BIOCHEMICAL AND ULTRASTRUCTURAL CHANGES ASSOCIATED WITH CHILLING INJURY IN SOYBEAN SEEDS DURING IMBIBITION

BY

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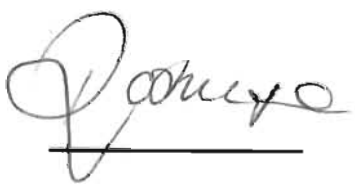
" Deep within the seed is a mysterious source of life- of a plant,

of more seeds, and of more plants and more seeds"

COPELAND(1976)

PREFACE

The experimental work described in this thesis was conducted in the Department of Botany, University of Natal, Pietermaritzburg, under the supervision of Dr M T Smith. These studies are the result of my own investigations except where the work of others is acknowledged.



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ABSTRACT

Biochemical and ultrastructural changes associated with chilling injury (CI) in soybean seeds imbibed at 5°C and 25°C were investigated. Soybean seed germination appeared to be affected by chilling temperatures and initial seed moisture content. Seeds with higher moisture contents exhibited 85% germination, while low moisture content seeds had a 32% germination.

Leakage rates were greater in chilled seeds, indicating that membrane integrity in the tissues was impaired at chilling. The low rates of potassium ion leakage between 6 and 24 hours of imbibition compared to the high peroxide levels observed during this period led to the suggestion that lipid peroxidation was a better marker of CI than leakage.

Transient changes in lipid hydroperoxide levels were observed in chilled and non-chilled seeds and axes. However, in axes, the increase in lipid hydroperoxides after 12 hours of imbibition at chilling temperatures was associated with an 18% decline in linoleic acid levels of total lipid fraction. Similarly, a 10% decline was observed in the polar lipid fraction. These results suggest that the capacity of seeds to control lipid peroxidation may be an important component in CI and that a consequence of peroxidation is likely to be a loss of fatty acid unsaturation.

Sugar levels were not affected by chilling and non-chilling temperatures and no relationship could be established with CI.

Antioxidant defense enzymes (catalase and superoxide dismutase) were expressed at chilling and non-chilling temperatures and increases were observed after 24 hours

of imbibition which showed an apparent correlation with increases to lipid hydroperoxide levels. Enzyme levels decreased after 48 hours of imbibition at a time which coincided with the decline observed in the peroxide levels. Overall, no marked differences were observed in chilled and non-chilled cells at the ultrastructural level, except that vacuolar reserve mobilization was markedly impeded.

LIST OF ABBREVIATIONS

APS	ammonium persulfate
ATP	adenosine triphosphate
$BaCl_2$	barium chloride
BHT	butylated hydroxytoluene
CI	chilling injury
CsCl	cesium chloride
Cu/Zn	copper/zinc
^oC	degrees celsius
DNA	deoxyribonucleic acid
DTT	dithiothrietol
EDTA	ethylenediamine tetra acetic acid
Fe	iron
g	grams per litre
g/ml	grams per millilitre
HCl	hydrochloric acid
HNO_3	nitric acid
H_2O_2	hydrogen peroxide
KCN	potassium cyanide
KFeCN	potassium ferricyanide
KH_2PO_4	potassium dihydrogen phosphate
KSCN	potassium thiocyanate
kV	kilovolts
$MgCl_2$	magnesium chloride
M	molarity
mA	milli-ampere

mg/ml	milligram per litre
ml	millilitre
ml/l	millilitre per litre
mM	millimolar
Mn	manganese
$NaCO_3$	sodium carbonate
NADP	nicotinamide adenine dinucleotide phosphate
Na_2HPO_4	disodium hydrogen phosphate
NBT	nitroblue tetrazolium
nm	nanometre
PEG	polyethyleneglycol
ppm	parts per million
PVPP	polyvinylpolypyrrolidone
rpm	revolutions per minute
SODs	superoxide dismutases
TCA	tricarboxylic acid
TEMED	N,N,N,N-tetramethyl ethylenediamine
TMAH	tetra-methylammonium hydroxide
TRIS	tris-(hydroxymethy) methylamine
µl	microlitre
v/v	volume per volume
V	voltage
w/v	weight per volume
%	percentage

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CHAPTER I

INTRODUCTION

The problem of chilling injury (CI) is of major agricultural importance since a large number of plants of economic importance are reported to be susceptible under field conditions. Low soil temperatures can lead to poor seedling establishment and reduced productivity. Plants can be divided into chilling-tolerant and chilling-sensitive species depending on their susceptibility to CI when exposed to temperatures between 0^{o}C and 10- 15^{o}C. Susceptibility is related to the place of origin of the crops, with tropical or subtropical crops being chilling-sensitive and those of temperate origin being chilling-resistant.

Several theories have been advanced to account for CI in vegetative tissues and seeds, but evidence for a primary determinant is lacking. LYONS (1973) hypothesized that the primary cause of CI was due to the physical response of membrane lipids to low temperature, resulting in a phase change in the membrane lipids of chilling-sensitive species.

Patterns of electrolyte leakage from chilled imbibing seeds have led to suggestions that membrane reorganization is impaired at low temperatures and is the primary cause of injury. Biochemical evidence for the leakage of cellular material from chilled seeds has been provided by several studies (POLLOCK and TOOLE,1966; BRAMLAGE, LEOPOLD and PARRISH,1978 ; LEOPOLD and

MUSGRAVE,1979).

Lipid peroxidation has been reported to be a common response of vegetative plant tissues subjected to environmental stresses, such as salinity (CHROMINSKI, KHAN, WEBER and SMITH,1986) and freezing (ELSTNER and KONZE,1976). Apart from initiating free radical processes, chilling may lead to a decline in enzymes which limit free radical damage, such as catalase (OMRAN,1980). However, to date, there has been no evidence implicating peroxidation in the CI response of seeds. Data obtained from a study on cotton seedling suggested a relationship between unsaturated fatty acids and chilling resistance (ST.JOHN and CHRISTIANSEN,1976). Likewise, DOGRAS, DILLEY and HERNER (1977) suggested that chilling resistance in seeds was dependent upon their ability to synthesize large amounts of fatty acids during the initial stages of germination.

Soybean seeds (Glycine max) have been shown to be susceptible to damage at low imbibitional temperatures. Injury was expressed as reduced respiratory rates (LEOPOLD and MUSGRAVE,1979) and faulty membrane reorganization (BRAMLAGE, LEOPOLD and PARRISH,1978) reflected by enhanced leakage patterns, amongst others. The following literature review presents current theories and research reports concerning injury which occurs in vegetative tissues and seeds of chilling-sensitive species at low, but non-freezing temperatures.

CHAPTER 2

LITERATURE REVIEW

2.1 CHILLING INJURY IN PLANTS

Chilling injury (CI) is a physiological defect of plants and their products that results in a reduction in product quality following exposure to low, but non-freezing temperatures (PARKIN, MARANGONI, JACKMAN, YADA and STANLEY,1988). Susceptibility to chilling temperatures also limits geographic distribution and affects the growth season and storage conditions of many crop plants and their products.

The following discussion presents current thoughts concerning the injury which occurs to plant vegetative tissues and seeds at low, but non-freezing temperatures. The theories advanced to account for the nature of CI are presumed to be similar in vegetative tissues and seeds, but in this review, vegetative tissues and seeds will be discussed separately. Although research has been conducted on both, more literature is available on vegetative tissues.

2.1.1 History of Chilling Injury

The term chilling injury (CI) was first used by MOLISCH (1897). Several studies were cited to demonstrate that a number of plant species were killed by low temperatures, above the freezing point, and it was suggested that this physiological harm should be referred to as CI to differentiate it from freezing injury. Since then, several theories have been advanced to account for the basis of CI in plant vegetative tissues and seeds, but support for a primary determinant is lacking.

The overall process of CI may be considered in two stages, namely, a primary event which might be a change in membrane lipid structure (RAISON,1974), a conformational change in some regulatory enzyme or structural protein (GRAHAM and PATTERSON,1982) followed by a series of secondary events which can include metabolic and ionic imbalances, loss of cellular integrity and later visible symptoms (RAISON and LYONS,1986). It was reported that primary events occur at some critical or threshold temperature which correlates with the onset of CI and are reversible. Secondary events are temperature- and time-dependent and prolonging of the stress makes the process irreversible (LYONS and RAISON,1986).

2.2 MECHANISM OF CHILLING INJURY

A number of mechanisms have been put forward to accomodate the physiological and biochemical changes associated with CI.

It has frequently been shown that cellular membranes in sensitive plants undergo a physical phase-transition from a normal flexible liquid to a solid gel structure at chilling temperatures (LYONS,1973), resulting in increased permeability and leading to ion leakage. The phase-transition also increases the activation energy of membrane-bound enzyme systems, establishing an imbalance with non membrane-bound enzyme systems. As the temperature is decreased, the rate of reaction of the soluble enzyme systems such as glycolysis will decrease. Similarly, the rate of reaction of the membrane-associated enzymes of mitochondrial respiration will decrease. This would lead to a major imbalance in the two systems leading to an accumulation of metabolites such as pyruvate, acetaldehyde and ethanol at the interface between glycolysis and respiration. Ultimately, the plant capitulates to physiological dysfunction and death due to the inability to withstand increasing concentrations of these metabolites (Figure 1).

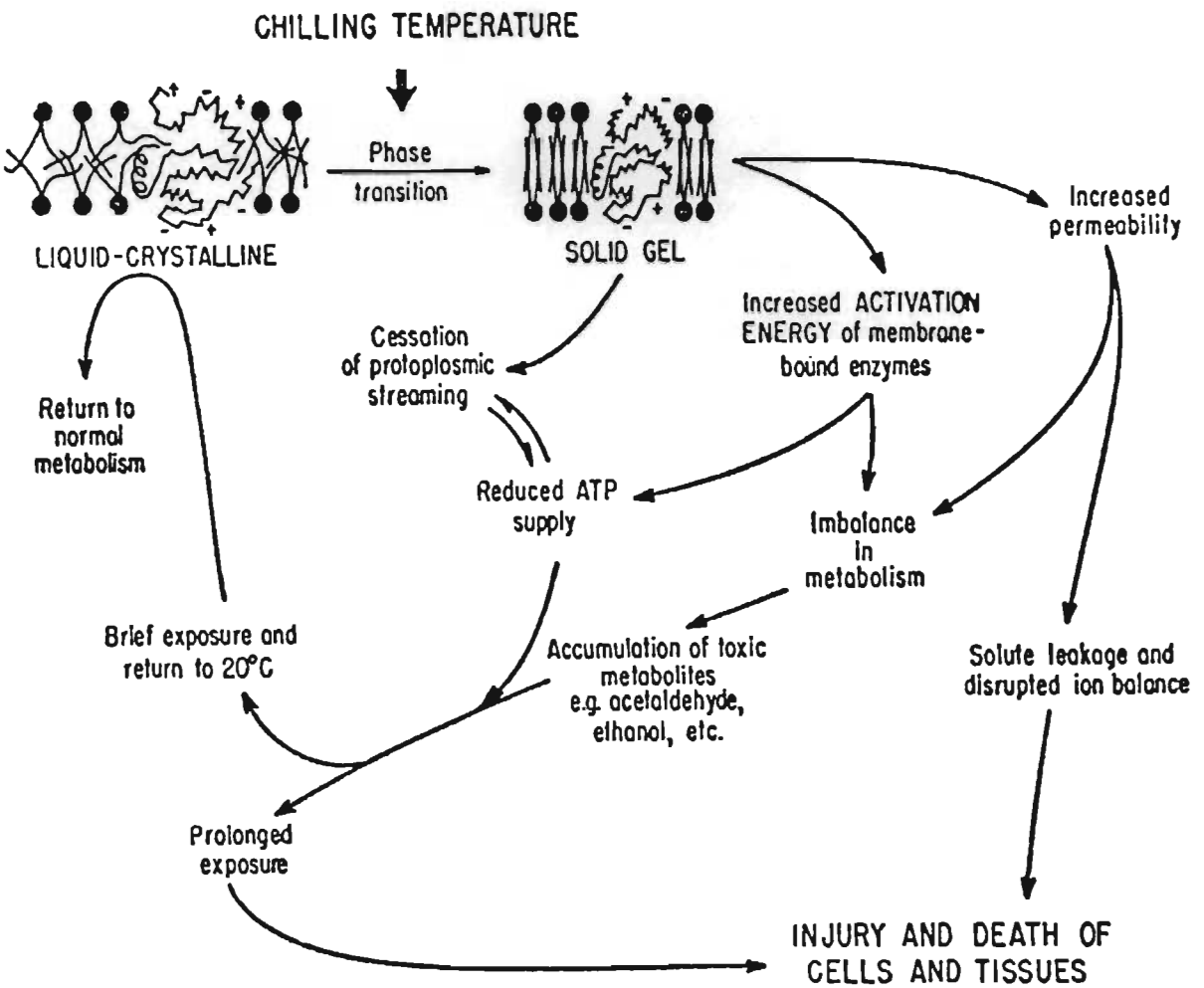


Figure 1 Schematic pathway of some of the events associated with chilling injury in sensitive plant tissues. After : LYONS (1973).

2.3 THEORIES OF CHILLING INJURY

Several theories have been advanced to account for the nature of CI, such as bulk membrane lipid phase transitions (LYONS,1973), unfavourable low temperature effects on proteins and enzymes (GRAHAM and PATTERSON,1982) and a redistribution of cellular calcium (MINORSKY,1985), just to mention a few.

2.3.1 Membrane Phase Transitions

The membrane lipid phase-transition hypothesis is by far the most widely accepted theory to explain the molecular mechanisms leading to CI. It gained early support because of frequent breakpoints and discontinuities in Arrhenius plots of membrane lipids of chilling-sensitive species.

A part of this evidence is based on electron spin resonance studies by (SHNEYOUR, RAISON and SMILLIE,1973; MURATA and FORK,1975). Using spin-labelled compounds and mitochondria from chilling-resistant and chilling-sensitive species, they showed an alteration of the lipid phase of membranes at temperatures critical to injury in sensitive species only.

Evidence indicating that membranes of sensitive plant species undergo a phase change at critical temperatures, led to the further observation that membrane lipids from chilling-sensitive plants had a higher proportion of saturated to unsaturated fatty acids than chilling-resistant plants.

Chilled chloroplasts also showed correlative changes in activity and membrane

phase changes following a brief exposure to cold.

It has been speculated that chilling impairment of photosynthesis in sensitive plants results from a minor phase transition at physiological temperatures of the thylakoid membrane lipid bilayer, rather than from a transition of the bulk lipid (MURATA and FORK,1975).

Although membrane lipids have extensively been reported to be affected by chilling temperatures, there is uncertainty as to whether the fatty acid composition of membrane lipids or other membrane components determine the physical state in response to low temperatures.

2.3.2 Permeability Changes

Changes in membrane permeability in response to chilling temperatures has often been assessed as a cause of CI. A number of researchers have reported increased membrane leakiness at low temperatures (GUINN,1971; LYONS,1973; TANCZOS,1977). The membrane permeability theory in response to chilling is discussed with respect to seeds in more detail in section 2.10.

2.3.3 The Calcium Hypothesis

It was MINORSKY (1985) who first hypothesized that chill-induced increases in cytosolic free calcium may serve as the primary physiological transducer of CI in plants.

Reduced rates at which chilling-sensitive plants exclude calcium from their cytosol and rapid cooling due to cold shock results in an increase in cytosolic free calcium. This may reflect either an inherent inability of chilling-sensitive plants to maintain homeostatic levels of calcium at chilling temperatures or the fact that increases in calcium represent a stress-induced adaptive response to protect plants against exposures to cold by protecting proteins that would otherwise be denatured due to weakening of hydrophobic interactions (MINORSKY, 1985).

On the other hand, LESHEM, SRIDHARA and THOMPSON (1984) reported that increased cytosolic calcium levels during chilling exposure caused an overstimulation of lipid-degrading enzymes and a loss of membrane integrity which may culminate in cell death.

These conflicting reports have yet to be resolved and consequently this theory has not become widely accepted.

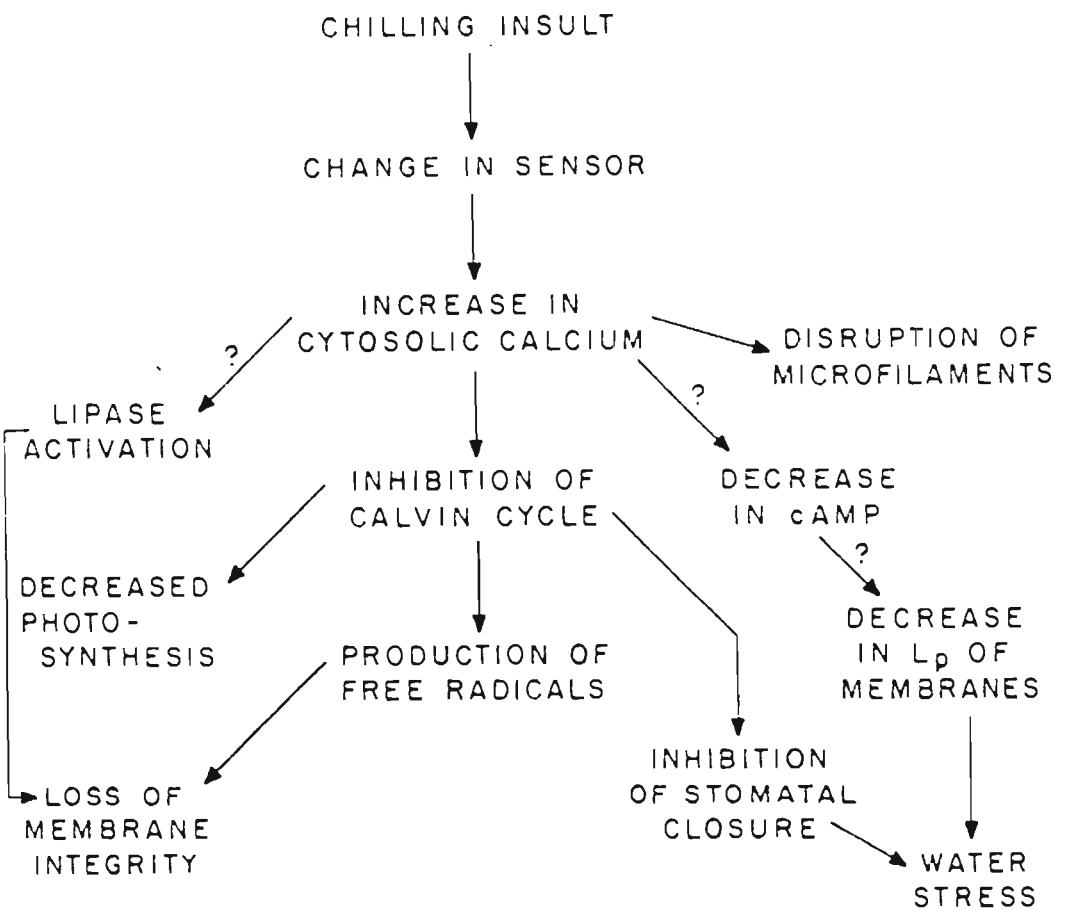


Figure 2 A schematic pathway of the main events leading to CI, as proposed by the Calcium Hypothesis. After : MINORSKY (1985).

2.4 THE EFFECTS OF CHILLING INJURY ON PLANT FUNCTION

Plant structure and function has been reported to be altered by chilling injury and ultrastructural studies have provided evidence for organelle damage.

2.4.1 Mitochondria

The effect of CI on mitochondria has attracted the most interest because this organelle is the site of cellular respiration.

Earlier studies by LIEBERMAN, CRAFT, AUDIA and WILCOX (1958) examined the effects of CI on respiration in mitochondria isolated from sweet potato roots that were stored at 7.5 and 25^{O}C. No differences were observed during the first four weeks of storage, but activity began to decline after five weeks and chilling inactivated the mitochondria completely by the tenth week.

Likewise, MINAMIKAWA, AKAZAWA and URITANI (1961) observed a decrease in oxidative activity at 25^{O}C of mitochondria isolated from sweet potato roots chilled at 0^{O}C . The oxidative and phosphorylative activities of mitochondria from chilled tissues were compared with those from the control and a sharp decline in oxidative activity was noted in chilled tissue after ten days.

