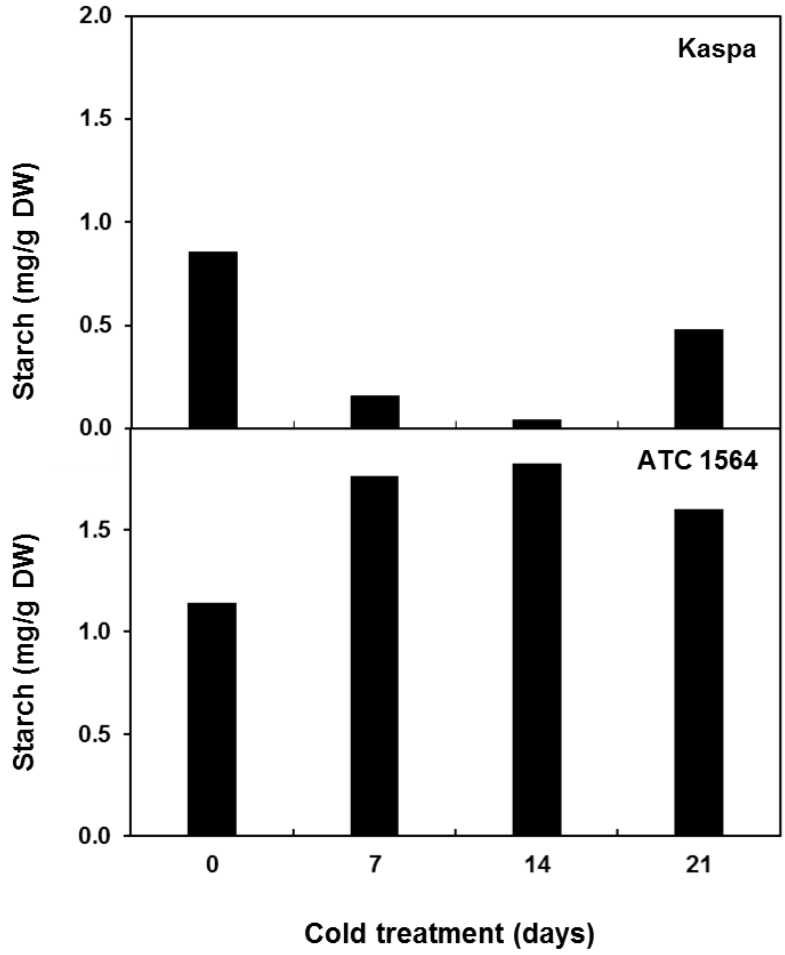


Fig. 6.10. The concentration of starch (mg g^{-1} DW) in stipules and leaflets of reproductive stage *Pisum sativum* L. plants of Kaspa (stipules) and ATC 1564 (leaflets), before cold treatment (0 day) and after 7, 14 and 21 days of cold treatment (10/5 ^{o}C).

6.4. Discussion

Similarities between Kaspa and ATC 1564 after cold treatment

Although Kaspa and ATC 1564 differed from each other in their physiological responses to cold and their frost sensitivity with or without prior cold treatment (Chapter 5 of this thesis), there were some similarities between them with respect to ultrastructural changes observed after cold treatment.

Modifications in the ultrastructure of parenchyma cells were found in both *P. sativum* genotypes, Kaspa and ATC 1564, when exposed to cold treatment at the reproductive stage for 7 days. Many small vesicles observed in both genotypes represented a cold-induced change. Formation of vesicles has also been observed in apple during cold treatment, and is considered to be involved in the mechanism of cold acclimation and associated with cold tolerance (Kuroda and Sagisaka 2001). Under natural conditions, small vesicles form in plant tissues as cold periods commence, and their presence in the vicinity of the plasma membrane may reflect their origin via invagination of the plasma membrane (Steer 1988).

The protrusion of vesicles into vacuoles or endocytosis, was observed in both genotypes after cold treatments (7, 14 or 21 days). The membrane-bound vesicles may have been derived from cytoplasmic and/or plasma membranes as described by Marchant and Robards (1968). Their presence during cold treatment may indicate the occurrence and/or continuation of important cellular functions, for example, uptake of extracellular nutrients and maintenance of cell polarity in leaf tissues (Mukherjee et al. 1997). In plants, there is limited information on this process (Robinson and Milliner 1990), however in mammals, it has been reported that endocytic pathways are also utilized by viruses, toxins, and symbiotic microorganisms to gain entry into cells (Mukherjee et al. 1997). In endocytosis, the origin of vesicles from the plasma membrane results in the invagination of the plasma membrane, which was observed in Kaspa plants after cold treatment. Invagination of the plasma membrane has been observed in *Arabidopsis thaliana* plants and found to be associated with the cold acclimation process and frost tolerance (Ristic and Ashworth 1993).

After 14 days of cold treatment, dark stained small globules were observed on the outer layer of chloroplasts in Kaspa, and on the vesicular membrane, mitochondrial layer and plasma membrane in ATC 1564. It is reported that during cold treatment, such small

globules represent structural changes in membranes of cellular organelles (Niki and Sakai 1981; Steer 1988). These small globules were similar to those observed in *Arabidopsis thaliana* during cold acclimation and described as 'membraglobuli' (Ristic and Ashworth 1993). Further, their appearance was associated with the maximum saturation of cold acclimation (cold-induced changes) after which extension of the cold treatment had no further effect on frost tolerance (Ristic and Ashworth 1993). If the same is true in field pea, this indicates that maximum saturation (of cold acclimation) may be achieved in pea after 14 days of cold treatment, and elevation in frost tolerance may be expected in (14 days-) cold treated plants (This was investigated in experiments that are reported in Chapter 7 of this thesis).

The membraglobuli found here are similar to the phenolic deposits observed in the leaf tissues of acclimated oilseed rape (*Brassica napus*, L.) (Stefanowska et al. 2002). In pea plants, these deposits may represent the accumulation of anthocyanins, which have previously been reported to accumulate in stress affected leaves under low temperature conditions (Dixon and Paiva 1995). Accumulation of anthocyanins may also be visualised by the change in leaf colour (purple-red) in field pea which is commonly seen in fields in winter months in southern Australia (personal observations).

Differences between Kaspa and ATC 1564 after cold treatment

Kaspa and ATC1564 also showed some differential responses to cold treatment. It is possible that some of these differences may be due to anatomical differences between stipules (in Kaspa) and leaflets (in ATC 1564). However, under control conditions (before cold treatment) no differences were observed in cellular structures between stipules and leaflets. According to Lecoeur (2010), the stipules of pea plants are functionally essential parts of the leaf, contributing the largest proportion of transpiring and photosynthetic area in *afila* type genotypes.

An increase in cytoplasmic volume and decrease in vacuolar volume observed after 7 days of cold treatment in ATC 1564 may be an important cold-induced change. A previous report showed that increase in cytoplasmic volume provided a mechanism for increasing the activity of enzymes and metabolites involved in the Calvin cycle and sucrose-biosynthesis pathway (Strand et al. 1999). The unaltered cytoplasmic volume during cold treatment may partially explain the greater sensitivity to frost at the reproductive stage of Kaspa than ATC 1564.

Modification in the structure of chloroplasts in Kaspa could be symptoms of chilling injury, which may have contributed towards the greater vulnerability to frost. The changes in the chloroplast of Kaspa (swollen stroma with large "vesicles" containing granular material, thylakoid system pushed to one side, thylakoid membranes dilated and patches of low electron density in chloroplasts) are similar to symptoms that have been reported in pea seedlings under stress conditions (cold: Ma et al. 1990) and (UV: He et al. 1994) and in chilling-sensitive *Cucumis sativus* L. (Xu et al. 2008) and other plants (Kratsch and Wise 2000). Dilation of thylakoids has been reported to be related to photo-oxidative conditions that are produced under low light and temperature conditions (Kratsch and Wise 2000), but the lack of cell plasmolysis in Kaspa indicates that the stress symptoms in Kaspa were moderate and not severe. No such modifications in the structure of chloroplasts were observed in ATC 1564.

The disappearance of starch grains in chloroplasts in Kaspa was probably also a symptom of chilling injury. In contrast, the apparent accumulation of starch grains in chloroplasts and the significant increase in leaf starch concentration in ATC 1564 may be associated with its better tolerance to frost relative to Kaspa. Disappearance of starch grains from chloroplasts has previously been reported as a symptom of chilling injury in plants (Kratsch and Wise 2000), and the accumulation of starch in cells has been found to be associated with cold acclimation and frost tolerance (Ristic and Ashworth 1993). This trend is similar to what has been observed in chilling resistant (Ma et al. 1990) and frost resistant (Bourion et al. 2003) pea seedlings under cold conditions.

Cold-induced cellular changes and frost tolerance in field pea

After 21 days of cold treatment, the cold-treated plants of Kaspa and ATC 1564 were exposed to frost (results are presented in Chapter 5 of this thesis). The cold-treated plants of both genotypes were more susceptible to frost than non-cold-treated plants, however frost-sensitive Kaspa was found to be more responsive to cold treatment (prior to frost) than ATC 1564 (Chapter 5 of this thesis).

The cold-induced modifications in cellular ultrastructures observed here, for example, appearance of small vesicles, invagination of the plasma membrane and accumulation of phenolic deposits, were not associated with cold acclimation and reproductive-stage frost tolerance. These results are in contrast to previous studies where such changes were reported to be associated with cold acclimation and frost tolerance (Ristic and

Ashworth 1993; Kuroda and Sagisaka 2001; Stefanowska et al. 2002). This indicates reproductive-stage pea plants are inherently sensitive to cold temperatures, and that cold temperatures induce physiological and cellular changes which may in turn weaken plant defence against frost. However, from the structural modifications in chloroplasts in Kaspa and starch accumulation in chloroplasts in ATC 1564, it is evident that there is variation between frost tolerant and frost-sensitive genotypes for sensitivity to cold treatment at the reproductive stage.

Chapter 7

Effect of low temperatures at the reproductive stage on photosynthetic characteristics and partitioning of photo-assimilates in two field pea genotypes

7.1. Introduction

In field pea, flowering depends on temperature and photoperiod. Under optimal conditions, plants tend to progress towards the reproductive stage without delay (Lejeune-Henaut et al. 1999). During the reproductive phase, vegetative growth and the development of reproductive organs such as buds, flowers and pods continue simultaneously. During the reproductive phase of plant growth, environmental conditions affect the regulation of active photosynthesis and distribution of photo-assimilates from vegetative tissues to reproductive organs or fruits through transport systems (Marcelis 1996). Under stress conditions, plants may adjust photosynthesis and maintain the distribution of assimilates between source and sink organs.

During cold treatment of field pea seedlings, decreases in the rate of photosynthesis have been observed (Yordanov et al. 1996), however some genotypes have shown an ability to maintain the rate of photosynthesis under low temperatures (Georgieva and Lichtenthaler 2006). Moreover, the accumulation of carbohydrates (soluble sugars) during cold treatment apparently improved the survival of field pea plants exposed to sub-zero temperatures at the vegetative stage (Bourion et al. 2003).

The physiological effects of cold treatment at the reproductive stage have not been studied. The response to cold treatment at the reproductive stage in plants might be more complicated than at the vegetative stage due to the presence of both vegetative and reproductive organs and the balance between sources and sinks for photo-assimilates. In Chapter 5 of this thesis, changes in photosynthesis and concentration of soluble sugars were observed in leaf tissues during the cold treatment. In the research reported in this chapter, photosynthetic characteristics were studied in detail, and partitioning of photo-assimilates between vegetative and reproductive organs, were monitored in reproductive-phase plants of two field pea genotypes during 7- and 14-day cold

treatments. The impact of these cold treatments on the tolerance of reproductive organs (buds, flowers and pods) to subsequent frost exposure was investigated.

7.2. Materials and Methods

Plant material

Two field pea genotypes, ATC 968 and Kaspa, were studied in the present experiment. ATC 968 is a conventional-leaf, white-flowered type from Italy. Kaspa is an *afila*-type cultivar with pink flowers. It yields well in southern Australia, but is sensitive to frost at the reproductive stage. ATC 968 was chosen for comparison with Kaspa because in an earlier experiment (Shafiq et al. 2012) it commenced flowering at about the same time as Kaspa (68 days from sowing, Appendix 1) and exhibited better frost survival of buds, flowers and set pods than Kaspa.

Growth conditions and experimental design

Both genotypes were sown on the same day under natural growth and photoperiod conditions (about 12 hr) in a glasshouse. The temperature and relative humidity inside the glasshouse were recorded every 30 min during a 24 hr period and monitored throughout the experiment, using Tinytag data loggers (TGP-4500, Gemini Data Loggers, UK Ltd.) (Fig 7.1). Each seed was germinated in a 200 mm pot containing coco peat. Twenty-four pots of each genotype were arranged in the glasshouse in a completely randomised design (CRD). Ten days after sowing, another batch of seeds was sown in the same glasshouse under the same growth conditions in order to provide another 12 pots of each genotype. These pots were arranged in a second CRD. Three weeks after sowing, the soil around each emerging plant was inoculated with commercially available pea *Rhizobium* culture (*Nodulaid 100*, Bio-care Tech Pty Ltd. NSW, Australia) and Osmocote (17.2 g per pot). Wooden stakes were placed in pots one month after sowing, to support plants. Three weeks after the appearance of the first flower on all plants in the first batch, when each plant in that batch had buds, flowers, set pods and developing pods, 12 plants of each genotype were transferred to a cold chamber. After seven days these plants were transferred to a frost chamber.