Data obtained by LYONS and RAISON (1970) showed that there was no difference in phosphorylative efficiency in either chilling-sensitive and chilling-resistant plant species. They also studied the effect of a wide range of temperatures on

oxidative activity in isolated mitochondria. Arrhenius plots showed that the mitochondria from chilling-resistant plant species exhibited a linear plot with a constant activation energy from 1°C to 25°C. Mitochondria from chilling-sensitive species exhibited a discontinuity in plot at 10°C to 12°C, with a marked increase in activation energy below the break temperature, indicating that a direct effect of low temperature on these species was the suppression of mitochondrial respiration.

2.4.2 Chloroplasts

The effects of chilling on the photosynthetic apparatus of tomato, bean and cucumber has been studied by KANIUGA and MICHALSKI (1978). Their work confirmed that the Hill reaction was inactivated by chilling temperatures due to a release of free fatty acids (linoleic acid) and damage to the thylakoid structure, which ultimately affected the electron transport chain within photosystem II.

Studies by PEOPLES and KOCH (1978) also confirmed the effects of chilling on photosynthetic apparatus by demonstrating a 69% reduction in the carbon dioxide exchange ratio after chilling at 5°C overnight.

Studies on chloroplast structure and function support the theory that the primary response of plants to CI is a phase change in the membrane lipids. For example, electron spin resonance labels of the membrane lipids from chilling-sensitive plants showed a temperature-induced change in molecular ordering of the lipids that corresponded with the critical temperature for membrane-associated

enzymatic photoreduction of NADP (RAISON and CHAPMAN,1976). These changes were not observed in the membranes of chilling-resistant plants.

NOLAN and SMILLIE (1977) compared temperature-induced changes in the Hill activity of chloroplasts isolated from chilling-sensitive and chilling-resistant plants. An increase in activation energy for the Hill reaction activity at low temperatures was observed in chloroplasts isolated from both chilling-resistant and chilling-sensitive plants. However, the temperature at which the change occurred was characteristic for each plant, but did not correlate with chilling sensitivity.

Other changes in plant function during CI, such as membrane permeability and membrane composition will be discussed with respect to seeds in more detail in sections 2.10.2 and 2.10.3.devoted to seed chilling injury.

2.5 BIOCHEMICAL CHANGES ASSOCIATED WITH CHILLING INJURY

2.5.1 Chemical Modification of Lipids

Numerous studies have compared chilling-sensitive and chilling-resistant plant species and shown that chilling-resistant plants have a greater proportion of unsaturated fatty acids in membranes. Also correlated with this is the observation that as the growth temperature decreases, lipid unsaturation increases with the predominant increase being linolenic acid (ST JOHN and CHRISTIANSEN,1976).

The lipid composition of cellular membranes from higher plant cells is diverse. The chloroplast membranes are characterised by a high content of glycolipids and phospholipids, while the mitochondrial and endoplasmic reticulum membranes contain predominantly phospholipids. The plasmalemma and tonoplast membranes are rich in sterols, sphingolipids and phospholipids.

The effect of fatty acid unsaturation on the thermotropic phase behaviour of glycerolipids has been intensively studied in bilayer membranes (SILVIUS,1982). Fatty acids of glycerolipids are unique in having a high degree of unsaturates as represented by linoleic and linolenic acids.

Recently, the phase transition temperatures of a major molecular species of plant sphingolipid, cerebroside, was determined. MURATA and NISHIDI (1990) demonstrated that sphingolipids exist in tonoplast and plasma membranes and suggested that this class of lipids may induce a phase transition in these membranes at chilling temperatures.

Reconstituted proteoliposomes were used by LEE (1977) to investigate the effect of proteins on the thermotropic phase behaviour of membranes, based on lipid-protein interactions and according to PAPAHADJOPOULOS, MOSCARELLO, EYLAR and ISAC (1975), these are classified into three groups. In the first group, an extrinsic protein, such as ribonuclease, binds to the surface of the lipid bilayer through electrostatic forces, thus increasing the enthalpy of the phase transition

of the lipid bilayer. In the second group, an extrinsic protein, such as cytochrome c, binds to the lipid bilayer surface by partial penetration or deformation of the bilayer, thus decreasing the temperature and enthalpy of the lipid phase transition. The third group interaction is caused by an intrinsic protein, such as the apoprotein of myelin, which is non-polar. This type of interaction does not affect the phase transition temperature of the lipid bilayer, but reduces the enthalpy of the phase transition.

2.5.2 Effect of Low Temperature on Membrane Proteins and Enzymes

It has been proposed that changes in enzyme levels and kinetic properties of cold labile proteins could account for the disturbances brought about by low temperature exposure (GRAHAM and PATTERSON,1982.

Protein-protein and protein-lipid interactions may be disturbed at low temperatures by reducing the relative strength of hydrophobic bonding. This may explain the dysfunction of soluble enzymes as a result of subunit dissociation and polypeptide unfolding (PARKIN, MARANGONI, JACKMAN, YADA and STANLEY,1988).

Solidification of membrane lipids at chilling temperatures could inhibit enzyme activity by inducing conformational changes in the protein (WOLFE,1978). Low temperature exposure is also associated with a decrease of certain enzyme activities, such as superoxide dismutase in chilled tomato leaves, and catalase in chilled cucumber seedlings (OMRAN,1980). These studies indicated that

tissues may be incapable of ameliorating oxidative stress at chilling temperatures.

2.5.3 The Role of Sugars in Chilling Injury

An increase in soluble carbohydrate accumulation in plant tissues in response to low temperatures has been reported in several studies (RAESE, WILLIAMS and BILLINGSLEY, 1978 ; PURVIS, KAWADA and GRIERSON,1979). However, few studies have shown a positive correlation between carbohydrate level or composition and resistance to CI.

It has been found that low field and storage temperatures induced invertase activity in grapefruit peel, and caused starch hydrolysis to soluble carbohydrates, although it is uncertain whether reducing sugar accumulation is part of the chilling-resistance mechanism or simply a consequence of chilling stress (PURVIS and RICE,1983).

The primary sugar and the level of soluble carbohydrates responsible for reduced sensitivity of plant tissues to low temperatures have not been established because the mechanism of CI is not fully understood. Although no specific mechanism has been proposed, there is a general agreement that the initial event is a direct effect of low temperature on cellular constituents, leading to the alteration of several biochemical and physiological processes (LYONS,1973).

Soluble carbohydrates may protect cellular constituents of chilling-sensitive plants directly or indirectly and can influence the chilling resistance mechanism

in three ways.

Firstly, carbohydrates can contribute to the osmotic potential of the cell thus decreasing cell water potential and water loss from the tissue (YOUNG and PEYNADO,1965), although it has been shown that osmotic agents such as mannitol and inorganic salts were ineffective in ameliorating CI in cotton cotyledon discs and tomato seedlings (KING, JOYCE and REID,1988).

Secondly, certain carbohydrates are capable of stabilizing cell membranes and enzymes by binding directly to constituted molecules. Sugars are almost universally present in plant tissues (CAFFREY, FONSECA and LEOPOLD,1988) and trehalose, a non-reducing disaccharide can provide membrane protection (CROWE and CROWE,1986) by inhibiting hydration-dependent phase transitions (CROWE, CROWE and JACKSON,1983), but since it is not a common sugar in plant tissues, its function in preserving the intactness of membranes may be performed by other sugars, such as sucrose. Thirdly, carbohydrates serve as a respiratory substrate.

Chilling-sensitive and chilling-resistant plants have been distinguished on the basis of their respiratory response to low temperatures (LYONS,1973 ; GRAHAM and PATTERSON,1982). Sugars have a central role in metabolism and several studies have shown a positive correlation between respiratory rates and usable tissue sugar content (CRAWFORD and HUXTER,1977 ; DAY and LAMBERS,1983 ; BRYCE and ap REES,1985).

2.6 LIPID PEROXIDATION AND ANTIOXIDANT ENZYMES

A widely promoted hypothesis for seed deterioration is lipid peroxidation (SENARATNA, GUSSE and McKERSIE,1988). In the presence of oxygen, fatty acid hydrocarbon chains oxidize and produce highly reactive free radical intermediates called hydroperoxides.

Lipid oxidation has received much attention due to increased awareness of the importance of polyunsaturated lipids in foods and biological systems, and especially since the production of flavours and odours by oxidation have negative effects on food quality.

The main cellular components susceptible to peroxidative damage are : membranes lipids (peroxidation of unsaturated fatty acids in membranes, leading to loss of function) ; proteins (denaturation of enzymes and structural proteins) ; carbohydrates (scission of polysaccharides) and nucleic acids which involves scission of DNA strands : which ultimately leads to mutations (FRANK,1985).

2.6.1 Lipid Peroxidation and Chilling Injury

Lipid peroxidation has been reported to be a common response of plant tissues to environmental stress. This has been noted for plant tissues subjected to low temperature (MACRAE and FERGUSON,1985) , salinity (CHROMINSKI, KHAN, WEBER and SMITH,1986) and water stress during germination (LEPRINCE,

DELTOUR, THORPE, ATHERTON and HENRY, 1990). Although lipid peroxidation has not been examined as a primary response to chilling stress, there are some studies implicating its role in the development of CI. For example, WANG and BAKER (1979) demonstrated that the severity of low temperature injury was delayed or reduced in cucumber and pepper fruits after the application of antioxidants. Chilling also decreased the activity of catalase in cucumber seedlings (OMRAN,1980) indicating that prolonged peroxidative stress may deplete the antioxidant enzyme systems.

2.6.2 Free Radical Processes and Lipid Peroxidation

Although lipid peroxidation chemistry is well established, the involvement of free radicals in the initiation of peroxidation is less certain. In its ground state, molecular oxygen is unreactive, yet capable of producing reactive excited states, such as, free radicals and derivatives. The reactive species of reduced dioxygen include the superoxide radical, hydrogen peroxide and the hydroxyl radical which readily combines with most molecules present in living tissues, including nucleic acids, proteins and lipids (SCANDALIOS,1990).

In the initiation event, the attack by the highly reactive hydroxyl radical, which is the most damaging species of oxygen, results in the formation of a lipid radical which undergoes random rearrangement and attack by molecular oxygen. The oxygen molecule is incorporated into the lipid to form the first of a series of lipid peroxy radicals (HENDRY,1993).

2.6.3 Biophysical Changes in Membranes upon Lipid Peroxidation

Unsaturated lipids are important for the structural and functional integrity of biological membranes and it is for these reasons that lipid oxidation can cause changes in membrane structure and function by inactivation of membrane-bound proteins, including transport proteins and receptors, and changes in membrane permeability. Lipid hydroperoxides can facilitate membrane deterioration by decomposing to ethane and several cytotoxic and unsaturated aldehydes (HENDRY,1993).

Details of changes associated with the physical structure of membranes and alterations to the liquid-crystalline state of membrane lipids following peroxidation are not fully known. In a review, O'BRIEN (1987) reports that the fluidity or viscosity of biological membranes is principally determined by their lipid and antioxidant composition. Several investigators have suggested that some membrane proteins, in order to perform their function of transporting substances into or out of the cell, must be free to rotate within their lipid matrix as well as to move in the plane of the membrane and changes from liquid crystal to the gel state, could inhibit this activity.

At physiological temperatures, the bulk of lipids are in a liquid-crystalline phase in which the lipid hydrocarbon chains are relatively fluid. Formation of the gel phase, during which the hydrocarbon chains lose mobility to form an ordered array, can be induced by lowering the temperatures and is accompanied by a

loss of membrane function.

2.6.4 The Role of Antioxidant Defense Enzymes

Degradative processes in membranes, such as lipid peroxidation, are normal metabolic events occurring in stressed and unstressed tissues. However, plants have evolved a complex antioxidant system to protect cellular membranes and organelles from the damaging effects of toxic activated oxygen species.

WISE and NAYLOR (1987) reported that exposure of tropical and subtropical plant species to low, but non-freezing temperatures and high irradiation results in the depletion of the antioxidant capacity of the chloroplast and an ever-increasing photo-oxidative damage to thylakoid membranes, but biosynthetic defense and repair mechanisms are able to prevent degradative changes and prevent or limit injury in unstressed tissue (PURVIS and SHEWFELT,1993).

Disorders result when either compromised defense or repair mechanisms fail to provide adequate protection, or an increase in degradative reactions exceed the capacity of defense and repair mechanisms to provide adequate protection; or a combination of these processes. Components of quenching mechanisms include the lipid-soluble antioxidants (tocopherol and carotene) , the water-soluble reductants (glutathione and ascorbate) as well as active oxygen scavenging enzymes such as superoxide dismutase, catalase and peroxidase (SCANDALIOS,1993). Tocopherol quenches singlet oxygen and is a free radical trap for terminating lipid peroxidation chain reactions. Carotenoids in the

thylakoid membrane quench excess chlorophyll excitation energy that is not passed to the photosystems (STEFFEN,1991). Ascorbate plays a central role in detoxification of activated oxygen (FOYER, LELANDAIS, EDWARDS and MULLINEAUX,1991). Glutathione can react directly with free radicals or dehydroascorbate to regenerate ascorbate. These antioxidant defense systems are interdependent and ultimately transfer reducing equivalents from photosystem 1 to activated oxygen to form water (WALKER and McKERSIE,1993).

Peroxidases, catalases and superoxide dismutases have been shown to play a major role in protecting cells from oxidative damage.

Catalases and peroxidases are classes of enzymes that remove peroxides very efficiently. Superoxide dismutases comprise a class of metal-combining proteins that are highly efficient at scavenging the superoxide radical. The combined action of catalase and superoxide dismutase converts the potentially toxic superoxide radical and hydrogen peroxide to water and molecular oxygen and in the process, prevents the formation of the hydroxyl radical, thus preventing cellular damage. Since the hydroxyl radical is the most toxic and highly reactive oxidant, its detoxification is more crucial than the elimination of superoxide and hydrogen peroxide. It is for these reasons that superoxide dismutases and catalases are regarded as the most important of the antioxidant enzymes (SCANDALIOS,1990).

The physiological role of superoxide dismutases in plant tissues has been the subject of a number of studies relating environmental stress with protection from oxidative injury. The copper, zinc and manganese superoxide dismutase isozymes have been reported to increase in activity in response to stress (MATTERS and SCANDALIOS,1986). Chilling injury has also been related to the level of superoxide dismutase activity in strains of Chlorella ellipsoidea : the higher the activity, the greater the resistance to stress (CLARE, RABINOWITCH and FRIDOVICH,1984).

Reductions in catalase activity in response to chilling have been found in leaves of a number of chilling-sensitive and insensitive species (OMRAN,1980 ; PATTERSON, MacRAE and FERGUSON,1984) and since the function of catalase is to degrade hydrogen peroxide, its accumulation could be a possible consequence of catalase inactivation.

It can be concluded that antioxidant defense enzymes are very important in the protection against harmful oxidative reactions brought about by various physiological stresses.

2.7 IMBIBITIONAL CHILLING INJURY IN GERMINATING SEEDS

Imbibitional chilling injury is defined as sensitivity to a combination of low seed-water content and imbibition at cold temperature.

Imbibition is an essential primary process during seed germination (McDONALD, VERTUCCI and ROOS,1988). Events associated with water uptake in seeds are not well understood, but the seed hydration curve has been described as triphasic (BEWLEY and BLACK,1983). Phase 1 involves physical hydration of seed tissues (ABU-SHAKRA and CHING,1967). Phase 2 is associated with a reduced rate of imbibition over time, thereafter phase 3 is characterised by germination.

It is well-documented that crops of tropical or subtropical origin have seeds that are severely affected by exposure to low, but non-freezing temperatures resulting in a poor seedling establishment and a reduction in yield.

Seeds are the first to encounter stress during a plant's life cycle (HERNER,1986) and the severity of injury depends on several factors such as (i) the species involved, (ii) the initial water content of the seed, (iii) the temperature to which the seed is exposed, (iv) the duration of chilling exposure and (v) the period during the course of germination when chilling exposure occurs (BEDI and BASRA,1993).

2.7.1 History of Chilling Injury in Seeds

According to CHRISTIANSEN and ST.JOHN (1981) the term "chilling injury" was first introduced in 1897 by MOLISCH to describe the injurious effects of low, but non-freezing temperatures on sensitive plant species, which was discovered fifty years later in germinating seeds. In 1919, KIDD and WEST reported that soaking seeds of beans at 10^{o}C increased the amount of material leached from them and decreased germination compared with seeds soaked at 20^{o}C.

Furthermore, it was noted that the seeds of several warm-season vegetable crops would not germinate below 18^{o}C (KOTOWSKI,1926) and it was thought that reduced temperature germination was a consequence of seed infestation by soil-borne pathogens.

Further studies by HARRINGTON and MINGES (1954) reported that several vegetable crops of tropical origin would not germinate at 10^{o}C, even in the absence of pathogens, thus confirming that low temperature injury was not a consequence of pathogen attack, and assigned the term "chilling injury" to seeds injured during imbibition and germination. Since then, a considerable amount of literature has been published on this aspect.

2.8 MEDIATING FACTORS

Several factors have been implicated in contributing to the response of seeds to low temperature injury during germination and include :

2.8.1 Temperature and Timing

The longer a germinating seed of a sensitive species is exposed to chilling temperatures, the greater injury it will sustain (POLLOCK and TOOLE,1966 ; CHRISTIANSEN,1967 ; BRAMLAGE, LEOPOLD and PARRISH,1978). For example, CHRISTIANSEN (1967) reported that an exposure for 30 minutes at 5°C resulted in a reduced rate of germination in cotton, while hydration for 12 hours at 5°C killed the seed (CHRISTIANSEN,1968). This depends on the amount of time exposed to chilling temperatures before returning to non-chilling temperatures and this aspect is particularly important in areas with fluctuating temperatures.

2.8.2 The initial water content of the seed

The sensitivity of a seed to chilling temperatures is highly dependent on its moisture content prior to germination.

Seeds with a high initial moisture content are protected from injury and seeds with a low moisture content, although more stable to long-term storage, are particularly susceptible to stress during imbibition (POLLOCK,1969). This has been shown in cotton (CHRISTIANSEN,1967), soybean (OBENDORF and HOBBS,1970), sorghum (PHILLIPS and YOUNGMAN,1971) and maize (CAL and OBENDORF,1972).

2.8.3 Imbibition Rate and Seed Coat Characteristics

POWELL and MATTHEWS (1978) strongly support the concept that rapid imbibition is related to poor germination at low temperatures. In their study of pea seeds, they reported that rapid water uptake during the first 2 minutes of imbibition led to cell death and attributed this to imbibitional damage, rather than the effects of low temperature.

Several reports have shown that intact seed coats retard water uptake, thus protecting seeds from imbibitional injury (POWELL and MATTHEWS,1978 ; TULLY, MUSGRAVE and LEOPOLD,1981 ; McDONALD, VERTUCCI and ROOS,1988). Using soybeans and peas, TULLEY, MUSGRAVE and LEOPOLD (1981) showed that damaged seed coats decreased the barrier to rapid water uptake and increased susceptibility to chilling. Furthermore, imbibition of pea embryos without seed coats resulted in more rapid water uptake, more potassium ion leakage and more damage in comparison to intact seeds (POLLOCK and TOOLE,1966).

There have been numerous reports about the role of pigmented seed coats in controlling the rate of imbibition at low temperatures. Snap beans with pigmented seed coats absorbed water more slowly and germinated better than those with white seed coats (WYATT,1977 ; POWELL, OLIVEIRA and MATTHEWS,1986) and this has been associated with imbibition rate (WYATT,1977), rate of leakage loss from the seed coat (POWELL, OLIVEIRA and MATTHEWS,1986) and phenolics in the seed coat (DICKSON and

PETZOLDT,1988). However, DICKSON (1973) could not relate seed coat pigmentation to cold temperature germination in lima beans. Since the seed coat is functional in controlling imbibitional chilling injury, it is important in seed industries to prevent damage during handling.

2.8.4 Seed Vigor and Cultivar Differences

Differences in chilling sensitivity have been observed between cultivars of soybeans (HOBBS and OBENDORF,1972) , corn (CAL and OBENDORF,1972) and cotton (CLAY, BARTKOWSKI and KATTERMAN,1976). Low vigor seeds have been reported to be particularly injured by imbibition at low temperatures (WOODSTOCK and TAO,1981). Seed vigor and the ability of seeds to germinate at low temperatures is greatly influenced by the environment in which the seed develops (BRAMLAGE, LEOPOLD and SPECHT,1979 ; ASHWORTH and OBENDORF,1980). For example, BRAMLAGE, LEOPOLD and SPECHT (1979) reported that soybean seeds which matured under cooler environmental conditions germinated better at low temperatures.