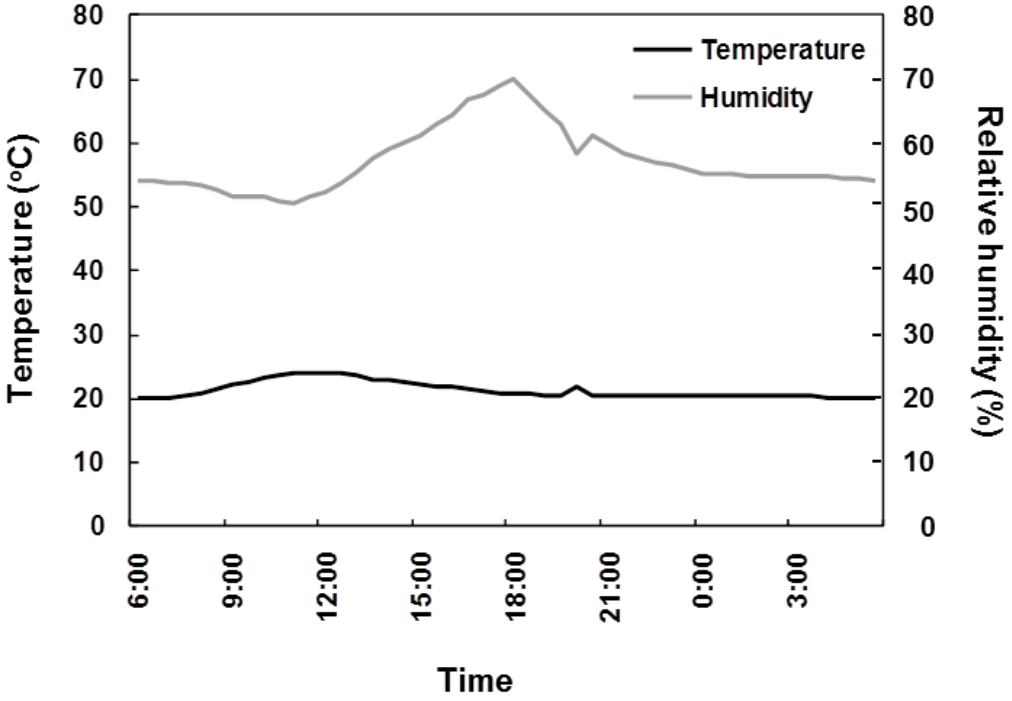


Fig. 7.1 Average temperature and relative humidity recorded in the glasshouse for 24 hr during the growth of field pea plants from March to June 2011

Cold and frost treatments

Plants placed in the cold chamber were arranged in a CRD and were kept for seven days at 10/5 ºC (day/night) temperature regime and 12 hr photoperiod at 250 µmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD) generated by high-pressure sodium lighting system (Vialox® 400 Watt NAV-T, OSRAM). The plants of Kaspa, which were shorter than those of ATC 968, were placed on polystyrene boxes so that the tops of all plants were at about an equal distance from the lights.

At the end of the cold treatment, eight plants of each genotype were transferred to the frost chamber. At the same time, eight plants of each genotype that had been retained in the glasshouse and were at the same growth stage as the cold-treated plants were also transferred to the frost chamber. Before exposure to frost, each bud, flower and pod on each of these plants was tagged to indicate its stage of development using the eight-category scale described by Knott (1987); immature bud: 201, mature bud: 202, open flower: 203, set pod: 204, flat pod: 205, swollen pod: 206, filled pod: 207 and mature pod: 208. All 32 plants were arranged in random positions within the frost chamber.

They were subjected to the same frost conditions as described in Chapter 3 of this thesis and by Shafiq et al. (2012). To simulate a frost event in the chamber, the temperature was controlled over a 24 hr period and dropped by 5 ^{o}C per hr from 10 ^{o}C to 3.5 ^{o}C and by 1^{o}C per hr from 3.5 ^{o}C to – 4.8 ^{o}C. The temperature was held at – 4.8 ^{o}C for 4 hr and then increased by 2 ^{o}C per hr to 3.5 ^{o}C and by 5 ^{o}C per hr to 20 ^{o}C. After the frost treatment, all plants were transferred to the growth room and kept under controlled conditions at 18/12 ^{o}C and 12 hr photoperiod for recovery in a CRD. After two days, all frost-treated plants were returned to the glasshouse under the same conditions as used before the cold treatment and were grown until the pods of the frost-treated plants were dry.

Symptom data were collected 72 hr after the frost exposure for all stages exposed to frost using the scoring key described in Table 3.3 in Chapter 3 of this thesis.. Further, symptoms on leaves that had been exposed to frost were scored using a five-category scale. A score of 1 was used for no frost symptoms, 2 for slightly pale leaf colour, 3 for dull leaves with slightly pale colour, 4 for crumbly curled leaves bleached from the base of the lamina and 5 for brittle curled leaves with or without completely bleached lamina. All seeds from pods that had been exposed to frost or that had developed from buds or flowers that were exposed to frost were harvested when dry, counted and weighed. Seeds from control plants were harvested at maturity, counted and weighed. The weight of 100 seeds was calculated for frost-treated and control plants for both genotypes.

A similar experiment was conducted later, commencing two months after the start of the first experiment, in the same glasshouse under the same conditions. The second experiment differed from the first one in that duration of cold treatment was 14 days in the second experiment, compared to only 7 days in the first experiment. Consequently, plants in the later experiment were one week older at the end of the cold treatment and their pods and seeds were at more advanced developmental stages when the plants were exposed to frost.

In both experiments, the physiological characteristics measured in leaves, flowers, pods and seeds were chlorophyll content, leaf thickness, photosynthesis rate and chlorophyll fluorescence parameters of leaves, and water content and concentration of soluble sugars of all organs. For plants that were exposed to cold and then frost, all variables were studied before cold, after cold and after frost recovery. For control plants,

measurements were taken at the same three times. For plants that were exposed to frost without prior cold treatment, measurements were taken before the frost treatment and after frost recovery. Control plants selected to study the above variables were at the same growth stage as treated plants. A schematic diagram indicating the sampling time for each treated set of plants is shown in Fig 7.2.

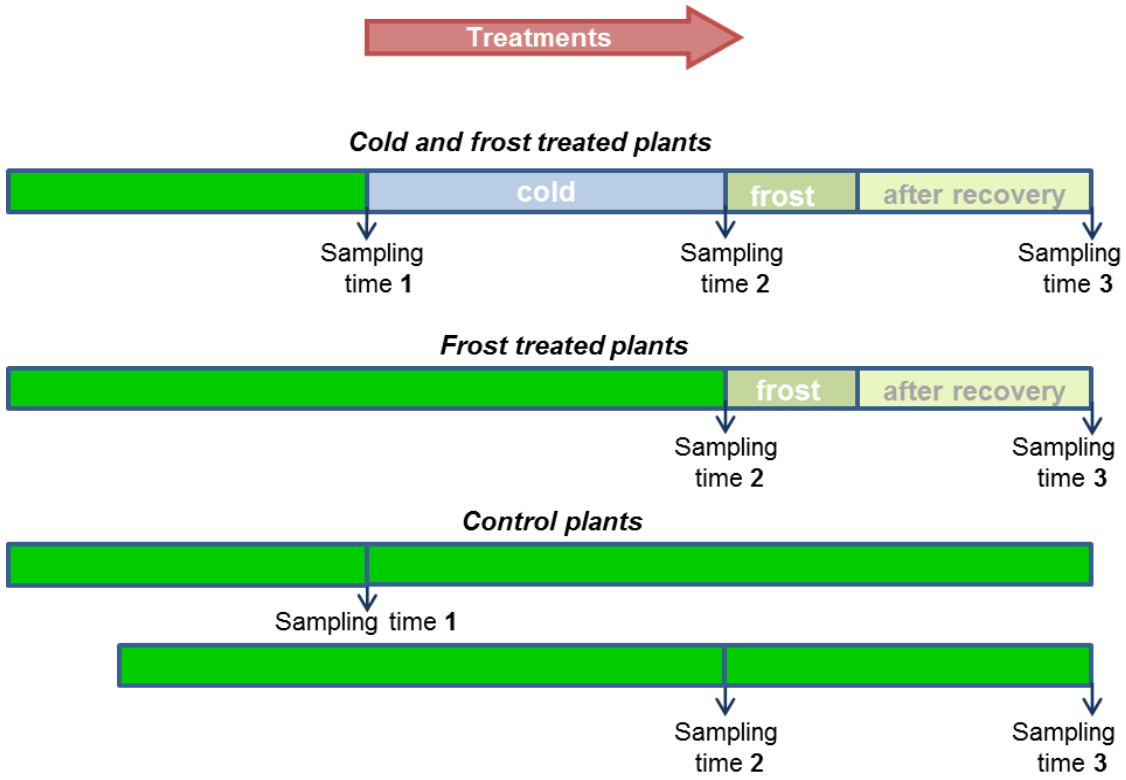
All measurements for the above variables were taken from stipules (leaf type) only. A few genotypic differences in the stipules were obvious to the naked eye; ATC 968 had longer and thinner stipules compared to Kaspa, and white patches were bigger and more apparent and frequent on the stipules of ATC 968 than Kaspa (Fig 7.3).

Measurement of chlorophyll content and leaf thickness

Chlorophyll content in the youngest fully expanded stipules of Kaspa (fourth and fifth stipules from the apex) and ATC 968 (second and third stipules from the apex) was estimated non-destructively using a hand-held SPAD-502 chlorophyll meter (Konica Minolta Sensing, Inc, Osaka Japan) (Feibo et al. 1998; Hoel and Solhaug 1998). This meter provides unitless SPAD (Soil-Plant Analysis Development) values ranging from 0 to 100 that are correlated with chlorophyll contents (Markwell et al. 1995). Three readings were taken from each stipule. The mean of six readings from two stipules was calculated for each plant. The thickness of stipules was measured non-destructively on the same stipules using a digital vernier caliper (Kincrome Australia Pty Ltd). Four readings were taken on each stipule; hypothetically the stipule was divided into right and left sides, and from each side readings were taken from the base and margin of the stipule. The mean of eight values from two stipules was calculated.

Measurement of photosynthesis and chlorophyll fluorescence

A LI-6400 portable photosynthesis system (Li-Cor, Inc., Lincoln, NE, USA) was used to measure the rate of photosynthesis and chlorophyll fluorescence parameters: efficiency of light harvesting (Fv'/Fm'), quantum yield of photosystem II electron transport (Φ_{PSII}), photochemical quenching (qP) and non-photochemical quenching



Sampling times for treated and control plants

Fig. 7.2. A schematic diagram of the experiment showing sampling/data recording times (down arrows), for the measurements of water content, sugar concentrations, leaf thickness and photosynthetic performance from control and treated plants. Two batches of control plants were grown with 10 days interval between sowing times, so the control (later batch) and cold-treated plants were at same growth stages. Seed weights were determined at maturity.

Fig. 7.3. The stipules of ATC 968 (conventional leaf type) and Kaspa (semi-leafless type); Note the large white patches on the stipules of ATC 968.

(NPQ). The photosynthesis system was coupled with an integrated fluorescence chamber head (Li-6400-40 leaf chamber fluorometer; Li-Cor Inc., Nebraska, USA). Plants were watered a day before the above variables were measured. Measurements were taken on the youngest fully expanded stipules in both genotypes and repeated at least three times. The rate of photosynthesis (*A*) was recorded at 20 ºC leaf temperature and 800 μmol photons m^{-2} s^{-1} light intensity with 10 % blue source (machine setting). The same light and temperature were used in the cold environment so that control and cold-treated plants could be compared under constant machine settings. Chlorophyll fluorescence was measured on light-adapted leaves under the prevailing light and temperature conditions in the glasshouse and cold chamber for control and cold-treated plants, respectively. Fluorescence parameters were defined as described by Genty et al. (1989), and estimated on light-adaptive leaves as follows; the efficiency of energy harvesting by open reaction centres of photosystem II for light-adapted leaves was calculated as:

$$\frac{F_v'}{F_m'} = \frac{F_o' - F_m'}{F_m'}$$

where F_v' is the variable fluorescence, F_o' is the minimal fluorescence of a momentarily darkened leaf, and F_m' is the maximal fluorescence during a saturating flash of light >7 mmol m^{-2} s^{-1}. The quantum yield of photosystem II (Φ_{PSII}), photochemical quenching (qP) and non-photochemical quenching (NPQ) was determined by formulae 1, 2 and 3, respectively;

$$\Phi_{PSII} = F_m' - \frac{F_s}{F_m'} \quad (1)$$

$$qP = \frac{F_m' - F_s}{F_m' - F_o'} \quad (2)$$

$$NPQ = \frac{F_m - F_m'}{F_m'} \quad (3)$$

F_s is steady-state fluorescence and F_m is maximum fluorescence during a light-saturating pulse of 8000 μmol m^{-2} s^{-1}.

Determination of water content

The relative water content (RWC) of youngest fully expanded stipules was determined as described by Weatherley (1950). One stipule per plant was excised and fresh weight was determined. The same stipule was allowed to hydrate in distilled water overnight and turgid weight was determined the next day. The hydrated stipule was then oven dried at 70 ^{o}C for two days to determine the dry weight. The RWC was calculated using the following formula;

$$RWC = \frac{(freshweight - dryweight)}{(turgidweight - dryweight)} \times 100$$

Moisture content (MC) of reproductive organs (flower, pod and seed) was determined following Deunff and Rachidian (1988). Two morphologically similar flowers and pods were harvested from each plant. Pods were dissected into pod walls and seeds. Fresh weights of flowers, pods and seeds were determined then samples were oven dried at 70 ^{o}C for two days and dry weights were recorded. Moisture content for each organ was calculated on the basis of fresh weight using the following formula;

$$MC = \left(\frac{freshweight - dryweight}{freshweight} \right) \times 100$$

Quantification of soluble sugars

Soluble carbohydrates (fructose, glucose, sucrose and raffinose) were extracted from leaf, flower, pod and seed tissues following Dumont et al. (2009) with some modifications. Tissues were excised from plants and kept at –20 ^{o}C until further use. The frozen samples were freeze-dried and ground to a fine powder. To 20 mg of this fine powder, 200 µl of 80 % ethanol was added in a 2 ml microfuge tube, vortexed and incubated at 60 ^{o}C for 30 min. Then samples were centrifuged at 15000 rcf for 15 min. The supernatant was removed from the pellet and transferred to a new microfuge tube. To the pellet, 100 µl of 80 % ethanol was added and vortexed, followed by incubation at 60 ^{o}C for 15 min. Samples were centrifuged at 15000 rcf for 15 min. The supernatant was collected and added to the previous supernatant extract. The pellet was resuspended in 50 µl of 80 % ethanol followed by the above process for incubation and centrifugation under the same conditions. The supernatants were combined and dried

using a speed vacuum. The dried residue was resuspended in 1 ml distilled water. Forty µl of this extract was analysed by normal phase high-performance liquid chromatography (HPLC) using a Hewlett-Packard 1090LC. Separations were performed using a Prevail Carbohydrate ES column (150 x 2 mm) with 90 % MeCN (A) and 10 mM NH_4OH (B) mobile phase. The ratio of eluent gradient was 94.5 % and 5.5 % for solvents A and B, respectively. The flow was 0.2 ml min^{-1} and the column was at room temperature, 22 ^{o}C. Runs lasted for 20 min. Peaks corresponding to fructose, glucose, sucrose and raffinose were detected with an evaporative light scattering detector (ALLTECH 800). The use of standards allowed determining the concentration of the sugars with the Chemstation software (Hewlett-Packard). For each sample, the sugar content was expressed as mg g^{-1} of dry matter.

Data analysis

The frost survival data on buds, flowers and set pods were combined and are presented as flowering stage (FS) survival and those on flat, swollen, filled and mature pods were combined and are presented as pod development stage (PDS) survival. To observe the variation among genotypes for frost survival at FS and PDS, REML analysis was used. For all other variables studied in this chapter, the significance of sources of variation (genotypes, treatments and their interaction) were tested using two-way analysis of variance (ANOVA) using GenStat (GenStat 13 Statistical software).

7.3. Results

Frost tolerance after 7 and 14 days of cold treatment

The 7-day pre-frost cold treatment somewhat reduced frost injury symptoms on the leaves of ATC 968 (Fig. 7.4 and Appendix 4), but neither the 7-day nor 14-day cold treatment had any significant effect on the frost survival of reproductive organs (flowering and pod development stages) or on seed weight (Appendices 4 and 5). Although the frost survival rates of reproductive organs after cold treatment were numerically higher (7-day cold treatment) or lower (14-day cold treatment) than control values (Fig. 7.5), these differences were not statistically significant (Appendix 5).

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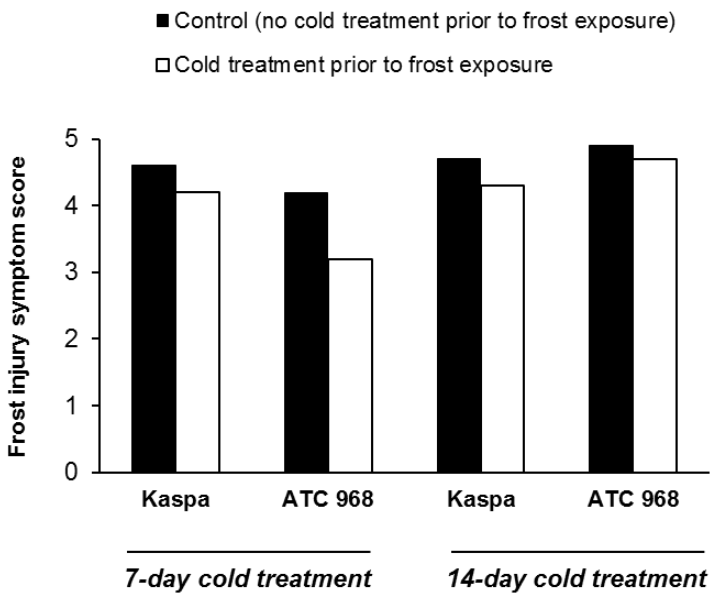

Fig. 7.4. Frost injury symptom scores on leaves of two field pea (*Pisum sativum* L.) genotypes after exposure to frost with or without preceding cold treatments of 7 and 14 days. Scores ranged from 1 (leaves with no frost damage) to 5 (totally damaged and brittle leaves).

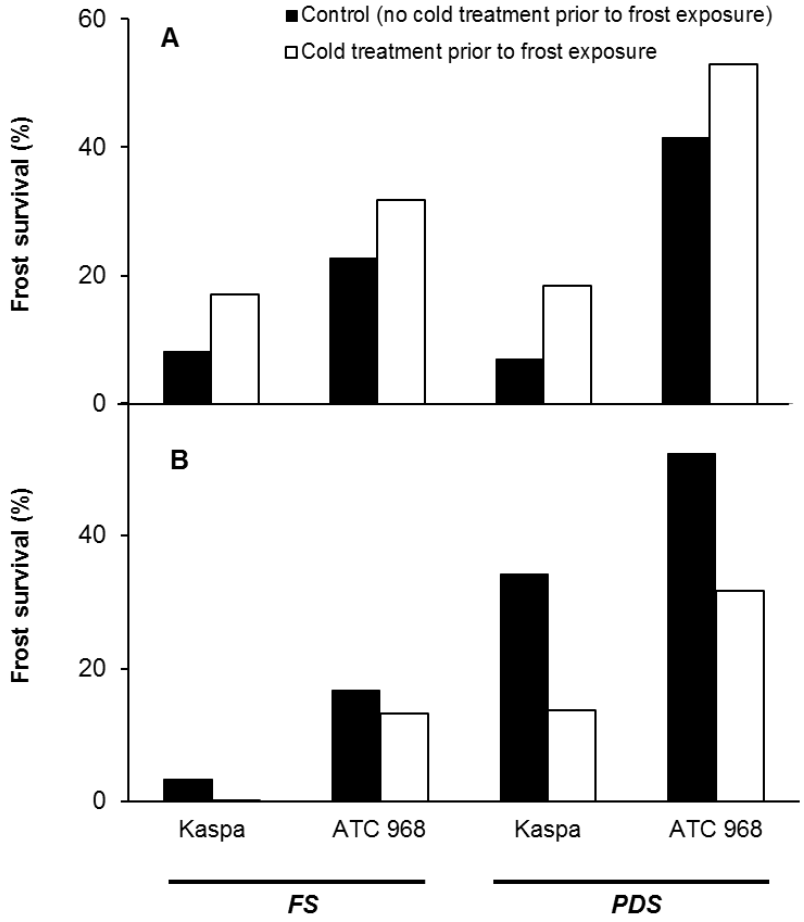

Fig. 7.5. Frost survival of flowering stage (FS) organs (buds, flowers and set pods) and pod development stage (PDS) organs (flat, swollen, filled and mature pods) of two field pea (*Pisum sativum* L.) genotypes with or without (**A**) 7-day prior cold treatment, and (**B**) 14-day prior cold treatment

Compared to Kaspa, ATC 968 showed better survival of FS organs on exposure to frost after a 14-day cold treatment, and of PDS organs after a 7-day cold treatment (Fig. 7.5 and Appendix 5). For both genotypes, the seeds harvested from frost-treated plants were much smaller than those from control plants (Fig. 7.6 and Appendix 4). In the first experiment, this effect was greater for Kaspa than for ATC 968. However in the second experiment, there was no significant effect of either genotype or treatment (i.e. with or without 14 days cold treatment prior to frost), possibly due to plants bearing seeds that

were more advanced in development at the time of frost exposure than in the first experiment (Fig. 7.6 and Appendix 4).

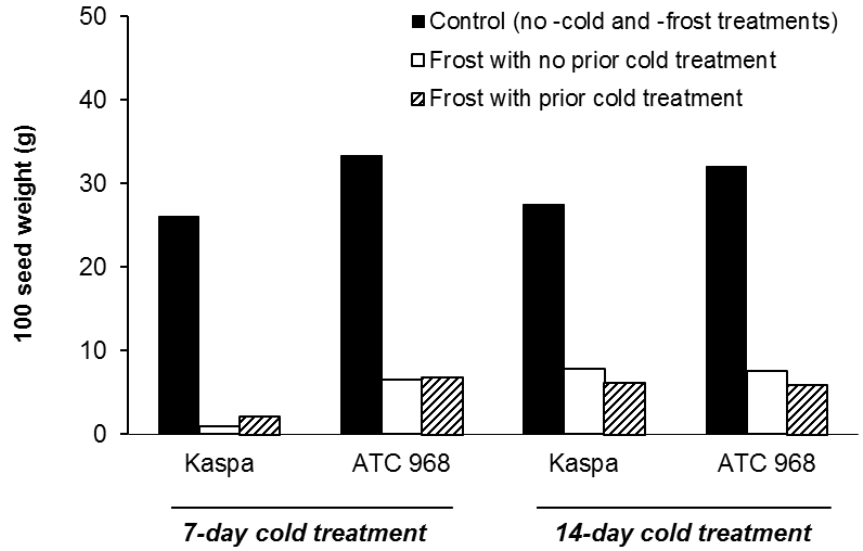

Fig. 7.6. 100 seed weight of control (no-cold, no-frost treated), and frosted plants with or without preceding cold treatments of 7 and 14 days, of two field pea (*Pisum sativum* L.) genotypes

At sampling time 1 (prior to cold treatment, Fig. 7.2), no significant difference was detected for the photosynthetic performance and physiological variables, between control plants and those allocated to the cold-frost treatment. Similarly, at sampling time 2 (prior to frost treatment, Fig. 7.2), no significant difference was detected between the control plants and those allocated to the frost treatment.

Photosynthetic performance at the reproductive stage under low temperatures

In both experiments, cold treatment reduced the rate of photosynthesis, quantum yield of PSII electron transport and photo- and non-photo-chemical quenching (Table 7.1 and Appendices 6 and 7). An increase in the chlorophyll content, and a decrease in the leaf thickness was observed after the 7-day cold treatment used in the first experiment (Table 7.1 and Appendix 6), and a decline in light harvesting efficiency in leaf tissues

was noted after the 14- day cold treatment used in the second experiment (Table 7.1 and Appendix 7).

The frost treatment reduced all of the above variables (Table 7.1 and Appendices 6 and 7). The only variable that was influenced by prior cold treatment was chlorophyll content, which was significantly higher in plants that were pre-treated with cold for 14 days than in plants that were treated directly with frost (Table 7.1 and Appendices 6 and 7).

Genotypes differed significantly from each other for the above variables after cold treatments. Kaspa had thicker leaves than ATC 968, regardless of duration of the cold treatment (Table 7.1, and Appendices 6 and 7). After the 7-day cold treatment, the quantum yield of PSII electron transport and photo- and non-photo-chemical quenching were higher in Kaspa than in ATC 968 (Table 7.1 and Appendix 6). After the 14-day cold treatment in the second experiment, Kaspa had higher chlorophyll content but a lower rate of photosynthesis than ATC 968 (Table 7.1 and Appendix 7).

After frost treatment without any prior cold treatment, Kaspa had higher chlorophyll content than ATC 968 in both experiments (Table 7.1, and Appendices 6 and 7). In experiment 1, the rate of photosynthesis, light harvesting efficiency and photochemical quenching were lower in Kaspa than in ATC 968 (Table 7.1 and Appendix 6). In the second experiment, Kaspa had thicker leaves than ATC 968 (Table 7.1 and Appendix 7).

After frost treatment with prior cold treatment for 7 days, Kaspa had higher chlorophyll content and lower light harvesting efficiency, quantum yield of PSII electron transport and photo- and non-photo-chemical quenching than ATC 968 (Table 7.1, and Appendix 6). After frost treatment with prior cold treatment for 14 days, Kaspa had thicker leaves and higher chlorophyll content than ATC 968 (Table 7.1 and Appendix 7).

Table 7.1. Mean values for chlorophyll content, rate of photosynthesis, leaf thickness and chlorophyll fluorescence parameters (efficiency of light harvesting, Fv'/Fm'; quantum yield of PSII electron transport, Φ_{PSII}; photochemical quenching, qP; non-photochemical quenching, NPQ), for two field pea genotypes observed after cold treatments of 7 and 14 days (at 10/5 ^{o}C) and subsequent frost exposure (-4.8 ^{o}C).

Genotype	Experiment with 7-day cold treatment					Experiment with 14-day cold treatment				
	After cold treatment[a]		After recovery from frost treatment[b]			After cold treatment[a]		After recovery from frost treatment[b]		
	Control	**Cold**	**Control**	**Frost**	**Cold-Frost**	**Control**	**Cold**	**Control**	**Frost**	**Cold-Frost**
Chlorophyll content (SPAD units)										
ATC 968	32.7	37.2	25.1	25.6	26.6	29.2	35.2	31.9	10.8	25.5
Kaspa	31.2	39.7	31.4	34.4	29.6	42.3	36.5	39.0	17.1	27.0
Rate of photosynthesis (µmole m^{-2} s^{-1})										
ATC 968	9.48	3.66	4.88	0.10	0.95	12.14	0.70	4.32	0.20	0.82
Kaspa	16.20	2.75	3.51	0.01	0.19	18.85	0.66	6.43	0.05	0.26
Leaf thickness (mm)										
ATC 968	0.18	0.16	0.13	0.07	0.10	0.12	0.13	0.10	0.05	0.07
Kaspa	0.22	0.19	0.13	0.09	0.07	0.18	0.17	0.13	0.09	0.09
Fv'/Fm'										
ATC 968	0.72	0.69	0.76	0.22	0.36	0.74	0.63	0.74	0.04	0.09
Kaspa	0.76	0.71	0.56	0.03	0.08	0.73	0.56	0.50	0.03	0.08

For [a]cold vs no-cold comparison, control (no-cold) and cold-treated plants were compared at sampling time 2, and for [b]control vs frost comparison, control (no-cold, no-frost) and frost-treated plants were compared at sampling time 3, and for [b]frost vs cold-frost comparison, frost treated and cold-frost treated plants were compared at sampling time 3 as indicated in Fig 7.2.

Table 7.1. continued.