2.9 SYMPTOMS

The symptoms of chilling injury in germinating seeds can be expressed immediately or over a season-long period. Reduced emergence has been reported to be the most immediate and severe expression of CI in seeds and

has been demonstrated in muskmelons and peppers (HARRINGTON and KIHARA,1960), soybean (HOBBS and OBENDORF,1972) and corn (CAL and OBENDORF,1972). CHRISTIANSEN (1968) observed an abortion of the radicle tip at the start of imbibition at chilling temperatures and after 24 hours, root cortex damage occurred. Stelar lesions (COHN and OBENDORF,1978) have also been observed as a symptom in the radicle of low moisture content maize kernels.

2.10 CAUSES AND UNDERLYING MECHANISMS

2.10.1 Solute Leakage

A number of compounds have been reported to leach out from seeds imbibed in water (KHAN,1982 ; SIMON,1984) and leakage rates have been used to assess seed quality (HENDRICKS and TAYLORSON,1976 ; VERMA and RAM,1987) and are interpreted as a sign of membrane damage. This has been shown in soybean seeds (BRAMLAGE, LEOPOLD and PARRISH,1978). Biochemical evidence for the leakage of cellular material from chilled seeds has also been provided by other studies (POLLOCK and TOOLE,1966 ; LEOPOLD and MUSGRAVE,1979). Although seed leakage results in damage, loss of materials from the seed is not the primary cause of injury, but a reflection of changes in membrane physiology.

2.10.2 Membrane Disruptions

A significant area of interest in chilling studies is that of damage to the plasma membrane, since leakage data primarily reflect changes in its integrity (BRAMLAGE, LEOPOLD and PARRISH,1978 ; LEOPOLD and MUSGRAVE,1979 ; LEOPOLD,1980). It is however, unlikely that the plasma membrane is the only membrane system affected by chilling, since LEOPOLD and MUSGRAVE (1979) have suggested susceptibility of mitochondrial membranes to chilling injury in soybean seeds.

2.10.3 Membrane Compositional Differences

Studies of chilling adaptation in biological organisms, has often led to suggestions that resistance to cold temperature is mediated through compositional changes in biological membranes.

LYONS (1973) hypothesized that the primary cause of chilling injury is the physical response of membrane lipids to low temperatures. It was suggested that cooling of membrane lipids would entail a sharp phase transition between a fluid liquid-crystalline state and a more rigid gel conformation at critical temperatures, which was not observed in the lipids of chilling-resistant species.

That a high degree of fatty acid unsaturation may be a feature of increased chilling resistance was first reported by LYONS (1973) and was later confirmed in cotton seedlings (ST.JOHN and CHRISTIANSEN,1976). Likewise, DOGRAS,

DILLEY and HERNER (1977) suggested that chilling resistance in seeds was dependent upon their ability to synthesize large amounts of unsaturated fatty acids during the initial stages of germination. In a similar study, BARTKOWSKI, BUXTON, KATTERMAN and KIRCHER (1977) showed a strong correlation between unsaturated/saturated fatty acid ratio of total seed and polar lipids. Based on the above findings, it is thought that a greater level of fatty acid unsaturation would confer increased lateral fluidity on bilayer components at chilling temperatures, thus allowing maintainance of normal membrane function. Despite evidence implicating fatty acid unsaturation as a central factor in chilling resistance, results are conflicting. For example, the difference in chilling sensitivity of pea and soybean could not be related to compositional differences in the major lipid components of seed membranes (PRIESTLEY and LEOPOLD,1980).

2.10.4 Membrane Reorganization Theory

There has been some evidence indicating that CI to seeds during imbibition might be attributed to disruptive effects on the reorganization of membranes, as water enters the dry seed (POLLOCK and TOOLE,1966 ; BRAMLAGE, LEOPOLD and PARRISH,1978). SIMON (1974) attributed the chilling damage to an interference with a supposed transition from a hexagonal organization of phospholipids in a dry state to the lamellar organization in the aqueous state. The membrane changes from a semi-permeable membrane to a porous, disorganized state as

water is lost from the seed during maturation. It was further suggested that as water enters dry seeds, the membranes become reorganized into a bilayer and leakage is reduced as the membranes regain their semi-permeability, but this concept has been set aside because several researchers have demonstrated the existence of the bilayer structure in the membranes of dry seeds.

2.10.5 The Calcium Hypothesis

That calcium is the primary physiological transducer of CI, with increased cytosolic levels mediating the cytological, physiological and metabolic changes in chill-induced plants, was first proposed by MINORSKY (1985). However, no work has been done to confirm this hypothesis in germinating seeds.

2.11 ULTRASTRUCTURAL ASPECTS OF CHILLING INJURY IN SEEDS

Evidence indicating that membrane damage may play a major role in chilling sensitivity during seed imbibition comes from studies showing increased amounts of solute leakage from chilled seeds (BRAMLAGE, LEOPOLD and PARRISH,1978). Studies during early imbibition (WEBSTER and LEOPOLD,1977) have shown extensive structural changes accompanying water entry into soybean seeds.

Studies indicating changes in membrane organization upon seed hydration were shown by WEBSTER and LEOPOLD (1977). Whereas, dry tissues showed a

disorganized plasma membrane and distorted mitochondria, hydrated tissues exhibited a more organized membrane and organellar structure.

At the ultrastructural level, mitochondria from chilled cells showed weakly defined envelopes and cristae in comparison to a well-defined internal structure seen in the controls (CHABOT and LEOPOLD,1985). Plasmalemma integrity was also studied using tannic acid as a probe. Control and chilled seeds were able to exclude tannin, from which it was concluded that CI effects did not damage the plasma membrane sufficiently to allow entry of these large molecules.
However, it is evident that structure-function relationships are important with respect to CI since most researchers have reported structural changes in response to CI at the ultrastructural level.

2.12 AMELIORATION OF CI DURING SEED GERMINATION

2.12.1 Hydration-Dehydration Treatments

The use of pre-sowing hydration-dehydration treatments to accelerate germination and improved performance at low temperatures has been reported (HENCKEL,1964 ; HAFEZ and HUDSON,1967). These treatments allow seeds to imbibe partially and undergo metabolic activation without radicle emergence. If radicle emergence has not occurred, seeds may be re-dried without any permanent deleterious effects upon subsequent germination.

2.12.2 Initial Seed Moisture Content

As mentioned in section 2.8.2., increasing the initial moisture content of seeds prior to planting in cold soils has increased germination rate, germination percentage and prevented imbibitional CI in cotton (CHRISTIANSEN,1967), corn (COHN and OBENDORF,1976) and soybeans (WOODSTOCK and TAYLORSON,1981). In general, as the moisture content of a susceptible seed increases, its chilling sensitivity decreases.

2.12.3 Alternating Temperatures

More injury occurs in chill-sensitive seeds if imbibition begins under low temperatures than if seeds are imbibed in moderate temperatures (POLLOCK and TOOLE,1966) and seeds may be irreversibly damaged if exposed to chilling temperatures for a period at the start of imbibition before being placed in normal incubation conditions (BEDI and BASRA,1993). Thus exposing seeds to a warm temperature during the initial stages of germination and subsequent transfer to a cold temperature, ameliorates damage or chilling injury.

2.12.4 Osmopriming

Osmopriming is a process of seed hydration followed by dehydration to the original moisture content (BROCKLEHURST and DEARMAN,1984). Seeds are imbibed in an osmoticum (usually polyethyleneglycol) of a concentration that allows water uptake and pre-germinative processes to be complete, except for radicle emergence (BRADFORD,1986). The seeds are redried without damage and resowing allows germination to proceed more rapidly and uniformly. The efficiency of this method has been confirmed in seeds of leek (BROCKLEHURST, DEARMAN and DREW,1984).

Amelioration of imbibitional CI in seeds during priming is manifested in activation of reserve mobilization enzymes (KHAN, TAO, KNYPL, BORKOWSKA and POWELL,1978), improved capacity for RNA and protein synthesis (COOLBEAR and GRIERSON,1979), increased accumulation of ATP (MAZOR, PERL and NEGBI,1984) and restoration of membrane integrity (BURGESS and POWELL,1984).

Growth regulator treatments have also been implicated in the ameliorating of CI in seeds. This process depends on high membrane permeability to facilitate penetration of chemicals into seeds during early imbibition (WOODSTOCK,1988). Further attempts to overcome or ameliorate imbibitional CI in seeds could include a better understanding of the role of environmental conditions, and mechanisms associated with seed germination under cold temperature conditions.

2.13 SCOPE OF THE PRESENT INVESTIGATION

From the above review it can be seen that:

1) the problem of chilling injury is of major agricultural importance since a large number of plants of economic importance are reported to be susceptible under field conditions, leading to reduced crop productivity;

2) biochemistry associated with CI has been studied extensively and results from these studies give possible explanations, such as, leakage studies reflecting membrane integrity.

3) ultrastructural studies have also shown organelles and other membranous structures to be damaged in response to chilling.

4) several theories have been advanced to account for CI in vegetative tissues and seeds, but evidence for a primary determinant is lacking;

The present study was undertaken on imbibing soybean seeds at chilling and non-chilling temperatures in order to :

Analyse leachates (electrolytes, potassium ions and amino acids) from soybean seeds, in order to determine aspects of membrane function by leakage patterns;

Investigate differences in lipid hydroperoxide levels of seeds and axes in an attempt to establish what relationship exists with chilling injury and peroxidation;

Determine differences in saturated and unsaturated fatty acid levels in seeds and axes as a possible indicator of peroxidation;

Monitor sugar levels in high and low moisture content axes in order to determine what changes in levels exist and examine the role of moisture content during CI; Study protein patterns and monitor the effect of chilling injury on antioxidant defense enzymes, namely, superoxide dismutase, catalase and peroxidase; Examine ultrastructural changes associated with CI in the axes of seeds in an attempt to localize cellular sites of possible damage.

CHAPTER 3

MATERIALS AND METHODS

3.1 Seed Material

Soybean seeds (Glycine max L.Merr,var.Forrest) were obtained from Pannar Seeds, Greytown. Germination was 83% and seeds were stored in a cold room at 10°C for the duration of the study period.

3.2 Moisture Content determination

Three replicates of 1g of intact seeds were dried in paper bags at 80°C overnight. Moisture contents were calculated as percentage of the seeds fresh weight.

3.3 Seed Equilibration

For certain experiments, soybean seeds were equilibrated to 6.2% and 20.9% moisture contents over saturated salt solutions of $MgCl_2$ and Na_2CO_3, respectively, for 3 weeks in air-tight containers. Four replicates of 10 seeds were removed from each container and dried to constant weight at 80°C and moisture content was calculated as a % of the seeds fresh weight.

3.4 Imbibition Curves

The germination characteristics of soybean seeds at 5°C and 25°C were established by subjecting the seeds to the following treatments :

(a) Four groups of 20 seeds each were massed. The seeds were then placed on Whatman No.1 filter paper in Petri dishes, 10ml of water added, and incubated at 5°C or 25°C. At regular time intervals thereafter, excess moisture was removed with absorbent towelling and their masses recorded. The seeds were then returned to Petri dishes at the appropriate conditions as soon as possible thereafter. The increase in fresh mass, as a percentage of the mass prior to imbibition, was determined for each group of seeds. The values obtained were averaged for each time interval and the standard errors calculated.

(b) The seeds were imbibed at 5°C and 25°C in an aqueous solution of fast green dye (0.1% w/v) in order to examine the path of water entry. The seeds were removed after 24 hours, rinsed to remove all surface accumulation of the dye, and viewed under a light microscope in order to determine the extent of dye penetration into the tissues.

3.5 BIOCHEMICAL ANALYSES

3.5.1 LEAKAGE STUDIES

Three groups of twenty seeds and isolated axes were placed in Petri dishes with 20ml of water. They were incubated at 5°C or 25°C and the supernatant was analysed at regular time intervals for the leakage of potassium ions, amino acids and electrolytes.

3.5.1.1 Potassium ions

Potassium ions were measured using flame photometry. Standards ranging from 0.5ppm - 7ppm were prepared from 1M HNO_3 (63ml/l) in a total volume of 100ml and were spiked with CsCl (1ml/100ml) before readings were taken. For sample analysis, 200µl of seed leachate was diluted with 10ml of HNO_3 and spiked with CsCl (100µl/10ml). Absorbance was recorded at 766.5nm.

3.5.1.2 Amino Acids

Amino acids were determined by the Ninhydrin Test (MOORE and STEIN,1948). 1ml of ninhydrin reagent was added to 200µl of leachate and after mixing, was heated at 80°C for 20 minutes, cooled and diluted with 5ml propanol : water (1:1

v/v) and absorbance read at 570nm. The propanol : water solution was used as a blank. Quantities of amino acids were read from the standard curve, using glycine as a standard.

3.5.1.3 Electrolyte Leakage

Electrolyte leakage was measured using a RM IOO multi-station conductivity meter (REID and ASSOCIATES, DURBAN).

3.5.2 LIPID EXTRACTION

Lipids were extracted using the solvent system of KHOR and CHAN (1985). Ten seeds and twenty axes were ground and extracted with 20ml and 10ml respectively of dichloromethane/methanol (2:1 v/v) containing BHT (0.005% w/v) for 20 minutes. After standing for 10 minutes, the upper phase was removed and centrifuged at 1500xg in a benchtop centrifuge at room temperature for 5 minutes. The supernatant was aspirated and then dried down at 45^{o}C under nitrogen.

3.5.2.1 Lipid Fractionation

Lipid was fractionated by silicic acid column chromatography (BEUTELMANN and KENDE,1977). Columns were filled with a silicic acid/chloroform slurry. A

sample of lipid dissolved in 1ml chloroform was placed onto the column and eluted with successive 20ml volumes of chloroform, acetone and methanol, giving the neutral, glycolipid and polar lipid fractions respectively. Eluates were dried down at 45°C under nitrogen and stored at 4°C until further use.

3.5.2.2 Hydroperoxide Determination

Reagents

a) Benzene/Methanol (7:3 v/v) Analytical grade.

b) $FeCl_2$ Solution. 0,4g of $BaCl_2$ was dissolved in 50ml water and added to 0,5g Fe_2SO_4 in 50ml water. 2ml of 10N HCl was then added and the precipitate cleared by brief centrifugation.

c) 10N HCl. 95,24ml of HCl was brought up to 100ml with distilled water.

d) KSCN solution. 15g of KSCN was dissolved in 50ml water, to obtain a 30% solution.

Hydroperoxide levels were determined using a modification of the test of STINE, HARLAND, COULTER and JENES (1953). 20µl of $FeCl_2$ solution was added to 5ml of benzene/methanol (7:3 v/v) and shaken. 20µl of lipid followed by 20µl of 30% KSCN was then added with shaking. The absorbance was read at 505nm against a blank of the reagents.

3.5.2.3 Lipid Esterification and Fatty Acid Determination

Methyl esters of the fatty acids were obtained using the organic base-catalysed technique of METCALFE and WANG (1981). An aliquot of lipid was dissolved in 1ml of diethyl ether, 0.5ml of 1M TMAH in methanol was added and the mixture was shaken for 1 minute at room temperature. 1ml of water was then added and 1µl of the upper ether phase was analysed using a Varian 3700 Gas Chromatograph fitted with a flame ionisation detector and a 1.8m x 2mm internal diameter glass column packed with 10% Silar 5CP on 100/120 mesh Supelco-port. During the analysis, injection port and detector temperatures were 270 and 300°C respectively, while the column was maintained isothermally at 185°C. Nitrogen was used as a carrier gas at 25ml/min and peaks were intergrated using a HP 3380A intergrator and expressed as area percentages. Tentative identification was achieved by comparison of retention times with those of an authentic fatty acid methyl esters mix (SIGMA) which included the esters of palmitic, stearic, oleic, linoleic and linolenic acids. There were three replicates of each sample.

3.5.3 SUGAR EXTRACTION

Seeds were imbibed at 5°C or 25°C for appropriate time intervals. Twenty axes were isolated from seeds and ground using mortar and pestle to which 10ml boiling methanol was added. The extract was shaken for 15 minutes, and the

supernatant was collected after centrifugation in a benchtop centrifuge. The supernatant was then taken to dryness at 45°C under nitrogen. There were 3 replicates of each sample.

3.5.3.1 Sugar Derivatisation

Sugars were analysed by Gas Liquid Chromatography, after conversion to volatile derivatives. This involved the production of oximes (TANOWITZ and SMITH,1984) followed by trimethylsilyl derivatisation of the oximes (SWEELEY, BENTLEY, MAKITA and WELLS,1963).

A solution containing 25mg/ml hydroxylamine monohydrochloride was dissolved in pyridine. 0.5ml of this solution was added to each dried vial of the methanol extract of axes. The vials were stoppered and heated at 40°C for 20 minutes. 100µl aliquots of the sample were taken to dryness under nitrogen and 100µl of the silylating reagent, Sylon BTZ (SUPELCO) added at room temperature for 15 minutes. The samples and standards were now ready for separation by gas chromatography methods. 1µl of sample was injected into the gas chromatograph. The authentic standards (MERCK) of fructose, glucose, sucrose, raffinose and stachyose were used to establish retention times of trimethylsilyl-oximes.

3.5.3.2 Sugar Determination

For the separation, a Varian 3700 gas liquid chromatograph fitted with a flame ionisation detector and a 1.8m x 6mm I.D. glass column packed with 5% OV17 on chromosorb WHP 100/120 mesh was used. For the separation, a temperature programme was used :

Initially, the column was held at 150°C for 3 minutes, whereafter the temperature was increased to 300°C at a rate of 2 °C per minute. The final temperature was maintained for 15 minutes. Results were integrated electronically and expressed as area percentages.

3.5.4 PROTEIN EXTRACTION

Reagents

1) Extraction Buffer. 24,23g of TRIS was dissolved in 1 litre of water. 25ml of TRIS solution was added to 52ml HCl and brought to 100ml using distilled water. 2% PVPP insoluble (2g/100ml), 0,2mM EDTA (0.0074g/100ml) and 1mM DTT (0.015g/100ml) was added to the TRIS-HCl solution.

Soybean seeds were imbibed at 5°C and 25°C for 0, 24 and 48 hours. Seeds were blotted dry on filter paper to minimise the effects of water. After this, the seed coats were removed and 5g of the material was weighed out and ground in a prechilled mortar and pestle. 15ml of extraction buffer was added to the ground

seed material and it was homogenized. The extraction was allowed to stand for 1 hour at 10°C. The cell debris was removed by filtering through cheesecloth and the filtrate centrifuged for 20 minutes at 14000 rpm. The supernatant was then transferred to clean tubes and centrifuged for a further 15 minutes at 14000 rpm. The supernatant was removed and protein content was determined using the method of BRADFORD (1976). Absorbance was measured at 595nm. Albumin was used to prepare a standard curve. The samples were stored in small aliquots in Eppendorff tubes at -70°C until further use.

3.5.4.1 Polyacrylamide Gel Electrophoresis

Reagents

a) 28g Acrylamide was mixed with 0.735g bis-Acrylamide and made up to 100ml with distilled water.

b) 1N HCl. 9,52ml of concentrated HCl was made up to 100ml with distilled water.

c) 36,3g TRIS was dissolved in 48ml 1N HCl and 230µl TEMED was added. The solution was brought to 100ml with distilled water (pH 8.8- 9).

d) 0,280g of Ammonium persulfate (APS) was dissolved in 50ml distilled water. This solution was freshly prepared for all experiments.

A 7,5% Acrylamide Gel was prepared according to ACQUAAH (1992) as follows :

TRIS-HCl = 5ml

bis-Acrylamide = 10ml

Distilled water = 5ml

TEMED = 28µl

APS = 20ml

e) TRIS-GLYCINE STOCK Buffer (pH 8.3) was prepared by dissolving 6g TRIS and 29g glycine in 1 liter of distilled water.

f) A 10x diluted TRIS-GLYCINE RUNNING Buffer was prepared for running the gels by adding 4500ml of distilled water to 500ml of TRIS-GLYCINE STOCK Buffer.

A 7.5% Acrylamide gel was prepared as above, and poured into the assembled electrophoresis plates. The gel was overlayed with a thin layer of butanol : water (1:1 v/v) and allowed to polymerize. After this, a pre-electrophoresis run was performed for 1 hour at 100V and 25mA. For sample preparation, 0.4g of sucrose was added to 1ml protein samples in Eppendorffs tubes and samples were shaken until the sucrose dissolved. 25µl of 0,1% bromophenol blue was added as a dye marker. The dye was prepared by dissolving 0.1g bromophenol blue in 100ml of 10x diluted TRIS-GYCINE RUNNING Buffer). The samples were kept on ice throughout preparation.