Genotype	Experiment with 7-day cold treatment					Experiment with 14-day cold treatment				
	After cold treatment[a]		After recovery from frost treatment[b]			After cold treatment[a]		After recovery from frost treatment[b]		
	Control	**Cold**	**Control**	**Frost**	**Cold-Frost**	**Control**	**Cold**	**Control**	**Frost**	**Cold-Frost**
Φ_{PSII}										
ATC 968	0.66	0.55	0.70	0.16	0.30	0.65	0.46	0.68	0.01	0.05
Kaspa	0.75	0.62	0.52	0.00	0.03	0.65	0.42	0.46	0.00	0.03
qP										
ATC 968	0.90	0.80	0.92	0.42	0.58	0.87	0.73	0.92	0.25	0.23
Kaspa	0.97	0.87	0.74	0.11	0.15	0.89	0.71	0.72	0.14	0.20
NPQ										
ATC 968	3.74	0.15	4.12	1.39	1.94	3.94	2.72	3.96	1.05	1.12
Kaspa	4.25	0.15	3.16	1.03	1.11	3.79	2.53	2.80	1.03	1.09

For [a]cold vs no-cold comparison, control (no-cold) and cold-treated plants were compared at sampling time 2, and for [b]control vs frost comparison, control (no-cold, no-frost) and frost-treated plants were compared at sampling time 3, and for [b]frost vs cold-frost comparison, frost treated and cold-frost treated plants were compared at sampling time 3 as indicated in Fig 7.2.

Partitioning of water content and carbohydrates in vegetative and reproductive tissues under low temperatures

In the HPLC analysis of sugars, the peaks for fructose and particularly raffinose were frequently just above the baseline on chromatograms for tissues from both control and treated plants. This did not allow quantities of these two sugars to be determined accurately. To demonstrate HPLC results, chromatograms for different treatments and tissues for ATC 968 are presented in Fig 7.7.

The cold treatment of 7 days reduced the water content in pods and seed, and elevated the glucose content in flowers and reduced the sucrose content in leaves (Table 7.2 and Appendix 8). The frost treatment reduced the water content in all tissues, and glucose and sucrose contents in seed, but elevated sucrose content in leaves and pods (Table 7.2 and Appendices 8 and 9). In plants that were treated for both cold and then for frost, effects of preliminary cold treatment were noticeable in leaf tissue only; the RWC was significantly reduced and sucrose content was increased after frost if pre-treated with cold for 7 and 14 days, respectively, compared to frost exposed plants without cold treatment (Table 7.2 and Appendices 8 and 9).

Genotypes differed significantly from each other for the above variables after cold treatments; in Kaspa, regardless of duration of the cold treatments, the water content in seed was lower, and sucrose content in pods was higher than ATC 968 (Table 7.2, and Appendices 8 and 9). After 7 days of cold treatment, the water content in pods, and sucrose content in flowers were lower in ATC 968 than in Kaspa (Table 7.2 and Appendix 8).

After frost treatment (without any prior cold treatment) in both experiments, the water content in seed was lower in Kaspa than ATC 968 (Table 7.2, and Appendices 8 and 9). In the first experiment, glucose and sucrose contents in pods and seeds were higher in Kaspa than in ATC968. In the second experiment, water content of flowers was higher in Kaspa than in ATC 968 (Table 7.2 and Appendices 8 and 9).

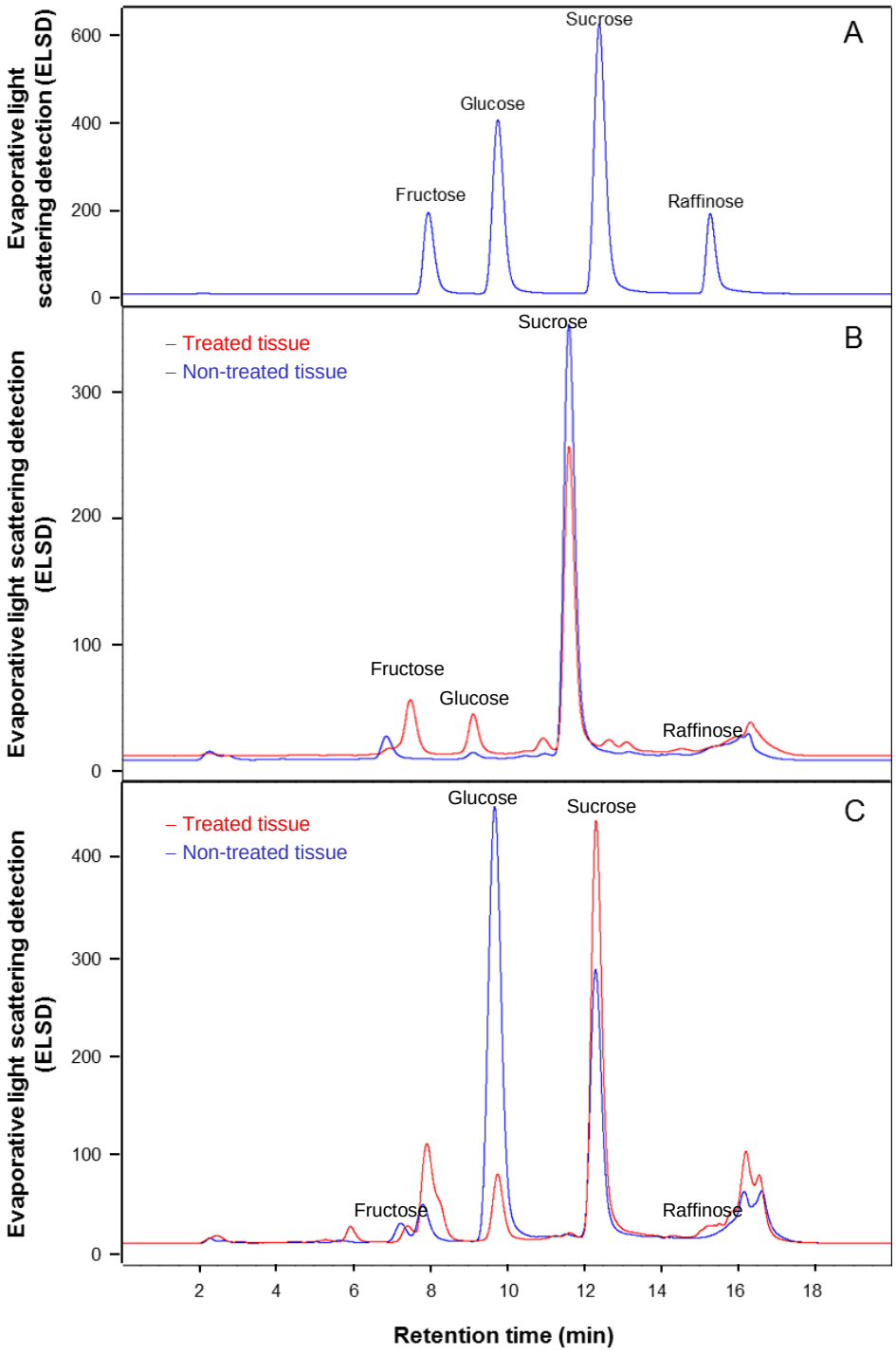

Fig. 7.7. HPLC profiles of *Pisum sativum* L. genotype ATC 968 showing fractionation of soluble sugars: (**A**) high strength standards for fructose, glucose, sucrose and raffinose; (**B**) leaf tissue profiles for control (blue) and after seven days cold treatment (red); (**C**) pod tissue profiles for control (blue) and after frost treatment (red)

Table 7.2 Mean values for moisture content and glucose and sucrose concentrations in leaf, flower, pod and seed, for two field pea genotypes after cold treatments of 7 and 14 days (at 10/5 ºC) and subsequent frost exposure (-4.8 ºC).

Genotype	Experiment with 7-day cold treatment					Experiment with 14-day cold treatment				
	After cold treatment[a]		After recovery from frost treatment[b]			After cold treatment[a]		After recovery from frost treatment[b]		
	Control	**Cold**	**Control**	**Frost**	**Cold-Frost**	**Control**	**Cold**	**Control**	**Frost**	**Cold-Frost**
Water content (%)										
					Leaf					
ATC 968	70.7	68.4	71.5	13.3	12.8	69.3	74.3	49.5	31.7	40.0
Kaspa	65.3	76.2	60.7	53.6	14.5	69.9	73.8	42.5	37.1	43.4
					Flower					
ATC 968	85.0	84.5	83.8	17.4	24.5	84.1	84.3	79.4	26.2	46.8
Kaspa	84.8	84.2	85.7	12.6	46.0	81.0	68.4	85.4	26.0	27.0
					Pod					
ATC 968	84.3	81.7	86.4	76.2	77.0	85.1	85.1	78.8	41.0	65.4
Kaspa	85.8	84.7	88.1	78.6	71.2	82.3	85.9	89.0	45.6	70.0
					Seed					
ATC 968	84.3	82.0	85.6	77.1	84.3	83.1	82.7	76.1	54.1	54.7
Kaspa	81.1	79.1	82.7	67.2	74.5	81.8	73.9	66.2	34.4	69.8

For [a]cold vs no-cold comparison, control (no-cold) and cold-treated plants were compared at sampling time 2, and for [b]control vs frost comparison, control (no-cold, no-frost) and frost-treated plants were compared at sampling time 3, and for [b]frost vs cold-frost comparison, frost treated and cold-frost treated plants were compared at sampling time 3 as indicated in Fig 7.2.

Table 7.2. continued.

Genotype	Experiment with 7-day cold treatment					Experiment with 14-day cold treatment				
	After cold treatment[a]		After recovery from frost treatment[b]			After cold treatment[a]		After recovery from frost treatment[b]		
	Control	**Cold**	**Control**	**Frost**	**Cold-Frost**	**Control**	**Cold**	**Control**	**Frost**	**Cold-Frost**
Glucose content (%)										
					Leaf					
ATC 968	0.92	0.68	0.56	0.68	0.58	0.59	0.61	0.58	0.68	0.58
Kaspa	0.77	0.62	0.52	0.57	0.58	1.88	0.80	0.01	0.54	0.73
					Flower					
ATC 968	0.88	3.29	0.93	0.80	0.83	0.74	2.30	-	1.52	0.99
Kaspa	2.42	4.16	-	3.50	-	2.52	2.33	0.99	3.91	1.50
					Pod					
ATC 968	11.99	10.75	8.13	3.16	5.88	8.72	5.28	1.61	1.07	1.63
Kaspa	10.49	10.34	11.75	6.96	7.82	3.72	8.43	0.25	0.93	1.26
					Seed					
ATC 968	1.90	1.09	1.40	0.22	0.34	0.96	0.56	0.25	0.21	0.58
Kaspa	0.87	-	0.87	0.67	0.79	0.58	0.38	0.17	0.46	0.53

For [a]cold vs no-cold comparison, control (no-cold) and cold-treated plants were compared at sampling time 2, and for [b]control vs frost comparison, control (no-cold, no-frost) and frost-treated plants were compared at sampling time 3, and for [b]frost vs cold-frost comparison, frost treated and cold-frost treated plants were compared at sampling time 3 as indicated in Fig 7.2. –indicates no data due to lack of replications.

Table 7.2. continued.

Genotype	Experiment with 7-day cold treatment					Experiment with 14-day cold treatment				
	After cold treatment[a]		After recovery from frost treatment[b]			After cold treatment[a]		After recovery from frost treatment[b]		
	Control	**Cold**	**Control**	**Frost**	**Cold-Frost**	**Control**	**Cold**	**Control**	**Frost**	**Cold-Frost**
Sucrose content (%)										
					Leaf					
ATC 968	6.65	4.98	4.15	5.78	6.99	4.68	5.07	1.92	2.87	4.74
Kaspa	6.44	4.98	3.19	5.05	5.90	9.18	5.39	1.68	1.54	4.64
					Flower					
ATC 968	1.38	1.72	1.25	3.63	2.70	1.72	2.49	-	3.14	2.16
Kaspa	3.02	5.06	-	4.39	-	3.05	2.84	1.30	1.98	3.34
					Pod					
ATC 968	5.17	5.20	3.67	6.74	7.35	3.61	4.16	1.21	5.43	10.69
Kaspa	16.38	10.93	9.06	20.66	20.46	19.70	12.98	1.09	8.29	4.19
					Seed					
ATC 968	19.84	21.07	17.96	2.66	7.10	13.29	10.29	1.56	1.16	3.36
Kaspa	13.15	-	11.43	3.87	11.77	16.56	8.33	1.27	1.60	6.37

For [a]cold vs no-cold comparison, control (no-cold) and cold-treated plants were compared at sampling time 2, and for [b]control vs frost comparison, control (no-cold, no-frost) and frost-treated plants were compared at sampling time 3, and for [b]frost vs cold-frost comparison, frost treated and cold-frost treated plants were compared at sampling time 3 as indicated in Fig 7.2. –indicates no data due to lack of replications.

The RWC, after frost treatment with prior cold treatment of 7 days, was higher in leaves but lower in seeds of Kaspa than ATC 968 (Table 7.2, and Appendix 8). The glucose and sucrose contents in pods and seed were higher in Kaspa than ATC 968 (Table 7.2, and Appendix 8).

7.4 Discussion

Studies presented in this thesis demonstrate that in reproductive stage plants, changes in physiology (chlorophyll content, and concentration of soluble sugars), and cellular ultrastructure (structural changes in chloroplasts, and starch contents in chloroplasts) occur in leaf tissues during the cold treatment (Chapters 5 and 6). Further, the cold treatment is effective in prevention of frost injury of vegetative tissues (for ATC 968) (Chapter 7). However, cold pre-treatment has a non-significant effect on the survival of reproductive organs (buds, flowers, set pods and maturing pods) following frost stress. These findings are similar to those reported in Chapter 5, where 21 days cold treatment at the reproductive stage promoted sensitivity to frost in reproductive organs.

From these results, it is suggested that reproductive organs of pea plants have a natural sensitivity to radiant frost, and an earlier alert/signalling (low positive temperatures) in reproductive-stage plants does not activate a defensive system against frost. Instead, long duration cold treatments, such as 21 days, effect chilling stress (Chapters 5 and 6) and can increase vulnerability to frost. As expected based on the results reported in Chapter 3 and Shafiq et al. (2012), frost survival of FS and PDS organs and seed weight from frost-treated plants were higher for ATC 968 than for Kaspa. However, it appears that short duration cold treatments such as 7 days as studied in this experiment, might induce positive effects on the frost survival of FS and PDS organs, if genotypes had exhibited some survival against frost in more than just couple of plants (like no survival in cold-and-frost-treated-Kaspa for 75 % of the total plants in this experiment) (Fig 7.5a). There was no survival of reproductive stage organs for many plants of Kaspa, therefore, REML analysis was used instead of ANOVA so that data with zeroes would be analysed appropriately with normal distribution of residuals achieved.

Previously, it was observed that Kaspa did not set seeds at all during cold treatment for 21 days (Chapter 5 of this thesis). Based on these result in Chapter 5, it was made sure in this study that Kaspa plants were at the seed developmental stage when treated for cold, so that the effect of frost after cold treatment could be observed. The results showed that frost with or without prior cold treatments, significantly reduced the seed weight in both genotypes, and there were also significant genotype and treatment effects. However, after cold treatment the loss in seed weight was higher in Kaspa compared to ATC 968. This is consistent with the previous findings of Chapter 5 of this thesis. In the second experiment, after frost exposure with 14 days prior cold treatment, there was no genotypic difference observed for seed weight, possibly due to the more advanced stage of development of seeds at the time of exposure to frost than in the first experiment.

Low temperatures significantly reduced the photosynthetic performance of both genotypes. Photosynthetic related parameters (Fv'/Fm', Φ_{PSII}, qP, NPQ) are inter-related, therefore, a decline in any one factor is expected to result in reductions in the other factors. In this study, a decrease in PSII efficiency (Φ_{PSII}) was observed under low temperatures. According to Härtel et al. (1998), this indicates a decrease in the fraction of absorbed light that is used in PSII photochemistry. This could lead to the closure of a high proportion of the PSII reaction centres, low absorption of electrons by Q_A (primary quinone acceptor of PS II) and a decrease in photochemical quenching (qP) (Maxwell and Johnson 2000). Krause and Weis (1991) stated that environmental stresses could affect Φ_{PSII} and lead to a decrease in the efficiency of light harvesting (Fv'/Fm') in leaf tissues. In low-temperature conditions, in which photosynthesis rate declines and other photosynthetic characteristics are reduced, the chlorophyll fluorescence (light transmission) would be high for plant tissues to be protected from photo-damage. The decrease in photosynthesis and related parameters observed are similar to reports of Feierabend et al. (1992) and Yordanov et al. (1996) who reported photo-inactivation at <15 ^{o}C and a decrease in rate of photosynthesis in pea, respectively. Ying et al. (2000) also reported a decrease in rate of photosynthesis and related parameters in maize plants when exposed to low positive temperatures at the reproductive stage.

After cold and/or frost treatments, the rate of photosynthesis and photosynthesis-related characteristics were higher in ATC 968 than Kaspa. The decrease in photosynthesis rates that were observed here after cold treatments of 7 and 14 days, are consistent with

the initial decline observed during the first 10 days of a 21-day cold treatment in an earlier experiment (Chapter 5 of this thesis). In the earlier experiment, plants recovered to the control rate of photosynthesis by 20 days. Thus it seems that the photosynthetic apparatus of reproductive-stage pea plants may require quite long exposure to cold temperatures in order to adapt to cold conditions.

Regardless of the temperature treatments, the chlorophyll content of ATC 968 was lower than that of Kaspa. This may have been associated with its stipules being thinner than those of Kaspa, and with the presence of white patches on its stipules. These explanations are similar to the findings of Gianoli et al. (2004) and Bravo et al. (2007) who reported high chlorophyll content in plants with thick leaves. Consistent with the observations reported in Chapter 5 of this thesis, chlorophyll content increased after cold treatment. After the frost treatment, however, chlorophyll content and leaf thickness both declined. The reduction in chlorophyll content may represent photo-oxidation (Wise 1995).

The lack of any change in leaf RWC after cold treatment is consistent with the findings of Chapter 5 of this thesis but in contrast to the report of Bourion et al. (2003) that RWC decreases in pea seedlings after cold acclimation. The decrease in leaf RWC after the frost treatment observed here, is in line with what has been reported for chickpea, where leaf RWC decreased under chilling stress conditions (Nayyar et al. 2005a). In reproductive organs, the moisture content decreased after cold and frost treatments. The moisture content of seeds after cold and frost treatments was reduced more in Kaspa than ATC 968. This is consistent with the report of Baigorri et al. (1999) who found a greater decrease in water contents of a semi-leafless pea genotype during water stressed conditions at the reproductive stage, compared to a conventional type.

Analysis of soluble sugars (glucose and sucrose) in leaves, flowers, pods and seed provided insights into the physiological responses against low temperatures in the two genotypes. The cold treatment reduced the sucrose content in leaves, and increased it in flowers. Sucrose is critical for the retention of flowers on plants (Aloni et al. 1997). The increase in sucrose in flowers observed here may permit plants to retain flowers under low temperature conditions, as previously a decrease in sucrose content in chickpea flowers resulted in the abortion of flowers under chilling temperatures (Nayyar et al. 2005a). During the cold treatment, a decrease in rate of photosynthesis may result in the

decline in the leaf-sucrose content and leaf thickness. These findings are similar to those of Chabot and Chabot (1977a), who reported that accumulation of carbon assimilates increased the leaf thickness under high temperatures and light intensity. Further, these results are in contrast to reports of increases in the concentration of soluble sugars, including sucrose, after acclimation at the vegetative stage in pea (Bourion et al. 2003), spinach (*Spinacia oleracea*) (Guy et al. 1992), and *Arabidopsis thaliana* (Ristic and Ashworth 1993). Sugars play an important role in relation to freezing tolerance by protecting the plasma membrane from freezing on exposure to sub-zero temperatures (Sakai and Yoshida 1968; Steponkus 1984). The decrease in sucrose content in leaves found here may be related to the decline in frost tolerance as Sasaki et al. (1996; 1998) reported a positive relation between the concentration of sugars during cold acclimation and frost tolerance. The non-accumulation and/or decrease in the concentration of sucrose in leaf tissues in the present study represent the non-acclimation of pea after the cold treatment at the reproductive stage, and consequently no improvement in the freezing tolerance.

The decrease in glucose content in seeds after the frost treatment may represent the mobilization of seed reserves (1994), and contribute to the reduction in seed weight (Kaur et al. 2008). Under control conditions, the concentration of soluble sugars should increase in pea seeds with their development, and accumulate up to 3.8 % (Frias et al. 1996). A decline in glucose content in pods may represent higher strengths of other sinks in plants (Marcelis 1996), as the competition between sinks becomes crucial to attract assimilates at the seed filling stage (Jeuffroy and Warembourg 1991). These findings are similar to the concepts of Thakur et al. (2010) who described that low temperatures at the grain filling stage result in an alteration in source sink relationships and reduction in the grain filling rate and unfilled or aborted seed. The present results are similar to the report of Kaur et al. (2008) who found a decrease in the concentration of sugars in seeds when chickpea were exposed to chilling temperatures at pod filling stage. In Kaspa, the sucrose content was higher in pods before or after any treatment than ATC 968.

Of the two genotypes of field pea used here, ATC 968 had better reproductive-stage frost survival than Kaspa, regardless of whether the plants were exposed to cold in advance of the frost treatment. Cold pre-treatment for 7 or 14 days did not improve the frost survival of either genotype, but was effective in reducing frost symptoms on

vegetative tissues of ATC 968. Low temperatures elevated the chlorophyll content, and reduced the leaf thickness, rate of photosynthesis and all photosynthesis-related parameters. Water and glucose contents decreased in pods and seeds. Sucrose content decreased in leaves, and increased in flowers. During the cold treatment, chlorophyll content, leaf thickness and pod sucrose content in Kaspa were higher than in ATC 968, while seed moisture content and the rate of photosynthesis and related parameters were lower in Kaspa than in ATC 968. The response of low positive temperature at the reproductive stage in field pea was not similar to the response at the vegetative stage. Cold-induced changes are more complex at the reproductive stage. Non-accumulation and/or decrease in the concentration of glucose and sucrose, particularly in leaves, pods and seeds, may represent the non-acclimation of pea at the reproductive stage and consequently no improvement in the frost tolerance.

Chapter 8

General Discussion

Although more than two decades have passed since the sensitivity of reproductive-stage field pea plants against spring radiant frost was first recognised as a significant problem in Mediterranean environments, research on identification of reproductive frost tolerance (RFT) is lacking. Prior to this thesis research, the only report on reproductive-stage frost injury in field pea was that of Ridge and Pye (1985), who reported adverse effects on grain yield following radiant frost events after the commencement of flowering in field pea. In contrast, in northern France where frost is a significant problem at the vegetative stage in pea, much research has been carried on vegetative-stage frost tolerance. Genetic variation has been found among pea genotypes for vegetative stage-frost tolerance, and QTLs affecting winter frost tolerance have been mapped (Bourion et al. 2003; Lejeune-Henaut et al. 1999, 2008; Dumont et al. 2009). Due to the differences in the nature of frost between northern France (European countries) and southern Australia (Mediterranean environments), it is unlikely that material identified as frost tolerant at the vegetative stage would be useful to develop lines with RFT (Bond et al. 1994). Dedicated studies on RFT were needed. To carry out this research rapid and reliable methods were needed for frost screening at the reproductive stage.

8.1. Screening for frost tolerance at the reproductive stage in field pea

Frost screening of reproductive stage pea plants is complicated due to the presence of reproductive organs at several stages of development at the same time on a single plant. With the simplified screening method developed here, frost tolerance can be determined using a key for field pea developmental stages (Knott 1987), at reproductive stages, i.e. flowering and pod development stages, or for individual reproductive organs, i.e. buds, flowers, set pods, flat pods, filled pods, swollen pods and mature pods (Fig. 3.1 in Chapter 3). Tags were used in the present study to label reproductive organs present on individual plants (Fig 3.2 in Chapter 3).

Previously in vegetative stage frost studies, scoring keys were used to assess frost injuries on leaf tissues (Bourion et al. 2003). In the present study, a scoring key was developed to record frost symptoms at each reproductive organ from immature bud to mature pod (Table 3.3 in Chapter 3). Severe frost symptoms were observed on developing pods, and seeds within these pods were also affected by frost (Table 3.7 in Chapter 3). Therefore, damage from frost on seeds was also assessed in the present study and five categories were described based on modifications in seed colour, weight and seed coat texture on exposure to frost (Fig. 3.3 in Chapter 3).

The assessment of frost tolerance under natural conditions was previously found to be very complex because of natural variability in the intensity and timing of the frost events, and due to the involvement of several factors such as freezing-thawing sequences, soil hardening, humidity, waterlogging and soil pH (Levitt 1980; Blum 1988). In this study, a programmed temperature regime was imposed to create simulated frost conditions in a temperature controlled chamber that enabled frost screening with reproducibility of conditions between experiments (Table 3.2 in Chapter 3).

Exposure to a minimum temperature of – 4.8 ^{o}C for 4 hr was selected for frost screening. These conditions were selected on the basis of natural conditions observed in southern Australia in the past five years in areas of field pea cultivation. Under controlled conditions, most reproductive stage pea plants were susceptible to these sub-zero conditions, but variation among genotypes for RFT was observed.

8.2. Genetic variation for RFT in field pea genepool

This is the first study to report genetic variation in field pea for frost tolerance at the reproductive stage. A diverse collection of germplasm was screened, and included 83 accessions sourced from high altitude and frost prone areas in 34 countries. Two local adapted lines were used in the study; Kaspa and Mukta. Both are *afila* types, and high yielding varieties in southern Australia. However, both lines exhibited high susceptibility to frost at reproductive stages, and no reproductive organ survived frost in these two varieties (Table 3.6 in Chapter 3). In eight lines, (ATC 104, ATC 377, ATC 947, ATC 968, ATC 1564, ATC 3489, ATC 3992 and ATC 4204) each from a different

country, more than 20 % of flowering stage organs (immature and mature buds, flower and set pod) survived the frost treatment (Tables 3.1 and 3.6 in Chapter 3).

Future studies should be undertaken to validate the frost tolerance of these eight accessions under naturally occurring frost in the field in the target production area.

This is the first report that buds and set pods are the most frost-susceptible reproductive organs, and that mature pods are the most frost-tolerant reproductive organs (Fig 3.5 in Chapter 3). Further, the flowering stage was found to be more susceptible to frost than pod development stage (Fig 3.6 in Chapter 3).

With identification of RFT at the flowering stage, genetic improvement of RFT in locally adapted elite lines might be possible through classic or modern breeding techniques, and this could contribute considerably to increase grain yield and high biomass production in field pea cultivating areas.

8.3. Breeding potential

Based on the variation found for frost tolerance at the flowering stage among field pea genotypes, it seems likely that frost tolerance can be improved effectively by breeding using phenotypic selection. At present, varieties cultivated in southern Australia have inadequate tolerance to frost at the reproductive stage. For example, Kaspa is highly adapted and the most widely cultivated field pea variety in southern Australia but it has inadequate tolerance to radiant frost at the reproductive stage.

In the present study, a cross was made between the locally adapted frost-sensitive variety Kaspa, and the frost tolerant line ATC 1564 selected from frost tolerant materials identified in Chapter 3. On phenotypic evaluation, it was found that F_1 hybrid plants were susceptible to frost, consequently, the F_1 plants were crossed back to frost tolerant parent ATC 1564, and a backcross (BC_1F_1) population was derived. Most of the backcross plants exhibited susceptibility to frost at reproductive stages (Fig 4.3 in Chapter 4).