After the pre-electrophoresis run, protein samples in quantities of 25µl (for SOD detection) , 50µl (for catalase and peroxidase detection) and 100µl (for protein

detection) from each seed treatment were loaded into the wells using a Hamilton micro-syringe. Diluted TRIS-GLYCINE buffer was poured into the electrophoresis tank until the wells were completely covered. The apparatus was sealed and electrophoresis was allowed to proceed at 100V for the first 10 minutes and thereafter, at 340V for 4 hrs. After this, the gels were removed and washed twice with distilled water prior to staining.

3.5.4.2 Detection of Proteins

Reagents

a) 7% TCA was prepared by dissolving 7ml of TCA in 93ml water.

b) A Staining solution was prepared by mixing 227ml methanol with 46ml acetic acid and adding water to bring the volume to 500ml. 1.25g of Coomasie Blue R250 was then added.

c) A Destaining solution was prepared by mixing 140ml of methanol with 140ml acetic acid and made up to a final volume of 2 litres with distilled water.

Proteins were detected according to ACQUAAH (1992). Gels were fixed in 7% TCA for 15 minutes and then washed three times with distilled water. This was followed by fixing in a staining solution for 15 minutes and overnight destaining.

3.5.4.3 Detection of Catalase

Reagents

a) 1% H_2O_2 was prepared by bringing 1ml of H_2O_2 to 100ml with distilled water.

b) $FeCl_3$ and KFeCN was prepared by dissolving 1g of each in 50ml of distilled water. The two solutions were mixed to obtain a final volume of 100ml.

Catalase bands were detected according to the method described in ACQUAAH (1992). 100ml of 1% H_2O_2 was added to the gel for 10 minutes. The gel was then rinsed with distilled water and 100ml of the $FeCl_3$ and KFeCN solution added for 1-2 minutes.

3.5.4.4 Detection of Peroxidase

Reagents

a) 1N HCl. 9,52ml of concentrated HCl was brought to a final volume of 100ml with distilled water.

b) 3% H_2O_2 was prepared by bringing 3ml of H_2O_2 to 100ml with distilled water.

c) 50ml of 0,2M Acetate Buffer was prepared by adding 14,8ml of 0.2M acetic acid (2.88ml in 50ml distilled water) to 35.2ml of 0,2M sodium acetate (16.4g/l).

Peroxidase was detected according to BUTTERY and BUZZELL (1968). Benzidine (50mg) was dissolved in a few drops of IN HCl, then 100ml of 0,2M acetate buffer

was added, followed by 135µl guaiacol. The gel was incubated in this solution for 5 minutes. 1- 3ml of 3% H_2O_2 was added and the gel allowed to stand until bands appeared.

3.5.4.5 Detection of Superoxide Dismutase

Reagents

a) 112mg of NBT was weighed out in the dark and dissolved in a few drops ethanol, followed by 150ml of water.

b) 2mg Riboflavin was dissolved in 100ml of distilled water and 280µl of TEMED (0.025M) was added.

c) 0.036M Phosphate Buffer (pH 7.8) was prepared as follows :

0,07M KH_2PO_4 = 2.269g/250ml water.

0,07M Na_2HPO_4 = 11.948g/500ml water.

30ml of KH_2PO_4 was added to 470ml of Na_2HPO_4 followed by a further 500ml of distilled water.

d) 0,78g of KCN was dissolved in 2,5ml distilled water and 500µl concentrated HCl added to it to give 0,02M KCN.

e) 5mM H_2O_2 was prepared by mixing 30ml of H_2O_2 with 100ml distilled water.

Mn, Fe and Cu/Zn Superoxide dismutases were detected according to the method described in ACQUAAH (1992). Gels were incubated in 75ml NBT solution for 20 minutes in the dark.

100ml of riboflavin solution was then added and left for a further 15 minutes in the dark. 0,036M phosphate buffer was added and the gel illuminated until all bands appeared. Mn and Fe Superoxide dismutases were detected using the property of their insensitivity to cyanide. 750µl of 0,02M KCN was added to 75ml of NBT solution, poured over the gel and left for 20 minutes in the dark. The gel was then incubated for a further 15 minutes in a solution of 1ml 0,02M KCN in 100ml riboflavin solution. The gels were removed and 0.036M phosphate buffer was added and the gel illuminated until bands appeared. Mn Superoxide dismutase were detected using its insensitivity to hydrogen peroxide. 42µl of 30% H_2O_2 was added to 75ml NBT solution, poured over the gel and left in the dark for 20 minutes. 56µl of 30% H_2O_2 was added to 100ml of riboflavin solution. The gel was then incubated in this solution for a further 15 minutes in the dark. This was followed by the addition of 0,036M phosphate buffer and illumination until bands appeared.

All data was analysed statistically using the multiple analysis of variance test. The codebook procedure was used to calculate the standard errors.

3.6 ULTRASTRUCTURAL STUDIES

Soybean seeds were imbibed at 5°C and 25° C for 0, 6, 12, 24 and 48 hours. Thereafter, axes were excised and prepared for microscopy.

3.6.1 Transmission Electron Microscopy

Isolated axes were fixed in a 3% glutaraldehyde solution buffered to pH 6.9 to 7.2 with 0.05M sodium cacodylate buffer for 8 hours. Prior to post-fixation in 2% osmium tetroxide buffered with 0.05M sodium cacodylate, the specimens were rinsed 4 times in pure buffer (pH 6.2 to 7.9) 10 minutes each. A post-fixation time of 2 hours was employed. After this period, the embryos were washed again four times with buffer, and then twice with double distilled water, 10 minutes each. This was immediately followed by dehydration in a graded alcohol series (30%, 40%, 50%, 60%, 70%, 80%, 90%) and finally with three changes of 100% ethanol. Samples were prepared for embedding in resin (SPURRS,1969) as follows :

10g Vinyl cyclohexane dioxide (ERL 4206)

6g Diglycidyl ether of polypropylene glycol (DER 736), a plasticiser

26g Nonenyl succinic anhydride (NSA), a hardener

0,6g Dimethylaminoethanol (S-1), an accelerator.

These components were stirred for 1 hour at room temperature before use. The specimens were first placed in a 25% SPURR : 75% ETHANOL solution for 2 hours caps on, whereafter in a 50% SPURR : 50% ETHANOL solution for 2 hours caps on and then they were transferred to 75% SPURR : 25% ETHANOL for 2 hours caps off.

They were then transferred to 100% SPURR'S resin and left overnight. The resin was replaced by fresh 100% SPURR'S and the specimens were placed in moulds. The resin polymerized by exposure to 70^{o}C for 16 hours and after cooling, the

blocks were removed from the moulds and stored until required for sectioning. Sections were cut, using glass knives, on an LKB ultramicrotome and collected on copper grids. The specimens were stained with uranyl acetate (1g/30ml in 50% ethanol) and lead citrate for 10 minutes in each solution (REYNOLDS,1963). Thereafter, the sections were examined using a JOEL 100 CX transmission electron microscope, at an accelerating voltage of 80kV and the areas of interest were recorded photographically.

CHAPTER 4

RESULTS

4.1 Characteristics of Soybean Seed Imbibition

The overall rate of water uptake by soybean seeds was less during imbibition at chilling (5°C) than at non-chilling (25 °C) temperatures (Fig 3A), the final differences in the fresh weight of the seeds being 63% and 75% after 48 hours imbibition, respectively.

It was observed that at chilling temperatures, water uptake was more rapid between 6 and 12 hours of imbibition. At this stage of imbibition, the fresh weight of the seed increased from 51 to 57%. This was the greatest increase in fresh weight observed in the seeds. Thereafter, the rate of imbibition increased more gradually being 59% after 24 hours and 63% after 48 hours of imbibition. After 48 hours imbibition at 25°C, radicle emergence had begun. At non-chilling temperatures, the imbibition rate was much slower between 6 and 12 hours. This was followed by a rapid increase in seed mass after 12 hours, with the fresh weight of the seeds increasing from 62% to 68% after 24 hours and eventually 75% after 48 hours of imbibition. This value is much higher than for seeds that were imbibed similarly at chilling temperatures.

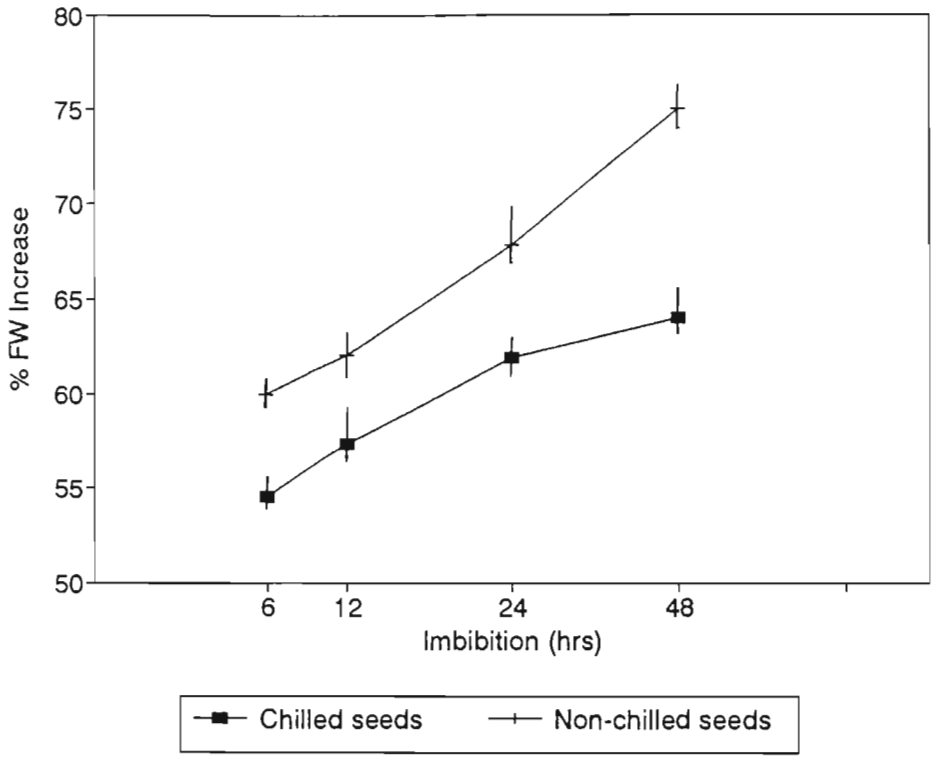


Figure 3A Water uptake patterns of soybean seeds at 5°C or 25°C for 48 hours. The vertical bars represent the standard errors of the means.

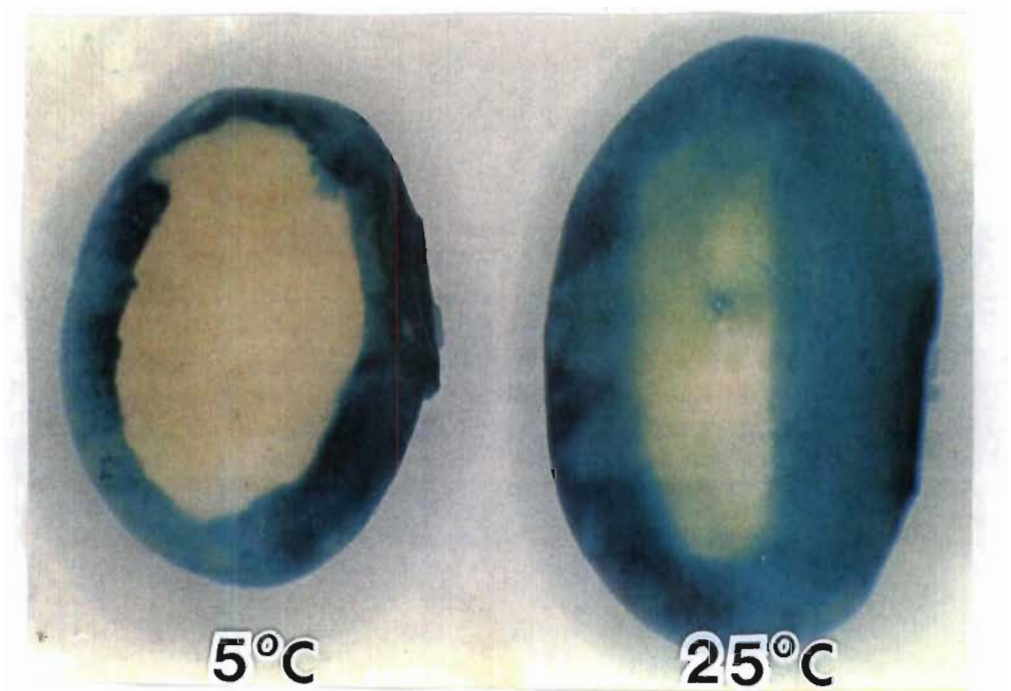


Figure 3B Soybean seeds imbibed for 24 hours at 5 °C and 25 °C in fast green dye in order to trace water uptake.

For example, after 48 hours of imbibition at chilling temperature, the fresh weight of the seeds were 63%, which is an 18% difference between the two treatments. A visual assessment of the seeds that were imbibed at 5°C showed that they did not germinate, even though they imbibed water and the fresh weight difference between chilled and non-chilled seeds was some 18%.

Studies using fast green dye, in order to trace water uptake, revealed that at non-chilling temperatures, after 24 hours imbibition, cotyledons were not only more swollen than seeds imbibed at chilling temperatures, but that dye had penetrated the tissues more completely after 24 hours imbibition (Fig.3B).
It was noted that hydration of the cells commenced at the periphery and slowly moved towards inner tissues, the rate of dye penetration being faster at 25°C.

The same trend was observed in the axes and whole seeds (Figs.4A and B). Soybean seeds were imbibed for 1, 24 and 48 hours at chilling and non-chilling temperatures. Axes were isolated and observed. It can be observed that chilled axes and seeds (top row) were less swollen than non-chilled axes and seeds (bottom row) , except that the differences in swelling were not as marked as that of seeds (Fig.3B).

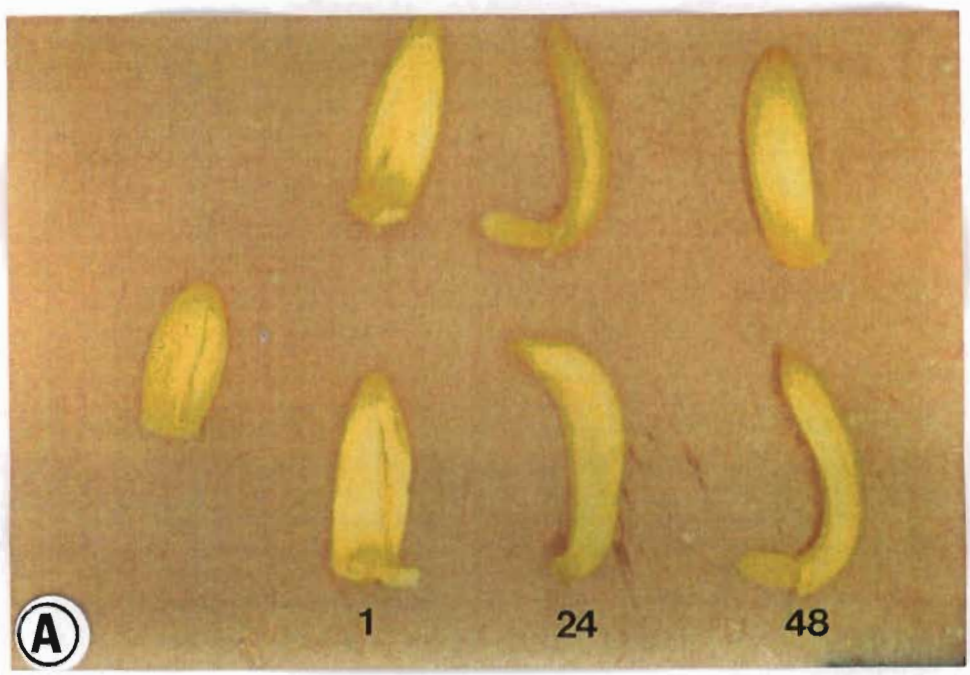


Figure 4A Axes isolated from soybean seeds imbibed at 5°C (top row) and 25°C (bottom row) after 1, 24 and 48 hours.

Figure 4B Soybean seeds imbibed at 5°C (top row) and 25°C (bottom row) after 1, 24 and 48 hours.

4.2 Effect of Seed Moisture Content on Temperature Sensitivity during Imbibition

It has been reported that low moisture content soybean seeds are more sensitive to cold temperatures during imbibition than high moisture content seeds. For this reason, it was decided to investigate the effect of soybean seed moisture content on temperature sensitivity during imbibition. Low moisture content (6.2%) and high moisture content seeds (20.9%) were used for this experiment. Chilled seeds of low moisture content imbibed water at a similar rate than seeds imbibed at 25^{o}C (Fig 5A) although imbibition was very slow between 6 and 12 hours of imbibition and increased gradually thereafter.

Although non-chilled seeds of low moisture content imbibed water at a faster rate than chilled, low moisture content seeds (Fig.5A), the overall fresh weight increase was much lower in comparison to high moisture content seeds imbibed at 25^{o}C (Fig.5B). Overall, low moisture content chilled seeds exhibited a 8% fresh weight increase and non-chilled seeds a 13% fresh weight increase between 6 and 48 hours imbibition.

However, high moisture content seeds imbibed greater amounts of water overall (Fig.5B). Water uptake in chilled seeds was rapid between 6 and 12 hours of imbibition (fresh weight increased from 44 to 47%), this is in contrast to water

uptake in low moisture content seeds where water uptake levels were low during the first twelve hours of imbibition (fresh weight values remained at 40%). Thereafter, water uptake in chilled seeds levelled off to a greater extent than seeds imbibed at 25°C. It was noticed that at chilling temperatures, high moisture content seeds survived the stress and radicle emergence occurred in 85% of the seeds. In contrast, radicle emergence occurred in 32% of low moisture content seeds.

Overall, water uptake occurred at a faster rate and was greatest in high moisture content seeds, leading to radicle emergence after 48 hours.

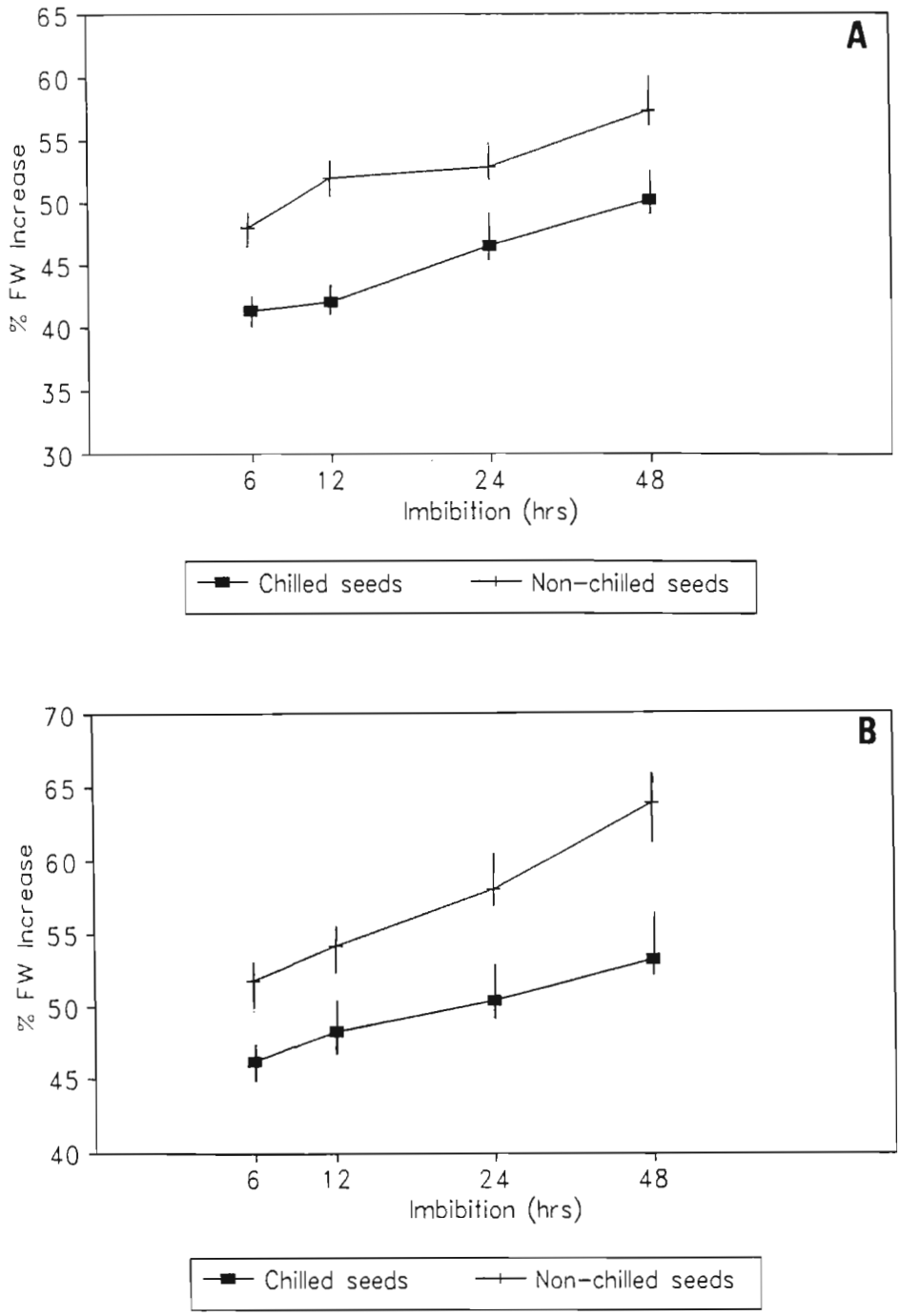


Figure 5 Rate of imbibition of soybean seeds at 5°C and 25°C in low moisture content (A) and high moisture content seeds (B). The vertical bars represent the standard errors of the means.