A total of 332 microsatellite primer pairs were assayed on DNA samples of the two parents, Kaspa and ATC 1564. Only 42 markers exhibited polymorphic products on gels (Table 4.1 in Chapter 4). After the genotypic evaluation of these polymorphic markers on the backcross progeny, an attempt was made to construct a linkage map.

Unfortunately, most of the markers were not linked with any other loci, and only two linkage groups were developed: one with three markers, and the other with only two (Fig 4.4 in Chapter 4).

Two markers, PGER-E62 and PGER-S139, were significantly associated with frost survival at the flowering stage (Table 4.3 in Chapter 4). For the marker PGER-E62 the favourable effect came from the frost tolerant parent ATC 1564 (Fig 4.5 in Chapter 4), but for the marker PGER-S139, the favourable effect came from the frost-sensitive parent Kaspa (Fig 4.6 in Chapter 4). This is the first report on segregation of frost survival trait and markers in field pea progeny. In future, other sources of frost tolerance identified in Chapter 3 could also be used, and instead of a BC_1F_1 population it would be advantageous to develop recombinant inbred lines (RILs) that would allow the frost screening (phenotyping) to be replicated and/or repeated. Further, field evaluation of the progeny for frost tolerance could be attempted.

8.4. Cold acclimation at the reproductive stage in field pea

Previously, field pea seedlings exposed to low positive temperature (cold treatment) were reported to exhibit acclimation and become more tolerant to vegetative-stage frost exposure (Bourion et al. 2003). Physiological changes induced by cold treatment were also investigated, and a decline in rate of photosynthesis (Yordanov et al. 1996), an increase in soluble sugar and starch, and decrease in relative water content (RWC) had been reported (Bourion et al. 2003). The response of field pea plants at the reproductive stage to cold treatment had not been reported, and it was not known whether exposure to cold at the reproductive stage would increase RFT. This thesis is the first report on cold-induced physiological and cellular changes during the cold treatment at the reproductive stage in pea plants, and its impact on RFT.

Cold-induced modification in cellular ultrastructure

Cold acclimation is a complex process, associated with fundamental changes in the cellular ultrastructures in plant tissues (Levitt 1980). In field pea, little was known about cold-induced modifications of cellular ultrastructure at the vegetative stage (Ma et al. 1990), and there is no literature about such changes at reproductive stages. Here, two genotypes (Kaspa and ATC 1564) were studied for cold-induced changes in leaf

parenchyma cells during a 21-day cold treatment at 10/5 ^{o}C and 150 µmole m^{-2} s^{-1} PPFD conditions. These genotypes exhibited differences in cellular ultrastructure with respect to each other and with respect to non-cold-treated controls.

Many cold-induced changes, such as appearance of vesicles in the cytoplasm, protrusion of vesicles into vacuoles, invagination of the plasma membrane, and phenolic deposits on cellular membranes, were observed in both genotypes (Figs 6.1 – 6.9 in Chapter 6). These changes were similar to those that have been observed in cold-treated plants of other crop species (Ristic and Ashworth 1993; Stefanowska et al. 2002).

In the present study, frost-sensitive and frost-tolerant genotypes differed in the changes in their cellular ultrastructure in response to cold treatment. In Kaspa, chloroplast structure was distorted, with less electron density and swelling of the stroma, widening of lacunae between thylakoid membranes, and formation of vesicles (Figs. 6.3 – 6.5). Further, in Kaspa, decreases were observed in the starch concentration in leaf tissues, and starch grains in chloroplasts (Fig. 6.10 in Chapter 6). These modifications have previously been reported to be associated with symptoms of chilling injuries in different crops (Kratsch and Wise 2000; Xu et al. 2008). In contrast, no modifications in the structure of chloroplasts were observed in ATC 1564. Further, the increase in the concentration of starch in leaf tissues, and starch grains in chloroplasts observed in ATC 1564 were previously reported to be associated with acclimation and frost tolerance (Ristic and Ashworth 1993; Strand et al. 1999; Bourion et al. 2003). Thus Kaspa seems to be more sensitive than ATC1564 to cold treatments, and the cellular responses (particularly chloroplasts) that resulted in sensitivity to chilling might also be a reason for greater sensitivity to frost.

Cold-induced physiological changes in reproductive stage field pea genotypes

Cold-induced physiological changes were studied in Kaspa and ATC 1564, after 7 and 14 days of cold treatment at 10/5 ^{o}C (D/N) and 250 m^{-2} s^{-1} PPFD conditions (Chapter 7 of this thesis). Cold-induced changes were also measured in four genotypes (ATC 968, ATC 1564, ATC 1040 and Kaspa) after 21 days of cold treatment under 10/5 ^{o}C (D/N) and 150 m^{-2} s^{-1} PPFD conditions (Chapter 5 of this thesis).

Exposure to these cold temperatures for 7 or 14 days significantly reduced the rate of photosynthesis in reproductive-stage plants (Table 7.1 of Chapter 7), but plants

exhibited the ability to adjust the rate of photosynthesis during a longer exposure to cold (Fig 5.2 in Chapter 5). The decline in rate of photosynthesis was compensated by an increase in the chlorophyll content (Fig. 5.2 in Chapter 5 and Table 7.1 in Chapter 7). During the 7-day and 14-day cold treatments, only a low fraction of absorbed light was used for photosynthesis. The efficiency of photosystem II declined and photo-, and non-photo-chemical quenching was observed (Table 7.1 in Chapter 7). A similar decrease in photosynthesis rate was previously reported for maize plants when exposed to low positive temperatures at the reproductive stage (Ying et al. 2000).

During cold treatments of 7, 14 and 21 days, decreases in soluble sugars including glucose and sucrose were observed in leaf tissues and reproductive organs (Fig. 5.2 in Chapter 5 and Table 7.2 in Chapter 7). This is in contrast to findings for pea seedlings, in which the sugar concentration doubled after acclimation (Bourion et al. 2003). The above results obtained here were also opposite to previous findings in other crops (Guy et al. 1992). Sugars play an important role in protecting cell membranes against frost damage (Sakai and Yoshida 1968; Steponkus 1984). The observed decline in sugar concentration might reflect the inability of pea plants to undergo modifications needed for acclimation to acquire tolerance against low temperatures.

The impact of cold treatments on reproductive frost tolerance

Two genotypes (Kaspa and ATC 1564) were exposed to frost after cold treatment of 7 and 14 days (Chapter 7 of this thesis), and four genotypes (ATC 968, ATC 1564, ATC 1040 and Kaspa) were exposed to frost after 21 days of cold treatment (Chapter 5 of this thesis). The 7-day cold treatment improved the frost tolerance of leaf tissues in ATC 968 (Fig. 7.4 in Chapter 7). Neither the 7-day nor 14-day cold treatments had any effect on RFT (Fig. 7.5 in Chapter 7), and the 21-day cold treatment induced more sensitivity against frost (Table 5.2 in Chapter 5).

Cold acclimation and increased frost tolerance was previously achieved in pea seedlings using conditions similar to those used here (Bourion et al. 2003). The fact that the opposite result was achieved here reflects the natural sensitivity of reproductive organs towards cold and frost. All genotypes were found to be sensitive to chilling, but the relatively frost-sensitive genotype Kaspa responded more to cold treatment than more

frost-tolerant genotypes, as reflected in the symptoms of chilling injury in Kaspa cells during 21 days of cold treatment (Chapters 5 – 7 of this thesis).

In conclusion, a drop in temperature under radiant frost conditions is lethal for reproductive stage-pea plants. Buds, flowers and set pods are more sensitive to frost than pods with seed developmental stages. Exposure to frost (– 4.8 ^{o}C for 4 hr), leads to the abortion of buds, flowers and set pods, and a significant reduction in the seed weight. Genetic variation is present among pea genotypes for frost tolerance at the flowering stage (FS). There is a potential to breed for RFT lines by introgressing frost tolerance from tolerant material identified (in this study) into locally adapted lines that were found to have inadequate RFT. Cold or low positive temperatures as pre-frost-alerts in plants at the reproductive stage, did not improve the tolerance of FS and PDS (pod development stage) organs against frost. Instead, exposure of pea plants at the reproductive stage to low positive temperature increased the vulnerability of reproductive organs to frost, and in frost-sensitive genotypes this expression of chilling sensitivity (distortion in the ultrastructure of chloroplasts, and no seed set) is more intense than frost tolerant genotypes.

Chapter 9

Conclusions

The present study is the first report on reproductive frost tolerance in field pea (*Pisum sativum* L.), and the main conclusions of the research reported in this thesis are:

Development of a methodology for frost screening at the reproductive stage

Simple methods were developed for screening flowering and podding stage organs at the same time (on one plant) for tolerance against frost and to score frost symptoms on individual reproductive organs from immature buds to mature pods.

Identification of the most sensitive and tolerant reproductive stages and organs

Flowering stage organs (bud, flower and set pod) are more frost-sensitive than pod development stage organs (flat, swollen, filled and mature pods). Immature buds and set pods are the most sensitive reproductive organs and mature pods are the most tolerant reproductive organs.

Variation for frost tolerance at the flowering stage in *P. sativum* genepool

Pea germplasm accessions vary in the tolerance of their flowering stage organs against frost. Eight accessions were found to exhibit tolerance of flowering stage organs with greater than 20 % survival under controlled conditions. It may be possible to improve reproductive frost tolerance by breeding.

Segregation of frost tolerance trait at the reproductive stage

In a BC_1F_1 population derived from relatively frost-tolerant and susceptible parents, very little polymorphism was detected for microsatellite markers, and no strong marker-trait associations were observed for frost tolerance.

Photosystem stability under low positive temperature

Field pea genotypes including frost-sensitive Kaspa are able to adjust the rate of photosynthesis under low positive temperature conditions at the reproductive stage.

Carbohydrate partitioning between vegetative and reproductive tissues

Cold treatment significantly reduces the concentration of soluble sugars, particularly sucrose, in vegetative and reproductive tissues but does not lead to acclimation of pea plants at the reproductive stage.

Chloroplast ultrastructure, as a frost sensitivity indicator

Cold treatment of a frost-sensitive genotype of field pea led to distortion in the chloroplast ultrastructure.

Cold acclimation at the reproductive stage

Cold treatment at the reproductive stage reduced frost injury symptoms in vegetative tissues, but not in the reproductive organs. Long exposure to low positive temperatures increased sensitivity to frost especially for frost-sensitive genotypes.

Chapter 10

Contributions to Knowledge

This research was focused on understanding frost sensitivity at the reproductive stage in field pea, screening germplasm for differences in frost tolerance and investigating whether frost tolerance would be improved by cold acclimation. The research reported in this thesis is the first dedicated study of radiant frost tolerance at the reproductive stage in field pea. It presents the following new contributions to knowledge:

1. Development of simple methods for evaluation of frost tolerance of reproductive organs from immature buds to mature pods, all of which can be present simultaneously on a single plant (Chapter 3). With these methods, sensitivity of flowering stage (buds, flower and set pod) and pod development stage (flat, swollen, filled and mature pods) organs to frost can be compared and understood, within or across stages. The methods overcome previous constraints due to lack of screening methods, and could be used in future research on sensitivity to frost at the reproductive stage.

2. Identification of the most frost sensitive and tolerant reproductive organs (Chapter 3). The study revealed that immature buds and set pods are the most sensitive reproductive organs and mature pods are the most tolerant. Further, the flowering stage is more sensitive to frost than pod development stage.

3. Information about genetic variation for reproductive frost tolerance in field pea (Chapter 3). This is the first study reporting variation for tolerance to frost at the flowering stage among field pea genotypes. A few accessions were found to have some frost tolerance at the flowering stage. These results show the possibility of increasing reproductive frost tolerance in field pea by breeding.

4. Information about changes in cellular ultrastructure and physiological characteristics in reproductive-stage pea plants exposed to cold (Chapters 5, 6 and 7). On exposure to low positive temperatures for a long duration, such as 21 days, field pea plants are able to maintain the photosynthetic rate (Chapter 5), but such long exposures induce

chilling injuries in frost-sensitive genotypes by affecting the ultrastructure of chloroplasts (Chapter 6). Under cold conditions, a decrease and/or non-accumulation of soluble sugar in vegetative and reproductive tissues in plants might be associated with the inability in reproductive stage plants to acclimate (Chapters 5 and 7).

5. Demonstration that cold treatment at the reproductive stage does not result in acclimation, and does not improve reproductive frost tolerance, and can increase sensitivity to frost (Chapters 5 and 7).

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Appendix

Appendix 1: The flowering time for 83 accessions of field pea (*Pisum sativum* L.), grown in the glasshouse under 18/12 ^{o}C (day/night), in the experiment reported in Chapter 3 of this thesis.

Accession	Days to flower	Accession	Days to flower
ATC 18	70	ATC 1605	64
ATC 49	59	ATC 1759	69
ATC 104	84	ATC 1791	54
ATC 377	139	ATC 1862	58
ATC 385	64	ATC 2201	41
ATC 514	71	ATC 2504	69
ATC 550	117	ATC 2549	76
ATC 872	130	ATC 2649	50
ATC 947	75	ATC 2702	87
ATC 968	68	ATC 2710	96
ATC 1026	56	ATC 3095	100
ATC 1036	87	ATC 3198	71
ATC 1039	62	ATC 3355	63
ATC 1040	48	ATC 3362	99
ATC 1211	97	ATC 3387	67
ATC 1263	73	ATC 3429	72
ATC 1436	66	ATC 3489	58
ATC 1498	68	ATC 3754	66
ATC 1502	78	ATC 3755	59
ATC 1510	91	ATC 3975	59
ATC 1517	77	ATC 3976	87
ATC 1541	72	ATC 3977	75
ATC 1564	52	ATC 3979	79

Appendix 1 continued.