4.3 Leakage Studies

Analysis of leachates using flame photometry showed that potassium ion leakage from seeds and isolated axes increased slowly over the first 24 hours of imbibition at chilling and non-chilling temperatures, but increased markedly after 48 hours (Figs.6 and 7).

Differences were not significant between chilled and non-chilled seeds (Fig.6). However, although a similar leakage pattern was observed for excised axes at chilling and non-chilling temperatures, leakage from chilled axes was significantly greater at chilling temperatures (Fig.7).

When the pattern of electrolyte leakage was monitored at half-hourly intervals for the first four hours of imbibition using a conductivity meter, dry seeds exhibited an initial rapid loss of electrolytes which became constant after 3 hours (Fig.8A). When seeds were imbibed for 24 hours at 25°C, and the patterns of electrolyte leakage were assessed over a further four hour period at 25°C, a similar pattern of leakage was seen (Fig.8C). Although seeds imbibed for 24 hours at 5°C before transferring to the conductivity cell showed the greatest loss of electrolytes over the four hour period, the final conductivity values were not significantly different from the other treatments (Fig.8B).

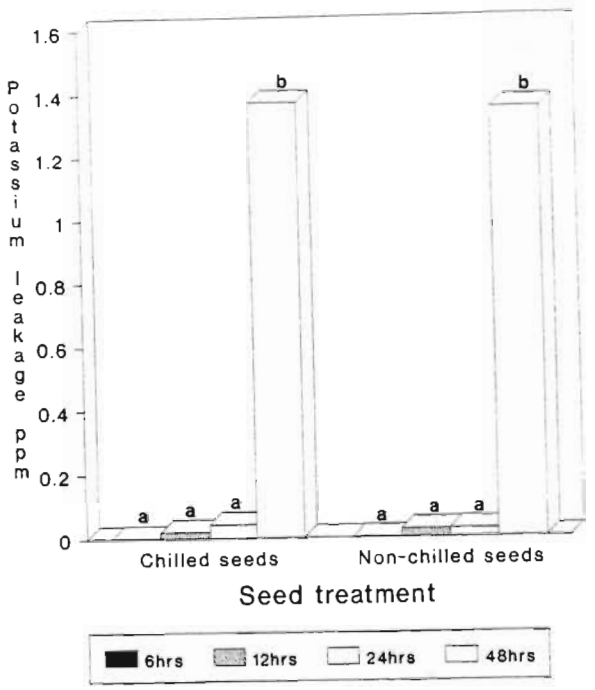


Figure 6 Potassium ion levels in the leachate of soybean seeds imbibed at chilling and non-chilling temperatures. Bars with the same letter are not significantly different from each other.

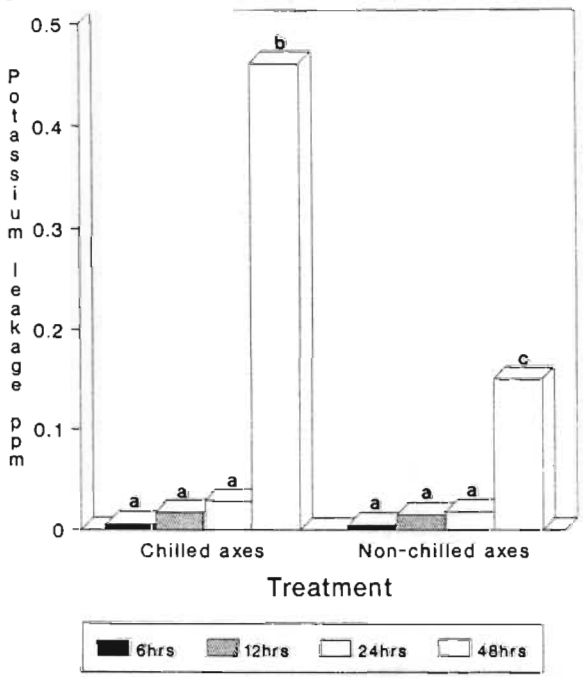


Figure 7 Potassium ion levels in the leachate of soybean axes imbibed at chilling and non-chilling temperatures. Bars with the same letter are not significantly different from each other.

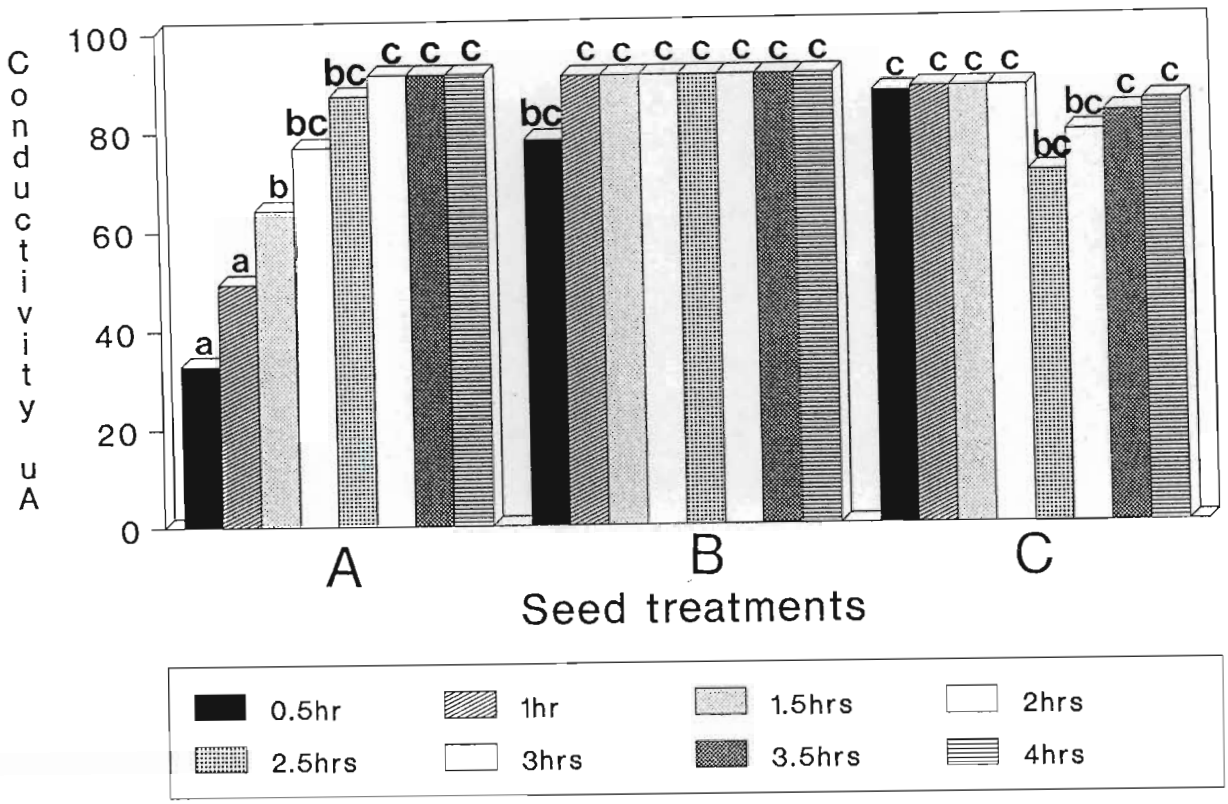


Figure 8 Conductivity values of leachate measured at half-hourly intervals from soybean seeds subjected to the following treatments: Dry seeds imbibed directly in conductivity cell (A); seeds imbibed at 5°C for 24 hours (B); and seeds imbibed at 25°C for 24 hours (C), before transfer to conductivity cell. Bars with the same letter are not significantly different from each other.

Greater amounts of amino acids leached out of chilled seeds than from non-chilled seeds, although both showed essentially linear increases between 6 and 48 hours imbibition (Fig.9).

The level of amino acids which leached out of chilled axes was greater than that of non-chilled axes, although the overall pattern was similar in that a transient decline in amino acid leakage was observed at 24 hours for both treatments (Fig.10).

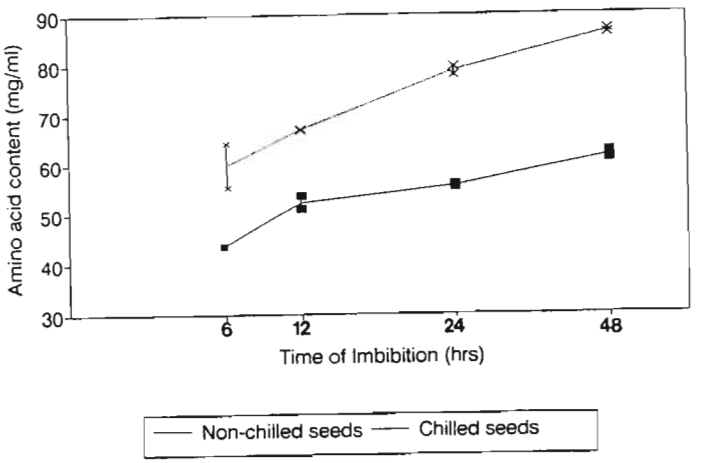


Figure 9 Total amino acid levels in the leachate of soybean seeds imbibed at chilling (.....) and non-chilling (____) temperatures. The vertical bars represent the standard errors of the means.

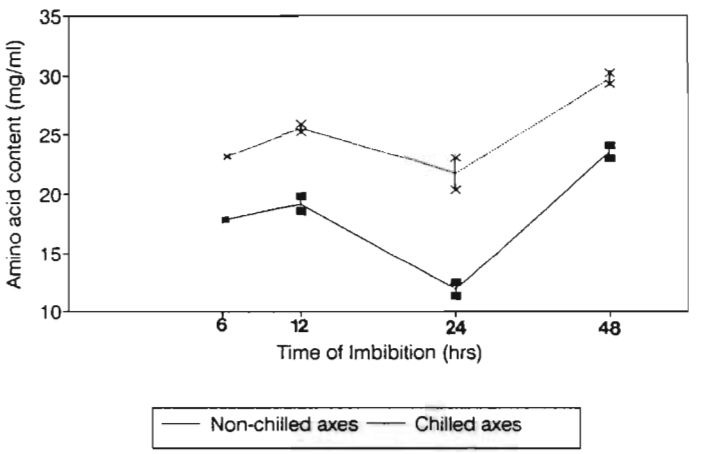


Figure 10 Total amino acid levels in the leachate of soybean axes imbibed at chilling (.....) and non-chilling (____) temperatures. The vertical bars represent the standard errors of the means.

4.4 Lipid Peroxidation during Imbibition

Transient changes in lipid hydroperoxide levels were observed during imbibition of both seeds and isolated axes at chilling and non-chilling temperatures over 36 hours (Fig.11).

Although changes in hydroperoxide levels were greater in isolated axes (Fig.11B) than in the seeds (Fig.11A), differences in lipid hydroperoxide levels between chilled and non-chilled axes were not significant. Significantly greater hydroperoxide levels were observed in chilled seeds than in non-chilled seeds (Fig.11A) and in some cases, these were almost doubled. The transient changes in lipid hydroperoxide levels observed in chilled seeds and isolated axes showed the same pattern; after 6 hours of imbibition levels peaked, falling to a lower level by 24 hours. A further increase in lipid hydroperoxides was noted in chilled seeds and axes between 24 and 36 hours. However, on the other hand, non-chilled seeds and their isolated axes showed somewhat different patterns, changes being more marked in the axes (Fig.11B).

4.5 The Effect of Temperature Changes on Lipid Peroxidation

It has been reported by several workers that exposing seeds to non-chilling temperatures prior to chilling, ameliorates chilling injury. This, however, depends on the duration of exposure to non-chilling temperatures, before transfer to chilling temperature.

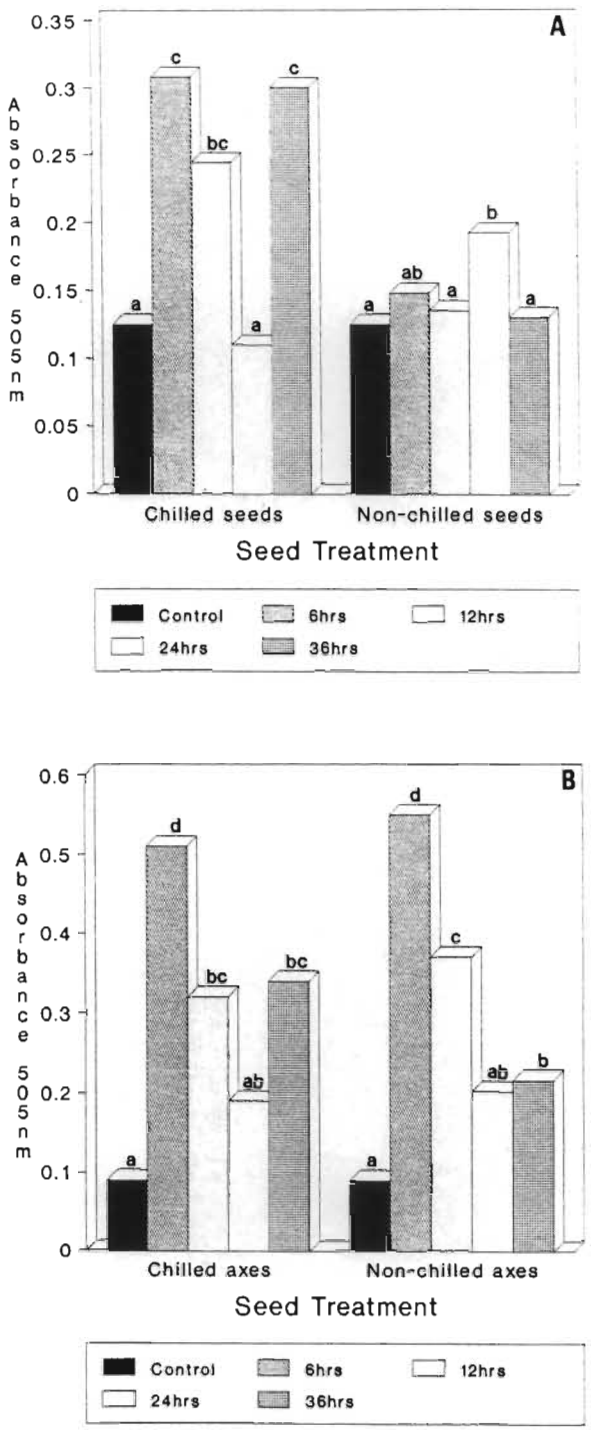


Figure 11 Lipid hydroperoxide levels in soybean seeds (A) and axes (B) following imbibition at chilling and non-chilling temperatures. Bars with the same letter are not significantly different from each other.

In this study, soybean seeds were imbibed at 25°C for 12 hours before transferring to 5°C for a further 12 and 24 hours, or imbibed at 5°C for 12 hours before transferring to 25°C for a further 12 and 24 hours.

This experiment was performed in order to see if chilling injury could be ameliorated by prior imbibition at 25°C before imbibing at chilling temperatures, or whether a return to 25°C from 5 °C allowed the seed to recover. It might further establish the amount of time necessary for non-chilling temperature exposure prior to exposure to chilling temperatures, for chilling injury to be ameliorated.

When soybean seeds were imbibed for 12 hours at 25°C followed by 12 hours at 5°C, the lipid hydroperoxide levels were higher than when seeds were imbibed for 12 hours at 5°C followed by 12 hours at 25 °C. Futhermore, seeds that were imbibed for 12 hours at 5°C and transferred to 25°C for a further 24 hours had greater hydroperoxide levels than seeds that were imbibed for 12 hours at 25°C then transferred to 24 hours at 5°C. The same trend was observed in the axes (Fig.12).

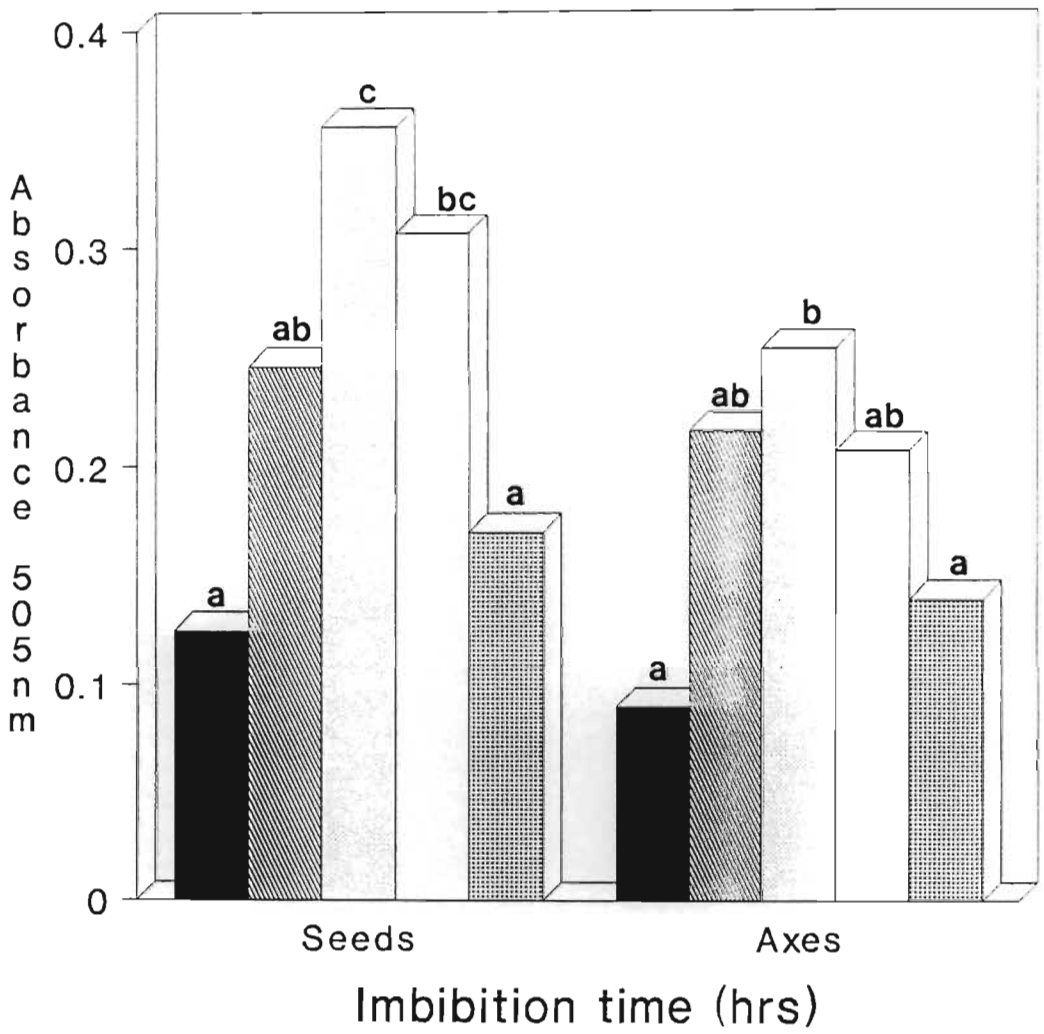


Figure 12 Lipid hydroperoxide levels in soybean seeds and axes following imbibition for 12 hours at :

5°C followed by 12 hours at 25°C ;

25°C followed by 12 hours at 5°C ;

5°C followed by 24 hours at 25°C ;

25°C followed by 24 hours at 5°C ;

(Control, non-imbibed seeds are denoted by).

4.6 Changes in Fatty Acid Levels during Imbibition

Several studies have been performed to examine fatty acid levels in seeds and axes in response to chilling. It was therefore decided to examine the fatty acid levels of the total lipids or lipid fractions, both for evidence of peroxidation and changes associated with chilling injury.

There were no marked differences between the fatty acid levels of chilled and non-chilled seeds during 36 hours of imbibition (Fig.13 A & B). Differences were evident between the levels of linoleic acid in chilled and non-chilled axes (Fig.14 A & B). Following a small increase in linoleic acid after 6 hours of chilling, there was a marked 18% decline in the levels of this fatty acid by 12 hours imbibition in the axes (Fig.14A).

In contrast, the linoleic acid levels of axes imbibed at non-chilling temperatures declined by 11% at 6 hours and 9% at 12 hours, followed by a gradual, but non-significant, increase (Fig.14B). Linolenic acid levels decreased by 7% after 12 hours in chilled axes, whereas changes were not as marked in axes at 25^{o}C.

Overall, levels of oleic acid were lower at chilling temperatures and showed only small changes, whereas at non-chilling temperatures a marked increase occurred between 6 and 12 hours (Figs.14A and B).

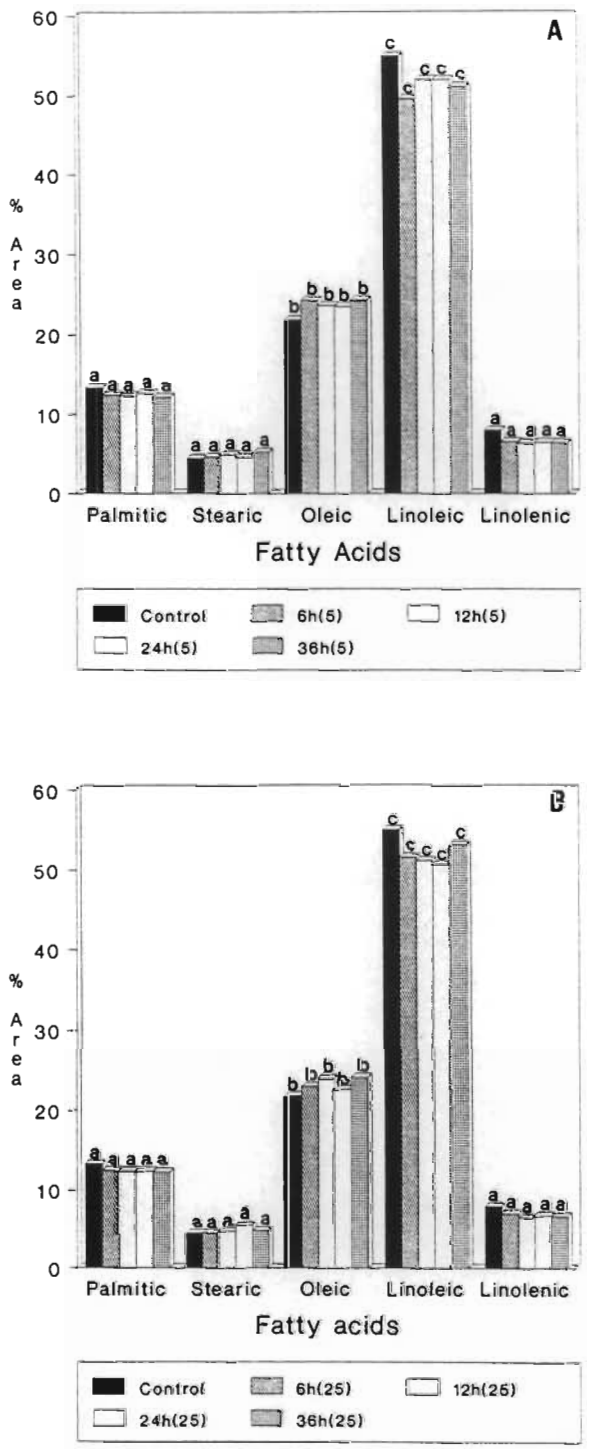


Figure 13 Percentage total fatty acid levels of soybean seeds following imbibition at chilling (A) and non-chilling (B). Bars with the same letter are not significantly different from each other.

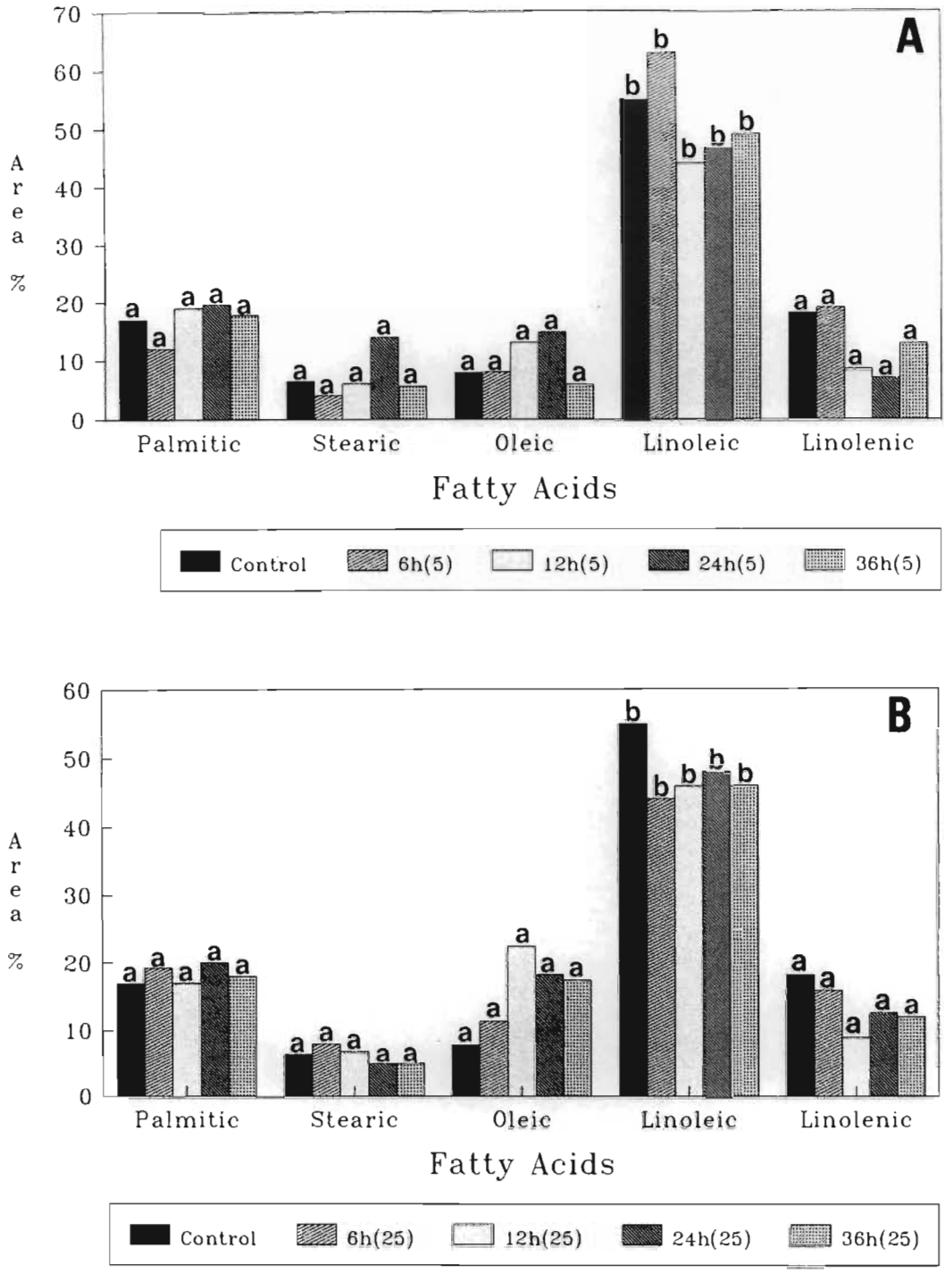


Figure 14 Percentage total fatty acid levels of soybean axes following imbibition at chilling (A) and non-chilling (B) temperatures. Bars with the same letter are not significantly different from each other.

4.7 Effect of Temperature Changes on Total Fatty Acid Levels during Imbibition

Small declines in linoleic acid were observed in seeds that were imbibed for 12 hours at 5°C, and then transferred to 25 °C for either 12 or 24 hours. A similar response was observed when seeds were imbibed for 12 hours at 25°C and then transferred to 5°C for a further 12 or 24 hours (Fig.15). By contrast, isolated axes showed more marked responses in comparison to the seeds especially with respect to linoleic acid levels (Fig.16). In comparison to seeds, changes in linoleic acid levels were strikingly different when the axes were subjected to 12 hours at 5°C followed by 12 hours at 25°C.

A significant decrease (23%) was observed in linoleic acid levels when axes were first imbibed for 12 hours at 5°C and then transferred to 25 °C for 12 hours (Fig.16). Levels of linoleic acid were also reduced, although not as markedly, when isolated axes were first imbibed for 12 hours at 25°C and then transferred to 5°C for a further 12 hours.

When axes were transferred after 12 hours at either 5°C or 25°C for a further 24 hours at 5°C and 25°C respectively, linoleic acid levels were significantly higher in comparison to the earlier transfer experiments. Thus, whereas seeds showed no significant changes in the fatty acid levels following temperature transfers, marked and significant changes were seen in the linoleic acid levels of axes similarly treated.

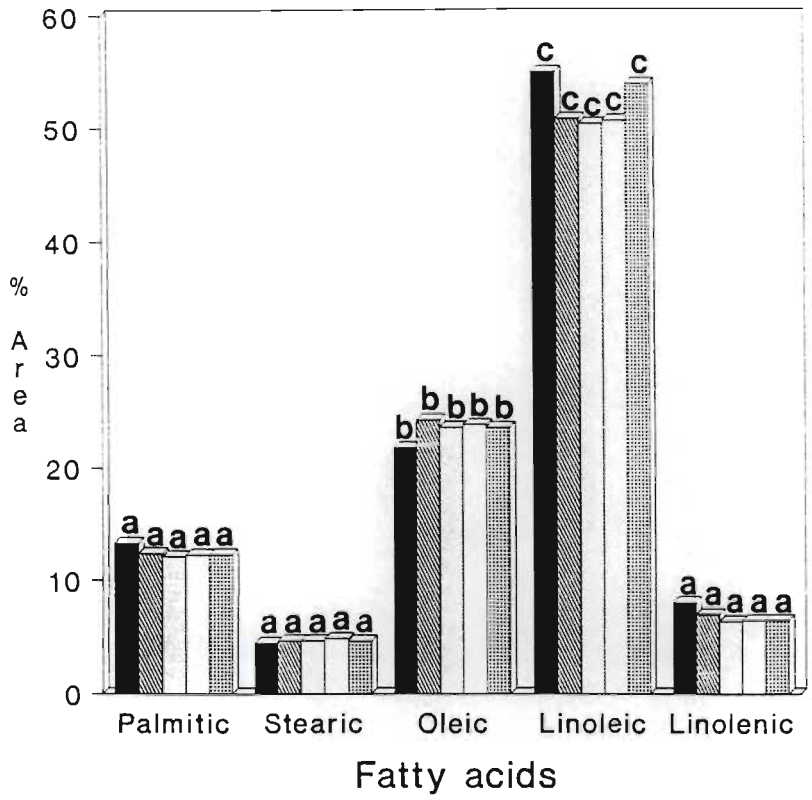


Figure 15 Percentage fatty acid levels of total lipid fraction of soybean seeds following imbibition for 12 hours at:

5°C followed by 12 hours at 25°C ;

25°C followed by 12 hours at 5°C ;

5°C followed by 24 hours at 25°C ;

25°C followed by 24 hours at 5°C ;

(Control, non-imbibed seeds are denoted by)

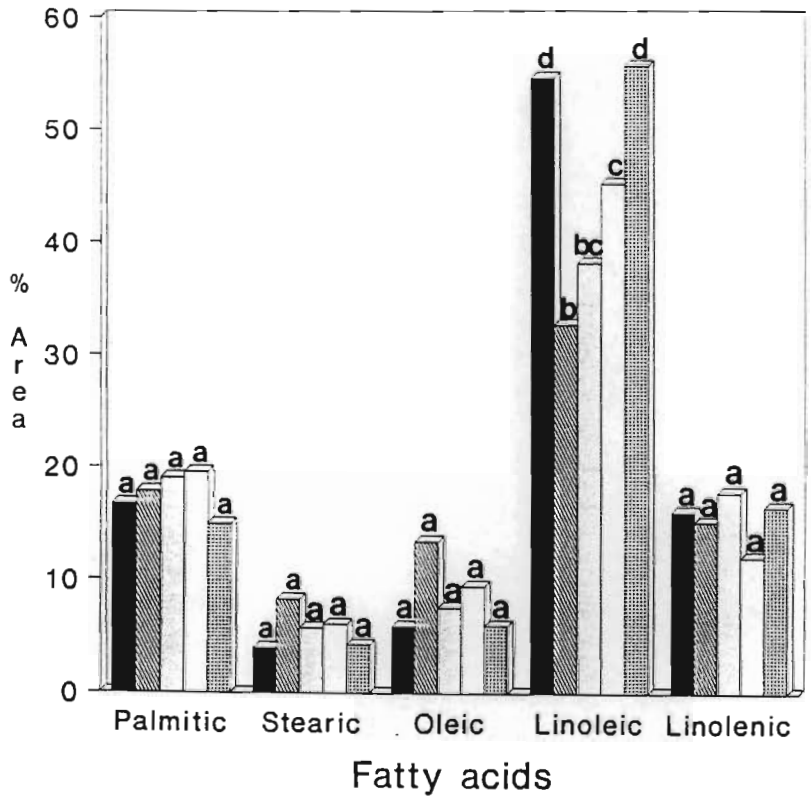


Figure 16 Percentage fatty acid levels of total lipid fraction of soybean axes following imbibition for 12 hours at :

5°C followed by 12 hours at 25°C ;

25°C followed by 12 hours at 5°C ;

5°C followed by 24 hours at 25°C ;

25°C followed by 24 hours at 5°C ;

(Control, non-imbibed seeds are denoted by)

4.8 Effect of Temperature on the Fatty Acid Levels of Different Lipid Fractions

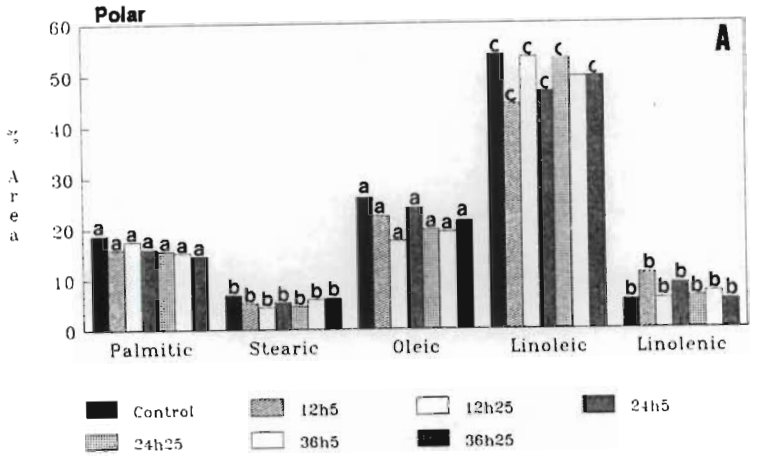
To examine whether membranes might be a possible site for chilling injury, analyses of fatty acids of the phospholipid fractions were made. In addition, the fatty acids of the glycolipid and neutral lipid fraction were also examined.

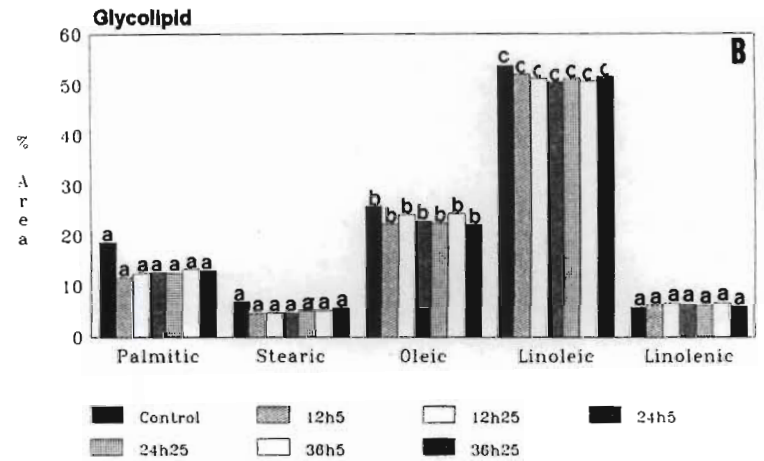
Gas-liquid chromatography analyses of the fatty acid methyl esters indicated that oleic and linoleic acids (unsaturated fatty acids) were the predominant fatty acids in all three lipid fractions of soybean seeds, while palmitic, stearic and linolenic fatty acids were present at much lower levels (Fig.17).
A typical gas chromatograph of the fatty acid methyl esters formed from the fatty acids of soybean seeds is given in Figure 18.

In the polar lipid fraction, a decline of 10% was observed in the linoleic acid levels after imbibition for 12 hours and 24 hours at chilling temperatures compared with the levels of seeds that were imbibed for 12 and 24 hours at 25°C (Fig.17A). No significant changes were observed in the fatty acid levels after 12 and 24 hours at 25°C. Likewise, non-significant differences in this fatty acid level were observed after imbibition for 36 hours at chilling and non-chilling temperatures. A 12% reduction in linoleic acid was observed in seeds that were first imbibed for 12 hours at 25°C before being transferred to 5°C for 12 hours in comparison to seeds that were first imbibed for 12 hours at 5°C and then transferred to 25°C for

12 hours (Fig.17D).

These results are supportive of earlier results obtained for lipid peroxidation studies for seeds in transfer experiments (Fig.12). It was observed that upon transferring seeds from non-chilling to chilling temperatures, peroxidation increased and fatty acid levels decreased but, upon transferring seeds from chilling to non-chilling temperatures, peroxidation levels decreased and fatty acid levels remained high.