Accession	Days to flower	Accession	Days to flower
ATC 3980	92	ATC 4258	77
ATC 3984	84	ATC 4259	96
ATC 3987	56	ATC 4262	80
ATC 3988	87	ATC 4263	80
ATC 3989	91	ATC 4388	65
ATC 3991	78	ATC 4471	68
ATC 3992	91	ATC 4472	87
ATC 4035	57	ATC 4519	72
ATC 4197	56	ATC 4542	56
ATC 4199	56	ATC 4557	73
ATC 4201	64	ATC 4906	55
ATC 4202	66	ATC 5744	57
ATC 4203	87	ATC 5745	66
ATC 4204	86	Pelikan-1	46
ATC 4206	91	Ps-05-01	77
ATC 4210	68	Mukta	71
ATC 4223	63	Nepal	75
ATC 4233	82	Kaspa	67
ATC 4257	48		

Appendix 2: The analysis of variance showing effect of reproductive timing (days from flowering to the frost exposure) on the frost survival of flowering stage organs (bud, flower and set pod) across 83 accessions of field pea (*Pisum sativum* L.)

Source	Degree of freedom	Sum of squares	Mean square	F	P
Rep. timing	36	8257.3	229.4	1.01^{NS}	0.465
Residual	86	19455.2	226.2		
Total	122	27712.5			

^{NS}= non-significant

Appendix 3: The analysis of variance showing effect of reproductive timing (days from flowering to the frost exposure) on the regrowth ability in plants after frost exposure across 83 accessions of field pea (*Pisum sativum* L.)

Source	Degree of freedom	Sum of squares	Mean square	F	P
Rep. timing	38	9.1328	0.2403	1.01^{NS}	0.472
Residual	93	22.1627	0.2383		
Total	131	31.2955			

^{NS}= non-significant

Appendix 4. Analysis of variance for the difference between two field pea (*Pisum sativum* L.) genotypes and two temperature treatments: control (20 ^{o}C) and cold (10/5 ^{o}C), for symptoms of frost injuries on leaves, and the 100 seed weight of seeds from plants exposed to subsequent frost after the pre-cold treatment of 7 and 14 days.

Sources of variation	**Frost symptoms on leaves**				**100 seed weight of seeds from frost-treated plants**			
	F value	*P*	F value	*P*	F value	*P*	F value	*P*
	(7- day cold treatment)		(14- day cold treatment)		(7- day cold treatment)		(14- day cold treatment)	
Genotype	6.05*	0.017	3.59	0.064	11.45*	0.003	0.01	0.915
Treatment (cold - no cold)	7.15*	0.010	3.06	0.086	0.66	0.425	0.29	0.596
Genotype x Treatment	6.38*	0.014	0.61	0.440	0.10	0.758	0.63	0.436

*indicates significant variation at $P < 0.05$ for respective source

Appendix 5. Observed variation in survival of flowering stage (FS) organs (buds, flowers and set pods) and pod development stage (PDS) organs (flat, swollen, filled and mature pods) against frost stress attributable to differences among two field pea genotypes (Kaspa and ATC 968), two temperatures prior to frost treatment (no-cold: 20 ºC and cold: 10/5 ºC)

Source of variation	Frost survival with 7- day pre-cold treatment				Frost survival with 14- day pre-cold treatment			
	Flowering stage		Pod development stage		Flowering stage		Pod development stage	
	F value	*P*	F value	*P*	F value	*P*	F value	*P*
Fixed effects								
Genotype	2.53	0.123	11.67*	0.002	6.43*	0.018	2.11	0.160
Treatment (cold – no-cold)	0.81	0.375	1.21	0.280	0.39	0.536	2.13	0.157

Results of REML analysis are shown here and *indicates significant variation at $P < 0.05$ for respective source

Appendix 6. Analysis of variance for chlorophyll content, rate of photosynthesis, leaf thickness and chlorophyll fluorescence parameters (efficiency of light harvesting, Fv'/Fm'; quantum yield of PSII electron transport, Φ_{PSII}; photochemical quenching, qP; non-photochemical quenching, NPQ) in two field pea (*Pisum sativum* L.) genotypes subjected to cold treatment for *7 days* and subsequent frost exposure.

Sources of variation	Cold vs no-cold[a]			Frost vs no-frost[b]			Frost vs cold-frost[c]		
	d.f	F value	*P*	d.f	F value	*P*	d.f	F value	*P*
Chlorophyll content									
Genotype	1	3.41	0.076	1	29.24*	<0.001	1	7.88*	0.010
Treatment	1	14.52*	<0.001	1	1.88	0.189	1	0.96	0.336
Genotype x Treatment	1	1.34	0.258	1	0.71	0.411	1	2.22	0.149
Residual	26			16			24		
Rate of Photosynthesis									
Genotype	1	0.20	0.656	1	4.65*	0.048	1	3.14	0.089
Treatment	1	41.27*	<0.001	1	110.81*	<0.001	1	3.58	0.07
Genotype x Treatment	1	7.51*	0.011	1	2.46	0.137	1	1.50	0.233
Residual	26			15			24		

For [a]cold vs no-cold comparison, control (no-cold) and cold-treated plants were compared at sampling time 2, and for [b]frost vs no-frost comparison, control (no-cold, no-frost) and frost-treated plants were compared at sampling time 3, and for [c]frost vs cold-frost comparison, frost treated and cold-frost treated plants were compared at sampling time 3 as indicated in Fig 7.2. *indicates significant variation at $P < 0.05$ for respective source

Appendix 6. continued.

Sources of variation	Cold vs no-cold[a]			Frost vs no-frost[b]			Frost vs cold-frost[c]		
	d.f	F value	*P*	d.f	F value	*P*	d.f	F value	*P*
Leaf thickness									
Genotype	1	25.81*	<0.001	1	0.72	0.411	1	1.28	0.269
Treatment	1	12.44*	0.001	1	10.35*	0.006	1	0.02	0.893
Genotype x Treatment	1	0.34	0.566	1	0.36	0.558	1	9.48*	0.005
Residual	33			14			24		
***Efficiency of light harvesting* (Fv'/Fm')**									
Genotype	1	0.74	0.397	1	4.53*	0.049	1	10.29*	0.004
Treatment	1	1.77	0.194	1	35.40*	<0.001	1	1.74	0.199
Genotype x Treatment	1	0.13	0.724	1	0.00	0.966	1	0.39	0.538
Residual	28			16			25		
***Quantum yield of PSII electron transport* (Φ_{PSII})**									
Genotype	1	8.04*	0.008	1	3.87	0.067	1	10.82*	0.003
Treatment	1	13.38*	0.001	1	38.42*	<0.001	1	1.70	0.204
Genotype x Treatment	1	0.08	0.784	1	0.01	0.905	1	0.73	0.402
Residual	28			16			25		

For [a]cold vs no-cold comparison, control (no-cold) and cold-treated plants were compared at sampling time 2, and for [b]frost vs no-frost comparison, control (no-cold, no-frost) and frost-treated plants were compared at sampling time 3, and for [c]frost vs cold-frost comparison, frost treated and cold-frost treated plants were compared at sampling time 3 as indicated in Fig 7.2. *indicates significant variation at $P < 0.05$ for respective source

Appendix 6. continued.

Sources of variation	Cold vs no-cold[a]			Frost vs no-frost[b]			Frost vs cold-frost[c]		
	d.f	F value	*P*	d.f	F value	*P*	d.f	F value	*P*
***Photochemical quenching* (qP)**									
Genotype	1	7.42*	0.011	1	4.49*	0.050	1	12.99*	0.001
Treatment	1	10.91*	0.003	1	20.17*	<0.001	1	1.14	0.295
Genotype x Treatment	1	0.00	0.969	1	0.25	0.624	1	0.29	0.596
Residual	28			16			25		
***Non-photochemical quenching* (NPQ)**									
Genotype	1	7.35*	0.011	1	3.79	0.069	1	7.81*	0.010
Treatment	1	12.06*	0.002	1	60.75*	<0.001	1	2.15	0.155
Genotype x Treatment	1	0.00	0.973	1	0.95	0.345	1	1.06	0.313
Residual	27			16			25		

For [a]cold vs no-cold comparison, control (no-cold) and cold-treated plants were compared at sampling time 2, and for [b]frost vs no-frost comparison, control (no-cold, no-frost) and frost-treated plants were compared at sampling time 3, and for [c]frost vs cold-frost comparison, frost treated and cold-frost treated plants were compared at sampling time 3 as indicated in Fig 7.2. *indicates significant variation at $P < 0.05$ for respective source

Appendix 7. Analysis of variance for chlorophyll content, rate of photosynthesis, leaf thickness and chlorophyll fluorescence parameters (efficiency of light harvesting, Fv'/Fm'; quantum yield of PSII electron transport, Φ_{PSII}; photochemical quenching, qP; non-photochemical quenching, NPQ) in two field pea (*Pisum sativum* L.) genotypes subjected to cold treatment for *14 days* and subsequent frost exposure.

Sources of variation	Cold vs no-cold[a]			Frost vs no-frost[b]			Frost vs cold-frost[c]		
	d.f	F value	*P*	d.f	F value	*P*	d.f	F value	*P*
Chlorophyll content									
Genotype	1	17.79*	<0.001	1	9.92*	0.012	1	7.45*	0.014
Treatment	1	0.49	0.493	1	39.91*	<0.001	1	33.44*	<0.001
Genotype x Treatment	1	17.77*	<0.001	1	0.01	0.907	1	1.04	0.323
Residual	22			9			17		
Rate of Photosynthesis									
Genotype	1	7.17*	0.013	1	1.47	0.265	1	2.90	0.109
Treatment	1	748.10*	<0.001	1	22.47*	0.002	1	2.76	0.117
Genotype x Treatment	1	0.22	0.64	1	0.94	0.365	1	0.75	0.402
Residual	23			7			15		

For [a]cold vs no-cold comparison, control (no-cold) and cold-treated plants were compared at sampling time 2, and for [b]frost vs no-frost comparison, control (no-cold, no-frost) and frost-treated plants were compared at sampling time 3, and for [c]frost vs cold-frost comparison, frost treated and cold-frost treated plants were compared at sampling time 3 as indicated in Fig 7.2. *indicates significant variation at $P < 0.05$ for respective source

Appendix 7. continued.

Sources of variation	Cold vs no-cold[a]			Frost vs no-frost[b]			Frost vs cold-frost[c]		
	d.f	F value	*P*	d.f	F value	*P*	d.f	F value	*P*
Leaf thickness									
Genotype	1	13.38*	0.001	1	14.71*	0.006	1	6.46*	0.022
Treatment	1	0.01	0.923	1	18.84*	0.003	1	0.31	0.587
Genotype x Treatment	1	0.54	0.471	1	0.02	0.887	1	1.85	0.192
Residual	25			7			16		
***Efficiency of light harvesting* (Fv'/Fm')**									
Genotype	1	1.61	0.217	1	0.78	0.406	1	0.14	0.713
Treatment	1	10.33*	0.004	1	19.32*	0.003	1	1.25	0.281
Genotype x Treatment	1	0.30	0.588	1	0.82	0.395	1	0.01	0.929
Residual	22			7			15		
***Quantum yield of PSII electron transport* (Φ_{PSII})**									
Genotype	1	0.54	0.468	1	0.82	0.388	1	0.53	0.478
Treatment	1	16.54*	<0.001	1	27.42*	<0.001	1	1.28	0.273
Genotype x Treatment	1	0.17	0.682	1	1.05	0.332	1	0.07	0.800
Residual	22			9			17		

For [a]cold vs no-cold comparison, control (no-cold) and cold-treated plants were compared at sampling time 2, and for [b]frost vs no-frost comparison, control (no-cold, no-frost) and frost-treated plants were compared at sampling time 3, and for [c]frost vs cold-frost comparison, frost treated and cold-frost treated plants were compared at sampling time 3 as indicated in Fig 7.2. *indicates significant variation at $P < 0.05$ for respective source

Appendix 7. continued.

Sources of variation	Cold vs no-cold[a]			Frost vs no-frost[b]			Frost vs cold-frost[c]		
	d.f	F value	*P*	d.f	F value	*P*	d.f	F value	*P*
***Photochemical quenching* (qP)**									
Genotype	1	0.16	0.692	1	1.39	0.269	1	0.56	0.464
Treatment	1	12.36*	0.002	1	26.13*	<0.001	1	0.06	0.804
Genotype x Treatment	1	0.21	0.655	1	0.13	0.732	1	0.19	0.669
Residual	22			9			17		
***Non-photochemical quenching* (NPQ)**									
Genotype	1	1.25	0.275	1	1.09	0.324	1	0.28	0.603
Treatment	1	23.57*	<0.001	1	26.41*	<0.001	1	1.67	0.213
Genotype x Treatment	1	0.01	0.936	1	1.61	0.236	1	0.05	0.828
Residual	22			9			17		

For [a]cold vs no-cold comparison, control (no-cold) and cold-treated plants were compared at sampling time 2, and for [b]frost vs no-frost comparison, control (no-cold, no-frost) and frost-treated plants were compared at sampling time 3, and for [c]frost vs cold-frost comparison, frost treated and cold-frost treated plants were compared at sampling time 3 as indicated in Fig 7.2. *indicates significant variation at $P < 0.05$ for respective source

Appendix 8. Analysis of variance for water content and glucose and sucrose concentrations in leaf, flower, pod and seed, in two field pea (*Pisum sativum* L.) genotypes subjected to cold treatment for *7 days* and subsequent frost exposure.