Polar
A
% Area
60
50
40
30
20
10
0
Palmitic
Stearic
Oleic
Linoleic
Linolenic
Control
12h5
12h25
24h5
24h25
36h5
36h25


Glycolipid
B
% Area
60
50
40
30
20
10
0
Palmitic
Stearic
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Linoleic
Linolenic
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12h5
12h25
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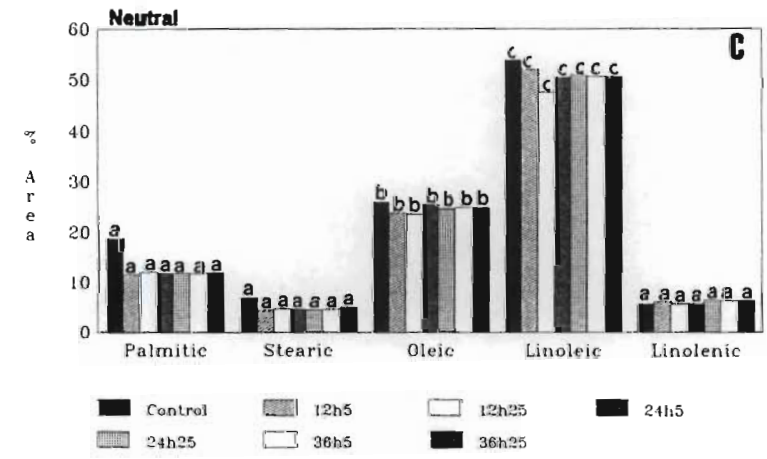
24h25
36h5
36h25


Neutral
C
% Area
60
50
40
30
20
10
0
Palmitic
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12h5
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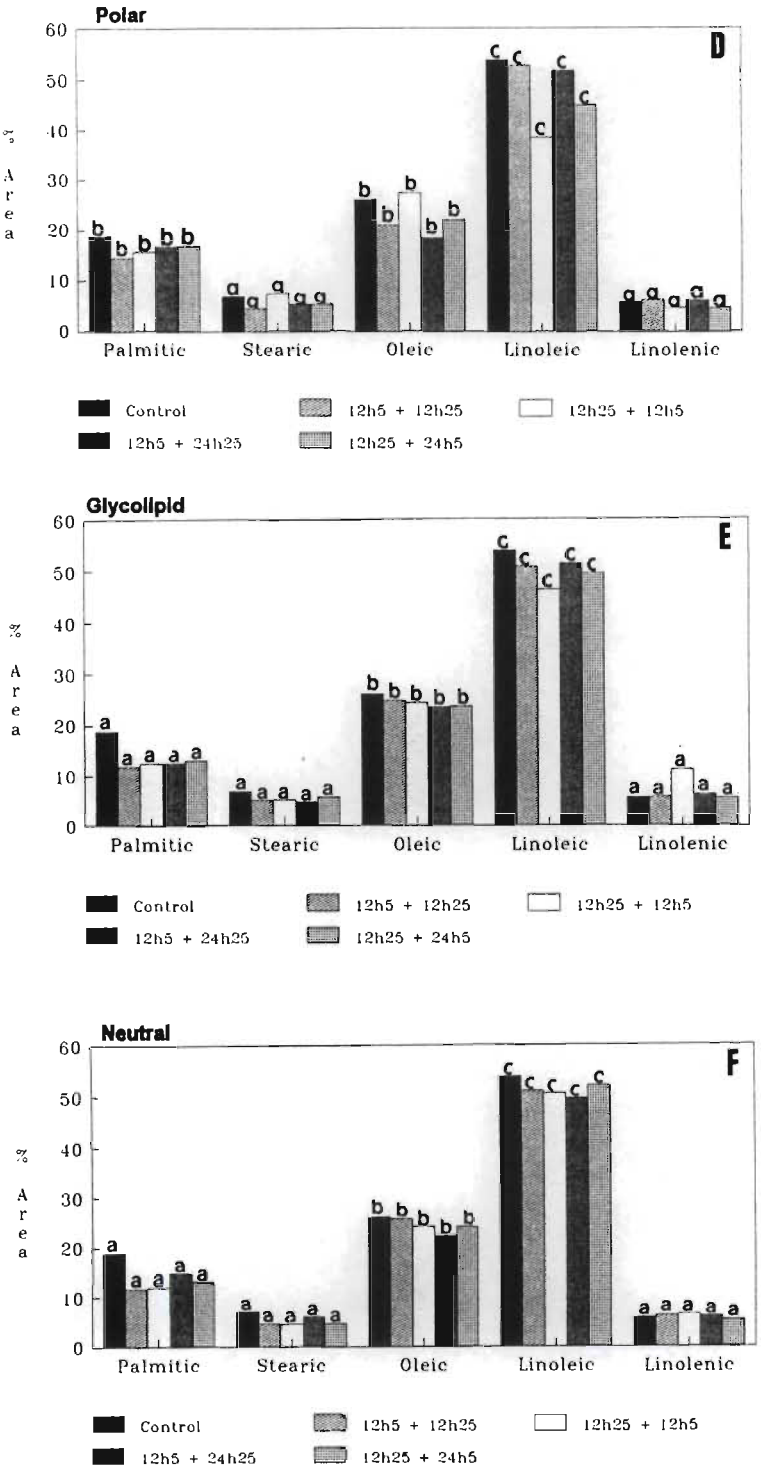


Figure 17 (a-f) Percentage composition of the fatty acids of the polar, neutral and glycolipid fractions of soybean seeds exposed to chilling and non-chilling temperatures. Bars with the same letter are not significantly different from each other.

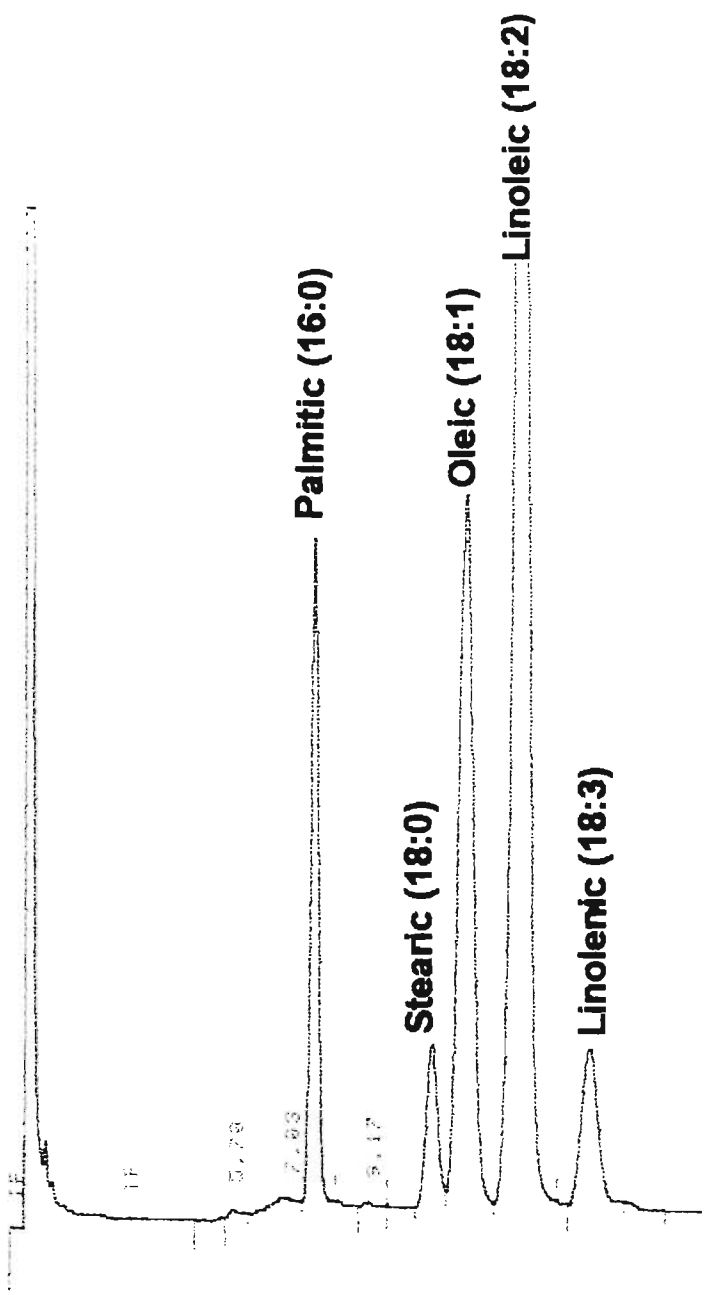


Figure 18 Typical gas-liquid chromatograph trace of the fatty acid methyl esters formed from the fatty acids of soybean seeds. The tentative identification was by comparison of retention times with those of authentic standards.

Differences in the fatty acid levels of the glycolipid fraction were not significant in seeds that were imbibed at chilling and non-chilling temperatures. Both linoleic and linolenic acid levels showed no marked differences (Fig.17B).
Upon transferring seeds from non-chilling to chilling temperatures (12 hours at each temperature) a 7% decrease in linoleic acid was observed (Fig.17E). On the other hand, a 2% increase in linolenic acid was observed. The decrease in the linoleic acid levels of the glycolipid fraction was greater in seeds that were first imbibed for 12 hours at 25^{o}C and then transferred to 5^{o}C for a further 12 hours. Overall, no statistically significant differences were observed.

In neutral lipids, fatty acid levels showed no statistically significant differences in seeds that were imbibed at chilling and non-chilling temperatures. Levels of linoleic acid in control (dry seeds) and in treated seeds remained unaffected, with the average level of this fatty acid being 50% (Fig.17C).
Unsaturated fatty acid levels were only slightly affected in transfer experiments (Fig.17F) and differences were non-significant.

4.9 Sugar Levels During Imbibitional Chilling Injury

Sugars serve as respiratory substrates in plants and sucrose is regarded as the major soluble carbohydrate reserve. Lipids are converted to carbohydrates, which are rapidly translocated from the cotyledons and used by the growing seed.

Sugar levels were determined by gas-liquid chromatography and co-chromatography with authentic standards. A representative chromatogram is given in Figure 20.

Results from this study indicated that sucrose, a disaccharide, was the dominant sugar present in axes isolated from seeds imbibed at chilling and non-chilling temperatures, ranging from 55% in the dry seed (control) to 21% in seeds after 36 hours imbibition (Figs.19, 21 and 22). Monosaccharides such as fructose and glucose were also present at an average of 15%. Oligosaccharides such as stachyose (14%) and raffinose (5%) were also present.

Axes from seeds that were imbibed at chilling and non-chilling temperatures, showed a marked decline in sucrose levels (Figs.19 A & B). After 6 hours at each temperature, a significant decrease (16%) in sucrose was observed but thereafter the levels remained relatively constant at 35% in non-chilled axes and after 24 hours, the decrease was not statistically significant in chilled axes.

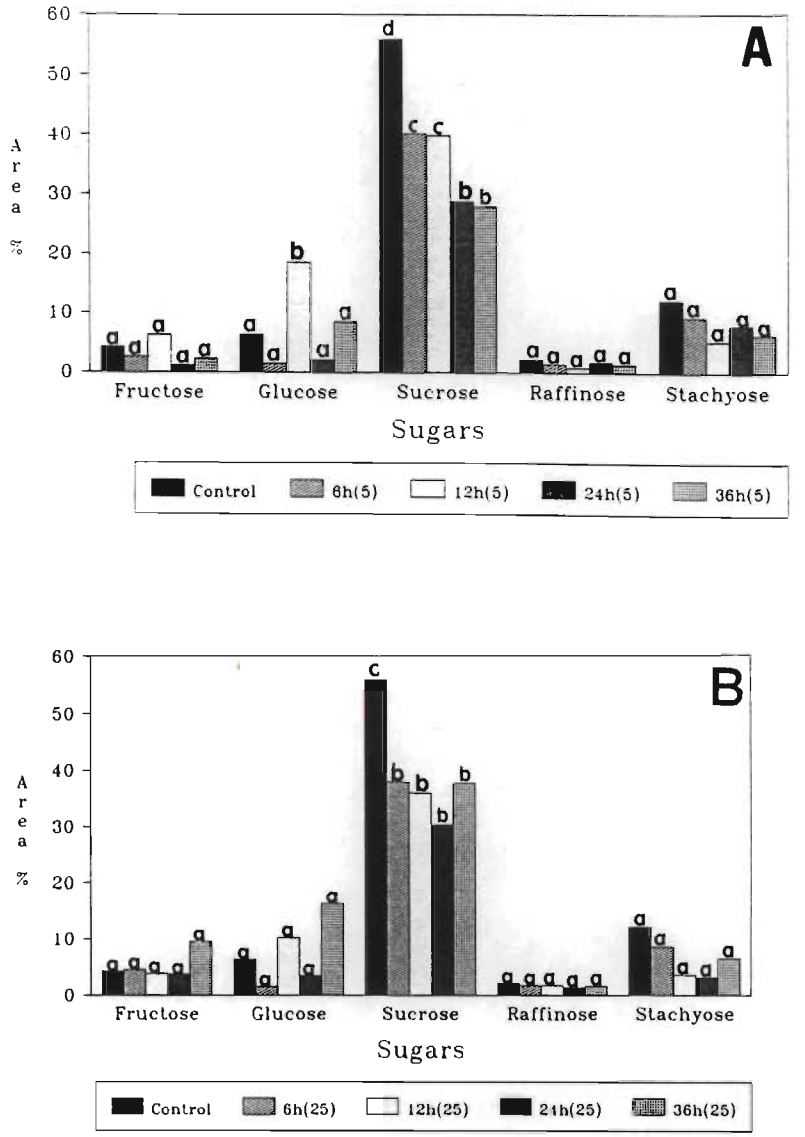


Figure 19 Percentage composition of the free sugars in axes isolated from soybean seeds after imbibition at chilling (A) and non-chilling (B) temperatures. The composition was determined by gas-liquid chromatography of the silylated sugars. Bars with the same letter are not significantly different from each other.

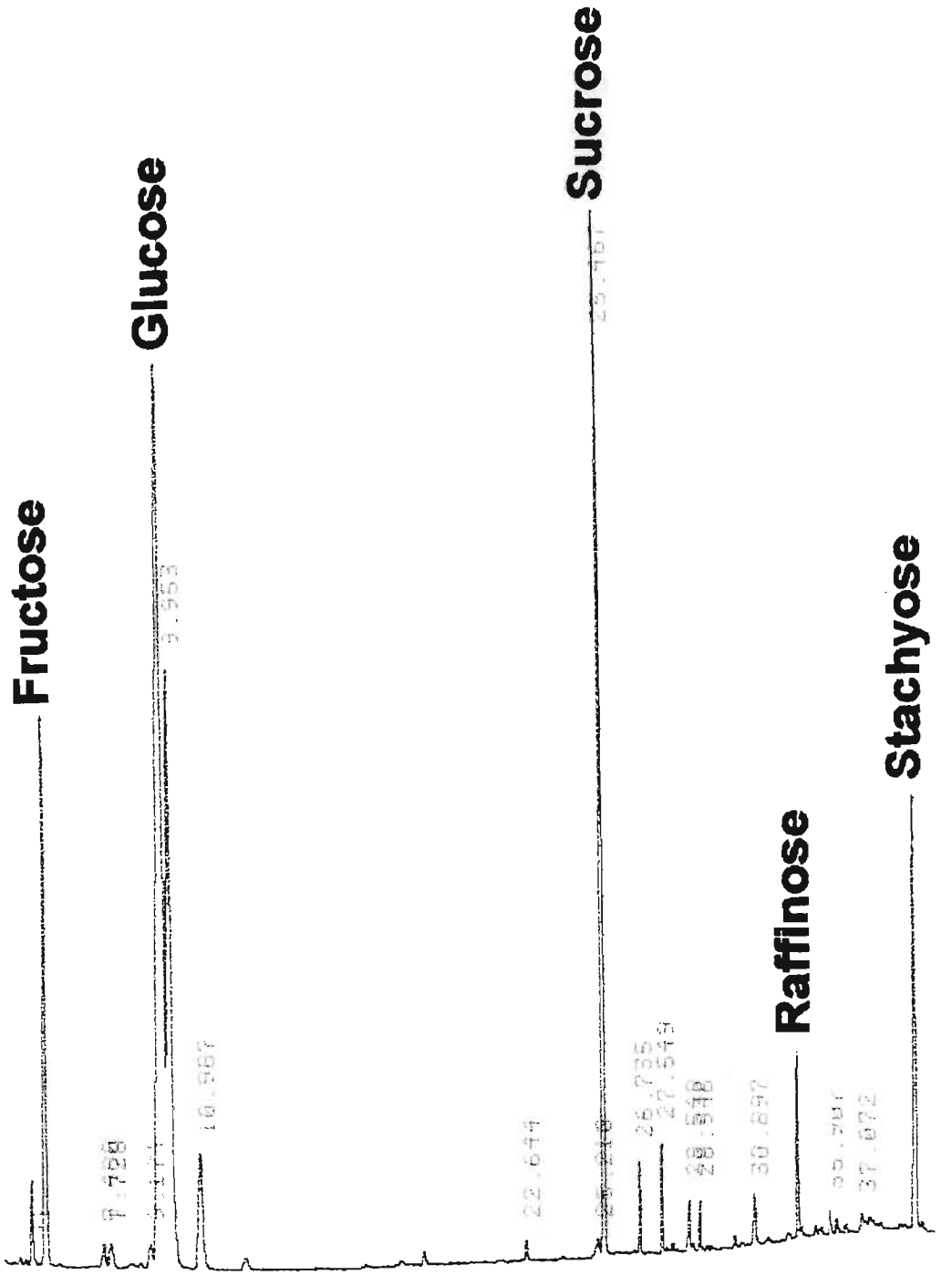


Figure 20 Typical gas liquid chromatograph trace of the silylated forms of the sugars of soybean axes imbibed at 5°C or 25°C. The tentative identification was by comparison of retention times with those of authentic standards.

In chilled axes, glucose levels showed a slight decline after 6 hours at 5°C, but showed a transient increase of 18% after 12 hours, followed by a marked decrease after 24 hours and an 8% increase by 36 hours (Fig.19A). The same pattern, with respect to glucose was observed in non-chilled axes. Levels of fructose, raffinose and stachyose levels remained low and showed no evident pattern changes.

As part of a further study on the effect of moisture content on temperature sensitivity, soybean seeds were equilibrated to low (6.2%) and high (20.9%) moisture contents and sugar levels were measured in axes isolated from chilled and non-chilled seeds (Figs. 21 and 22). As previously noted, sucrose was the most abundant sugar in the axes of low and high moisture content seeds. This was followed by stachyose, glucose and fructose, while the contribution of raffinose was low (2-4%).

In low moisture content axes, sucrose levels declined by 8% during the first 12 hours of imbibition at chilling temperatures, followed by a rapid decline of 30% after 36 hours (Fig.21A). Glucose levels of 10% were observed after 24 hours at 5°C, and increased to 15% after 36 hours imbibition. Non-chilled axes showed a rapid decline (20%) in sucrose levels after 6 hours, followed by a slight, non-significant increase after 12 hours and a decline thereafter (Fig.21B). Glucose levels were largely unchanged at 25°C.

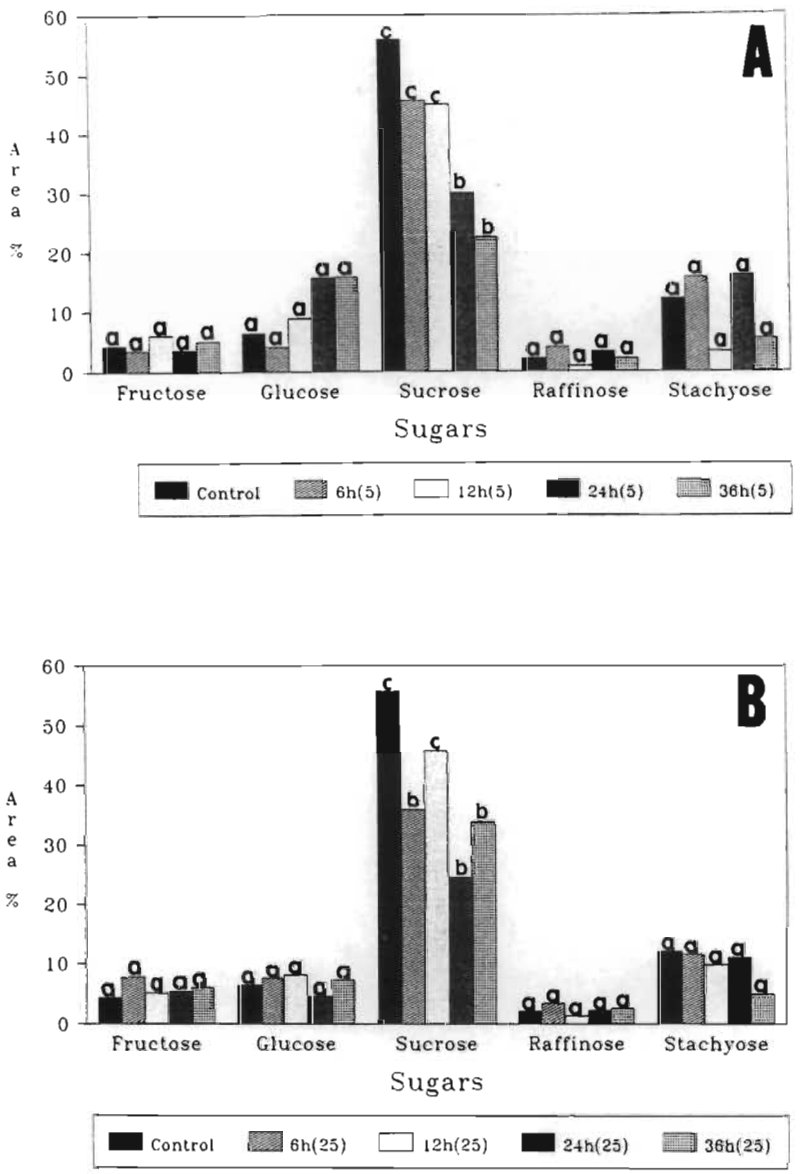


Figure 21 Percentage composition of the free sugars in axes isolated from low moisture content soybean seeds after imbibition at 5°C (A) and 25 °C (B). The composition was determined by gas-liquid chromatography of the silylated sugars. Bars with the same letters are not significantly different from each other.

However, low moisture content axes exhibited the greatest reduction in sucrose levels after imbibition for 36 hours at chilling and non-chilling temperatures. A 30% reduction was observed after 36 hours at 5°C in comparison to a 20% reduction at 25°C.

In high moisture content axes sucrose levels were generally higher (Fig.22) and axes from dry seeds (control) had 57% sucrose.