	Sources of variation	Cold vs no-cold[a]			Frost vs no-frost[b]			Frost vs cold-frost[c]		
		d.f	F value	*P*	d.f	F value	*P*	d.f	F value	*P*
Water content										
	[#]Leaf									
	Genotype	1	0.37	0.556	1	4.27	0.061	1	8.30[*]	0.014
	Treatment	1	0.47	0.510	1	21.06[*]	<0.001	1	7.41[*]	0.019
	Genotype x Treatment	1	1.61	0.233	1	12.88[*]	0.004	1	7.00[*]	0.021
	Residual	10			12			12		
	Flower									
	Genotype	1	0.24	0.649	1	0.04	0.847	1	0.25	0.644
	Treatment	1	1.10	0.354	1	101.03[*]	<0.001	1	1.46	0.293
	Genotype x Treatment	1	0.03	0.874	1	0.23	0.656	1	0.61	0.477
	Residual	4			4			4		

For [a]cold vs no-cold comparison, control (no-cold) and cold-treated plants were compared at sampling time 2, and for [b]frost vs no-frost comparison, control (no-cold, no-frost) and frost-treated plants were compared at sampling time 3, and for [c]frost vs cold-frost comparison, frost treated and cold-frost treated plants were compared at sampling time 3 as indicated in Fig 7.2. [*]indicates significant variation at $P < 0.05$ for respective source**,** [#] indicates that relative water content were measured for leaf

Appendix 8. continued.

	Sources of variation	Cold vs no-cold[a]			Frost vs no-frost[b]			Frost vs cold-frost[c]		
		d.f	F value	*P*	d.f	F value	*P*	d.f	F value	*P*
	Pod									
	Genotype	1	10.77*	0.007	1	1.09	0.318	1	0.28	0.608
	Treatment	1	6.55*	0.027	1	26.79*	<0.001	1	0.90	0.363
	Genotype x Treatment	1	1.52	0.244	1	0.04	0.845	1	1.15	0.306
	Residual	11			11			11		
	Seed									
	Genotype	1	19.83*	<0.001	1	7.36*	0.020	1	12.43*	0.005
	Treatment	1	11.80*	0.006	1	23.93*	<0.001	1	7.33*	0.020
	Genotype x Treatment	1	0.11	0.750	1	1.79	0.208	1	0.00	0.977
	Residual	11			11			11		
Glucose content										
	Leaf									
	Genotype	1	1.20	0.294	1	1.10	0.316	1	0.60	0.453
	Treatment	1	3.87	0.073	1	1.56	0.236	1	0.49	0.497
	Genotype x Treatment	1	0.22	0.651	1	0.29	0.597	1	0.65	0.434
	Residual	12			12			12		

For [a]cold vs no-cold comparison, control (no-cold) and cold-treated plants were compared at sampling time 2, and for [b]frost vs no-frost comparison, control (no-cold, no-frost) and frost-treated plants were compared at sampling time 3, and for [c]frost vs cold-frost comparison, frost treated and cold-frost treated plants were compared at sampling time 3 as indicated in Fig 7.2. *indicates significant variation at $P < 0.05$ for respective source

Appendix 8. continued.

Sources of variation	Cold vs no-cold[a]			Frost vs no-frost[b]			Frost vs cold-frost[c]		
	d.f	F value	*P*	d.f	F value	*P*	d.f	F value	*P*
Flower									
Genotype	1	2.22	0.167	1	3.99	0.064	-	-	-
Treatment	1	7.03*	0.024	1	0.47	0.505	-	-	-
Genotype x Treatment	1	0.18	0.683	1	0.00	0.990	-	-	-
Residual	10			15					
Pod									
Genotype	1	0.84	0.379	1	9.51*	0.009	1	12.26*	0.004
Treatment	1	0.58	0.463	1	16.46*	0.002	1	4.77*	0.050
Genotype x Treatment	1	0.27	0.615	1	0.01	0.943	1	1.29	0.278
Residual	11			12			12		
Seed									
Genotype	-	-	-	1	5.63*	0.033	1	14.45*	0.003
Treatment	-	-	-	1	17.50*	<0.001	1	1.07	0.325
Genotype x Treatment	-	-	-	1	8.98*	0.010	1	0.00	0.970
Residual				14			10		

For [a]cold vs no-cold comparison, control (no-cold) and cold-treated plants were compared at sampling time 2, and for [b]frost vs no-frost comparison, control (no-cold, no-frost) and frost-treated plants were compared at sampling time 3, and for [c]frost vs cold-frost comparison, frost treated and cold-frost treated plants were compared at sampling time 3 as indicated in Fig 7.2. *indicates significant variation at $P < 0.05$ for respective source, –indicates no analysis due to lack of replications.

Appendix 8. continued.

	Sources of variation	Cold vs no-cold[a]			Frost vs no-frost[b]			Frost vs cold-frost[c]		
		d.f	F value	*P*	d.f	F value	*P*	d.f	F value	*P*
Sucrose content										
	Leaf									
	Genotype	1	0.08	0.784	1	2.44	0.144	1	1.52	0.242
	Treatment	1	15.47*	0.002	1	10.53*	0.007	1	1.97	0.186
	Genotype x Treatment	1	0.07	0.794	1	0.05	0.834	1	0.06	0.809
	Residual	12			12			12		
	Flower									
	Genotype	1	6.57*	0.028	-	-	-	-	-	-
	Treatment	1	1.24	0.292	-	-	-	-	-	-
	Genotype x Treatment	1	0.76	0.404	-	-	-	-	-	-
	Residual	10								
	Pod									
	Genotype	1	14.77*	0.003	1	72.78*	<0.001	1	410.80*	<0.001
	Treatment	1	1.35	0.270	1	41.98*	<0.001	1	0.10	0.761
	Genotype x Treatment	1	1.62	0.229	1	14.19*	0.003	1	0.37	0.556
	Residual	11			12			12		

For [a]cold vs no-cold comparison, control (no-cold) and cold-treated plants were compared at sampling time 2, and for [b]frost vs no-frost comparison, control (no-cold, no-frost) and frost-treated plants were compared at sampling time 3, and for [c]frost vs cold-frost comparison, frost treated and cold-frost treated plants were compared at sampling time 3 as indicated in Fig 7.2. *indicates significant variation at $P < 0.05$ for respective source, –indicates no analysis due to lack of replications.

Appendix 8. continued.

Sources of variation	Cold vs no-cold[a]			Frost vs no-frost[b]			Frost vs cold-frost[c]		
	d.f	F value	*P*	d.f	F value	*P*	d.f	F value	*P*
Seed									
Genotype	-	-	-	1	7.47*	0.016	1	4.74*	0.054
Treatment	-	-	-	1	21.87*	<0.001	1	18.61*	0.002
Genotype x Treatment	-	-	-	1	2.16	0.163	1	1.26	0.288
Residual				14			10		

For [a]cold vs no-cold comparison, control (no-cold) and cold-treated plants were compared at sampling time 2, and for [b]frost vs no-frost comparison, control (no-cold, no-frost) and frost-treated plants were compared at sampling time 3, and for [c]frost vs cold-frost comparison, frost treated and cold-frost treated plants were compared at sampling time 3 as indicated in Fig 7.2. *indicates significant variation at $P < 0.05$ for respective source, –indicates no analysis due to lack of replications.

Appendix 9. Analysis of variance for water content and glucose and sucrose concentrations in leaf, flower, pod and seed, in two field pea (*Pisum sativum* L.) genotypes subjected to cold treatment for *14 days* and subsequent frost exposure.

	Sources of variation	Cold vs no-cold			Frost vs no-frost			Frost vs cold-frost		
		d.f	F value	*P*	d.f	F value	*P*	d.f	F value	*P*
Water content										
	[#]Leaf									
	Genotype	1	0.00	0.980	1	0.01	0.935	1	0.27	0.614
	Treatment	1	0.74	0.407	1	1.20	0.310	1	0.72	0.417
	Genotype x Treatment	1	0.01	0.914	1	0.36	0.565	1	0.01	0.908
	Residual	11			7			10		
	Flower									
	Genotype	1	3.47	0.092	1	102.99[*]	<0.001	1	0.95	0.362
	Treatment	1	1.10	0.320	1	23.29[*]	0.003	1	0.62	0.456
	Genotype x Treatment	1	1.55	0.241	1	101.74[*]	<0.001	1	0.82	0.395
	Residual	10			6			7		

For [a]cold vs no-cold comparison, control (no-cold) and cold-treated plants were compared at sampling time 2, and for [b]frost vs no-frost comparison, control (no-cold, no-frost) and frost-treated plants were compared at sampling time 3, and for [c]frost vs cold-frost comparison, frost treated and cold-frost treated plants were compared at sampling time 3 as indicated in Fig 7.2. [*]indicates significant variation at $P < 0.05$ for respective source, [#] indicates that relative water content were measured for leaf

Appendix 9. continued.

	Sources of variation	Cold vs no-cold[a]			Frost vs no-frost[b]			Frost vs cold-frost[c]		
		d.f	F value	*P*	d.f	F value	*P*	d.f	F value	*P*
	Pod									
	Genotype	1	1.37	0.262	1	0.46	0.517	1	0.23	0.645
	Treatment	1	3.57	0.081	1	16.34*	0.005	1	6.75*	0.029
	Genotype x Treatment	1	3.07	0.103	1	0.07	0.801	1	0.00	1.000
	Residual	13			7			9		
	Seed									
	Genotype	1	5.96*	0.030	1	5.33*	0.054	1	0.05	0.823
	Treatment	1	5.04*	0.043	1	16.65*	0.005	1	4.95*	0.053
	Genotype x Treatment	1	4.18	0.062	1	0.43	0.532	1	2.78	0.130
	Residual	13			7			9		
Glucose content										
	Leaf									
	Genotype	1	1.10	0.326	1	1.54	0.270	1	0.00	0.982
	Treatment	1	1.05	0.335	1	2.00	0.216	1	0.34	0.574
	Genotype x Treatment	1	0.74	0.414	1	0.88	0.392	1	1.51	0.248
	Residual	8			5			10		

For [a]cold vs no-cold comparison, control (no-cold) and cold-treated plants were compared at sampling time 2, and for [b]frost vs no-frost comparison, control (no-cold, no-frost) and frost-treated plants were compared at sampling time 3, and for [c]frost vs cold-frost comparison, frost treated and cold-frost treated plants were compared at sampling time 3 as indicated in Fig 7.2. *indicates significant variation at $P < 0.05$ for respective source

Appendix 9. continued.

Sources of variation	Cold vs no-cold[a]			Frost vs no-frost[b]			Frost vs cold-frost[c]		
	d.f	F value	*P*	d.f	F value	*P*	d.f	F value	*P*
Flower									
Genotype	1	0.88	0.385	-	-	-	1	1.69	0.250
Treatment	1	0.51	0.501	-	-	-	1	0.63	0.462
Genotype x Treatment	1	0.82	0.401	-	-	-	1	0.27	0.627
Residual	6						5		
Pod									
Genotype	1	0.00	0.963	1	2.57	0.148	1	0.44	0.522
Treatment	1	0.31	0.592	1	0.25	0.633	1	1.30	0.280
Genotype x Treatment	1	3.89	0.080	1	2.27	0.170	1	0.10	0.758
Residual	9			8			10		
Seed									
Genotype	1	1.07	0.327	1	0.30	0.602	1	0.43	0.528
Treatment	1	1.39	0.269	1	0.86	0.384	1	2.92	0.119
Genotype x Treatment	1	0.16	0.700	1	0.75	0.416	1	1.79	0.210
Residual	9			7			10		

For [a]cold vs no-cold comparison, control (no-cold) and cold-treated plants were compared at sampling time 2, and for [b]frost vs no-frost comparison, control (no-cold, no-frost) and frost-treated plants were compared at sampling time 3, and for [c]frost vs cold-frost comparison, frost treated and cold-frost treated plants were compared at sampling time 3 as indicated in Fig 7.2. [*]indicates significant variation at $P < 0.05$ for respective source, –indicates no analysis due to lack of replications.

Appendix 9. continued.

	Sources of variation	Cold vs no-cold[a]			Frost vs no-frost[b]			Frost vs cold-frost[c]		
		d.f	F value	*P*	d.f	F value	*P*	d.f	F value	*P*
Sucrose content										
	Leaf									
	Genotype	1	4.90	0.058	1	1.55	0.268	1	1.70	0.221
	Treatment	1	5.03	0.055	1	0.14	0.725	1	22.46*	<0.001
	Genotype x Treatment	1	4.71	0.062	1	0.27	0.623	1	1.24	0.292
	Residual	8			5			10		
	Flower									
	Genotype	1	3.11	0.128	-	-	-	1	0.16	0.708
	Treatment	1	0.35	0.575	-	-	-	1	0.00	0.955
	Genotype x Treatment	1	1.07	0.341	-	-	-	1	0.65	0.464
	Residual	6						4		
	Pod									
	Genotype	1	26.33*	<0.001	1	0.15	0.711	1	0.24	0.631
	Treatment	1	3.92	0.079	1	1.97	0.198	1	0.00	0.982
	Genotype x Treatment	1	2.29	0.165	1	0.12	0.740	1	1.62	0.232
	Residual	9			8			10		

For [a]cold vs no-cold comparison, control (no-cold) and cold-treated plants were compared at sampling time 2, and for [b]frost vs no-frost comparison, control (no-cold, no-frost) and frost-treated plants were compared at sampling time 3, and for [c]frost vs cold-frost comparison, frost treated and cold-frost treated plants were compared at sampling time 3 as indicated in Fig 7.2. *indicates significant variation at $P < 0.05$ for respective source, –indicates no analysis due to lack of replications.

Appendix 9. continued.

Sources of variation	Cold vs no-cold[a]			Frost vs no-frost[b]			Frost vs cold-frost[c]		
	d.f	F value	*P*	d.f	F value	*P*	d.f	F value	*P*
Seed									
Genotype	1	0.05	0.836	1	0.08	0.784	1	0.94	0.356
Treatment	1	4.79	0.056	1	0.03	0.861	1	3.35	0.097
Genotype x Treatment	1	0.91	0.366	1	1.13	0.323	1	0.38	0.552
Residual	9			7			10		

For [a]cold vs no-cold comparison, control (no-cold) and cold-treated plants were compared at sampling time 2, and for [b]frost vs no-frost comparison, control (no-cold, no-frost) and frost-treated plants were compared at sampling time 3, and for [c]frost vs cold-frost comparison, frost treated and cold-frost treated plants were compared at sampling time 3 as indicated in Fig 7.2. [*]indicates significant variation at $P < 0.05$ for respective source, –indicates no analysis due to lack of replication