At chilling temperatures, a rapid decline to 35% in sucrose levels was observed after 12 hours followed by a slight decline to 26% after 24 and 36 hours (Fig.22A). Fructose, raffinose and stachyose levels remained relatively constant, but a non-significant increase (9%) in glucose was observed after 6 hours at 5°C.

At non-chilling temperatures, sucrose levels decreased from 57% in the dry seed to 46% after 6 and 12 hours. Thereafter, sucrose levels declined rapidly to 33% then to 22% after 24 and 36 hours respectively (Fig.22B).

Fructose, glucose, raffinose and stachyose levels remained low, but a marked increase in glucose levels was observed after 6 hours at 25°C.

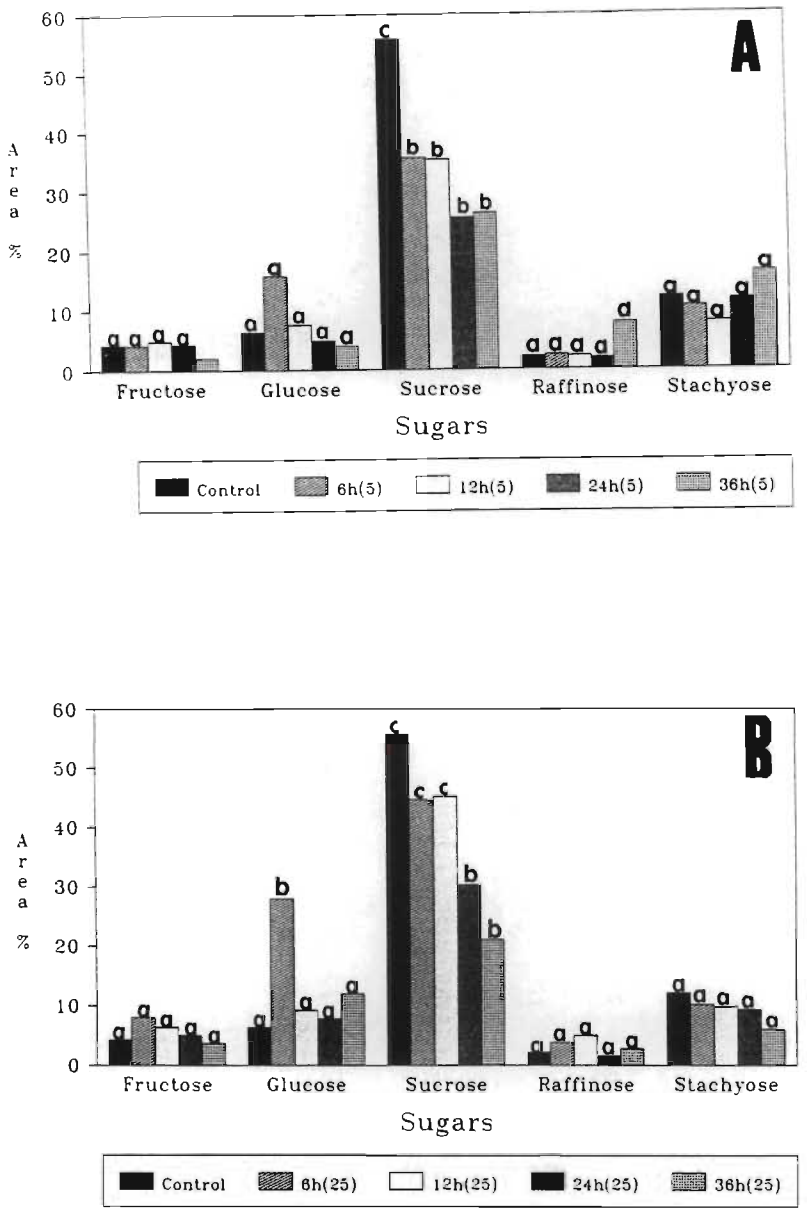


Figure 22 Percentage composition of the free sugars in axes isolated from high moisture content soybean seeds after imbibition at 5°C (A) and 25 °C (B). The composition was determined by gas-liquid chromatography of the silylated sugars. Bars with the same letter are not significantly different from each other.

4.10 Proteins and Antioxidant Defense Enzymes

An electrophoretic analysis of seed total protein patterns and the isozyme enzyme patterns of the antioxidant enzymes (catalase, peroxidase and superoxide dismutase) were examined in soybean seeds that were imbibed at chilling and non-chilling temperatures. Total protein patterns showed no apparent qualitative or quantitative changes (Data not presented). However, changes were observed in the antioxidant enzyme systems at chilling and non-chilling temperatures.

Expression of the catalase enzyme during imbibition at chilling and non-chilling temperatures over a 48 hour period is shown in figures 23 A & B. A visual assessment of the gels showed that the enzyme appeared as a single band at the top of the gel (Fig.23A), but scanning of the gels using densitometry, suggested that two isozyme bands were present in the dry seed (Fig.23B). The second band could be an artifact since no additional isozymes occurred during the subsequent sampling intervals studied (Fig.23 A).

In figure 23B, dry seeds (Lane 1) showed two catalase isozymes with very low enzyme activity. There was a marked increase in catalase activity following chilling for 24 hours (Lane 2), while non-chilled seeds showed only a smaller increase in activity after imbibition for 24 hours at non-chilling temperatures (Lane 3). Densitograms showed that the enzyme activity after 24 hours at non-chilling temperatures was similar to that seen in seeds

Figure 23 A Polyacrylamide gel electrophoresis of proteins after imbibition at chilling and non-chilling temperatures. Staining reveals catalase activity in dry seeds (1) , 24 hours at 5°C (2), 24 hours at 25°C (3), 48 hours at 5 °C (4) and 48 hours at 25°C (5).

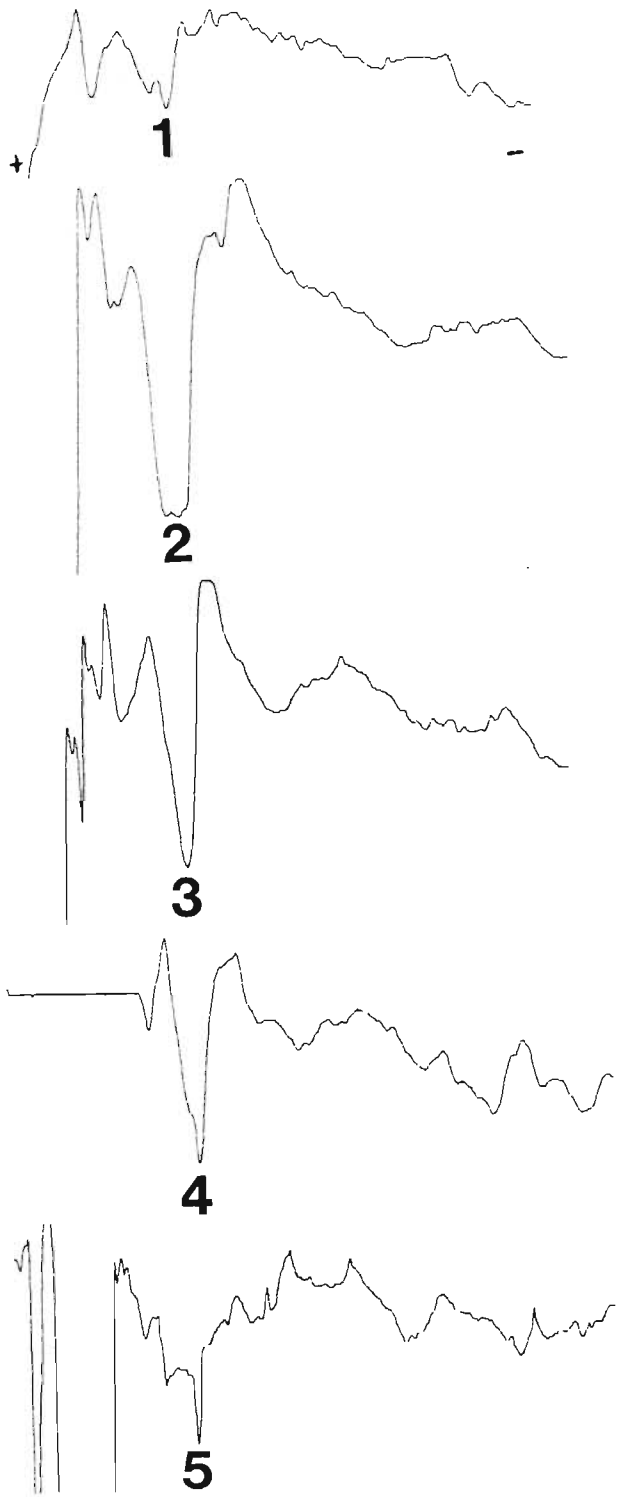


Figure 23 B Densitometric scans of catalase activity revealed by stained gel electrophoresis of soybean seed extracts from dry seeds (1), seeds imbibed for 24 hours at 5°C (2), 24 hours at 25°C (3), 48 hours at 5°C (4) and 48 hours at 25°C (5).

imbibed at chilling temperatures for 24 hours (Lane 2). However, enzyme activity was markedly reduced in seeds that were imbibed for 48 hours at 5°C (Lane 4) although these were not as low as those observed in seeds imbibed for 48 hours at 25°C (Lane 5).

The peroxidase activity in seeds that were imbibed at chilling and non-chilling temperatures is shown in figure 24. Bands were somewhat indistinct and no qualitative changes could be detected over the sampling periods. The only clear feature to emerge was that dry seeds exhibited a high level of peroxidase activity (Lane 1); this contrasts markedly with catalase activity which was very low or virtually absent in the dry seed (Lane 1, Fig.23).

A notable decline in peroxidase activity after imbibition for 24 hours at chilling temperature was observed (Lane 2) compared to a smaller decline after 24 hours at non-chilling temperatures (Lane 3). After 48 hours of chilling, there is evidence for slight peroxidase activity (Lane 4, Fig 24), whereas peroxidase was almost absent after 48 hours at non-chilling temperatures (Lane 5).

It can be seen from figure 24 (Lane 1) that peroxidase, unlike catalase, appeared to be a very stable enzyme in dry seeds.

On the other hand, catalase levels are low and virtually absent in the dry seed (Lane 1, Fig.23), being induced during subsequent imbibition.

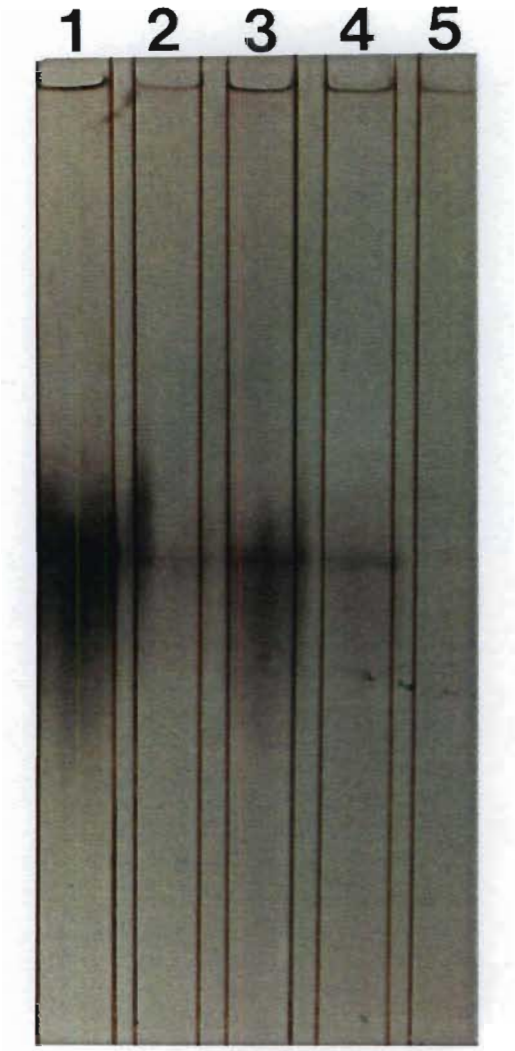


Figure 24 Polyacrylamide gel electrophoresis of proteins after imbibition at chilling and non-chilling temperatures. Staining reveals peroxidase activity in dry seeds (1), 24 hours at 5°C (2), 24 hours at 25 °C (3), 48 hours at 5 °C (4) and 48 hours at 25°C (5).

The results of studies on superoxide dismutase (SOD) activity in response to imbibition at chilling and non-chilling temperatures can be seen in figure 25.

The nitroblue tetrazolium staining technique revealed Mn, Fe and Cu/Zn superoxide dismutases (Fig.25A), with Cu/Zn SODs having the highest mobility and the lowest molecular weight.

Cu/Zn SODs are sensitive to KCN staining, therefore only Mn and Fe SODs were revealed when this was present in the incubation medium (Fig.25B) and Cu/Zn and Fe SODs are sensitive to H_2O_2, thus only Mn SODs, with the highest molecular weight and lowest mobility, were revealed when this was present in the incubation medium (Fig.25C).

No new isozymes were expressed in seeds over the time intervals examined. All lanes had the same number of isozymes : Mn SODs being the first single bands, followed by three light bands representing the Fe SODs. Immediately below the Fe SODs, three pronounced and more distinct bands were observed and identified as Cu/Zn SODs.

A visual assessment of the gel suggested that SOD activity was the same in lane 1 (dry seeds), lane 2 (24 hours at 5°C), lane 3 (24 hours at 25°C),and seeds that were imbibed for 48 hours at 5°C (lane 4) and 25°C (lane 5).

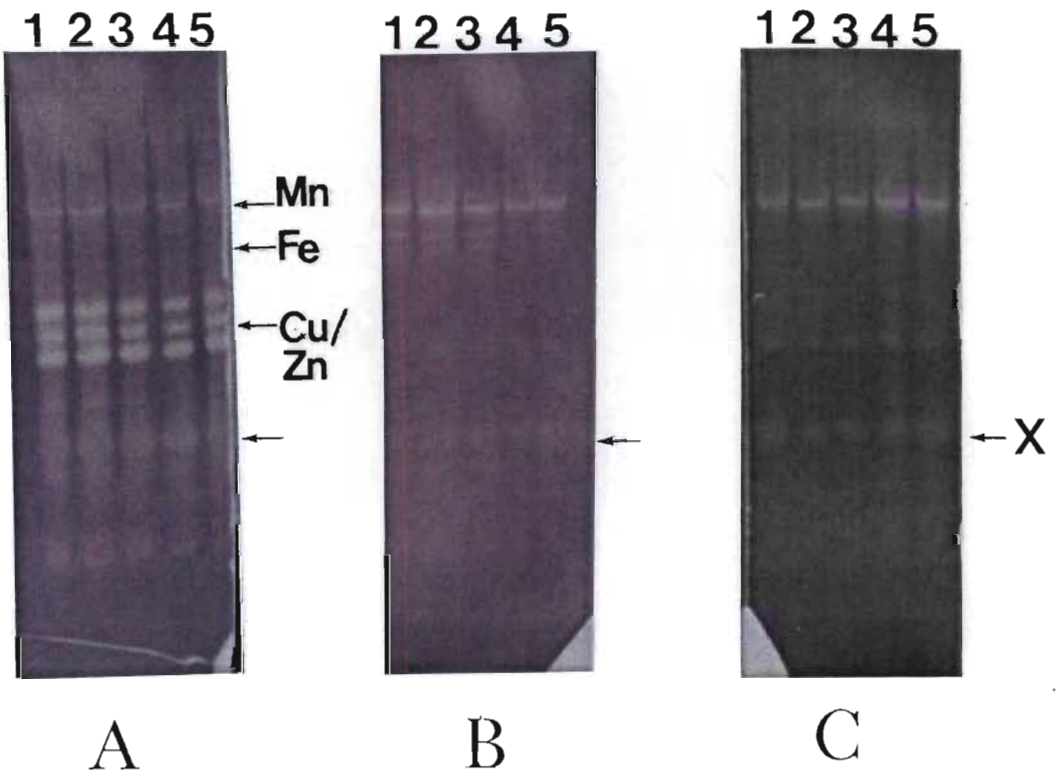


Figure 25 Polyacrylamide gel electrophoresis of proteins after imbibition at chilling and non-chilling temperatures. Staining reveals superoxide dismutase isozymes in dry seeds (1), 24 hours at 5°C (2), 24 hours at 25°C (3), 48 hours at 5°C (4) and 48 hours at 25°C (5). Mn, Fe and Cu/Zn SODs are shown in (A), Insensitivity of Mn and Fe SODs to cyanide (B) and insensitivity of Mn SODs to hydrogen peroxide (C).

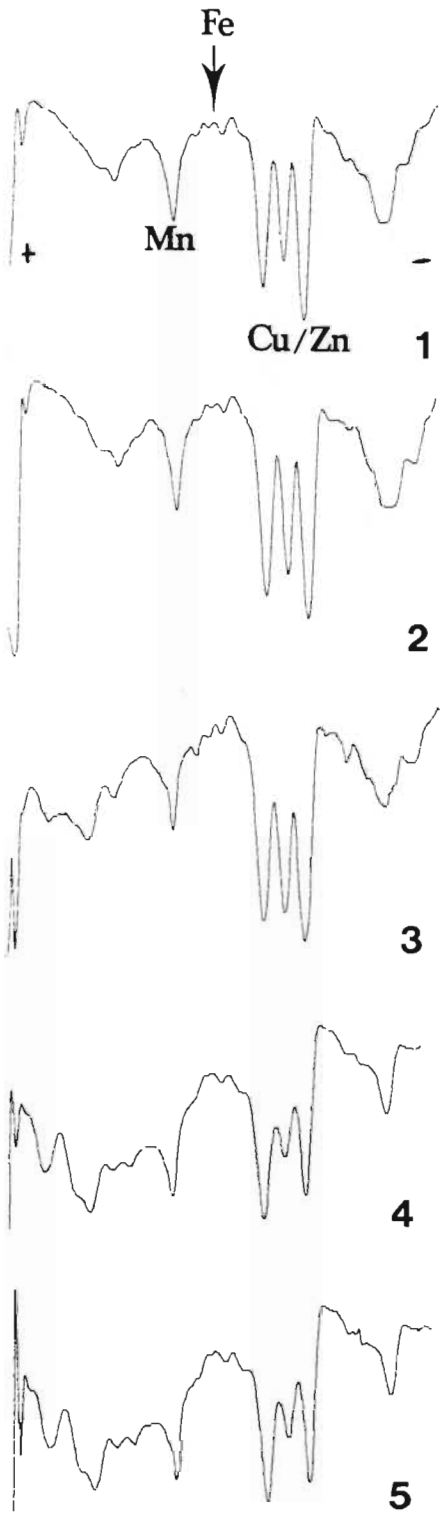


Figure 26 Densitometric scans of superoxide dismutase activity revealed by stained gel electrophoresis of soybean seed extracts from dry seeds (1), seeds imbibed for 24 hours at 5°C (2), 24 hours at 25°C (3), 48 hours at 5°C (4) and 48 hours at 25°C (5) containing levels of Mn, Fe and Cu/Zn SODs.

However, densitometric analysis of the gel showing superoxide dismutase activity (Fig.26, traces 1- 5), revealed that the activity of Mn SODs was initially high in the dry seed and seeds that were imbibed for 24 hours at 5°C. This was followed by a decrease in activity after imbibition for 24 hours at 25°C (3), 48 hours at 5°C (4) and 48 hours at 25 °C (5). Fe SOD levels were apparently unchanged in all treatments.

Overall, Cu/Zn superoxide dismutases showed the greatest amount of activity at chilling and non-chilling temperatures. Enzyme activity was highest in dry seeds (1), seeds imbibed for 24 hours at 5°C (2) and 24 hours at 25°C (3). Thereafter, activity decreased after 48 hours at chilling (4) and non-chilling (5) temperatures (Fig.26).

Additional bands of high mobility and low molecular weight were seen on the gels below the Cu/Zn SODs (X in Fig.25). These bands showed a different colour reaction (pinkish) in comparison to the typical colour of SOD isozymes. This raised a question as to whether they represented true SOD activity, lipids from the protein extract or other proteins. This was investigated by taking extracts from dry seeds and seeds that were imbibed for 24 hours at 5°C, and boiling them at 100°C for 5 minutes before electrophoresis. However, the two bands of high mobility persisted. It was concluded that the bands might represent dehydrogenases, since they considered thermostable and use tetrazolium salts as their substrates, but this will have to be confirmed by further tests.

4.11 Transmission Electron Microscopy

The ultrastructural characteristics of the embryonic axes of soybean seeds exposed to chilling (5°C) and non-chilling (25°C) temperatures are illustrated in Plates 1- 4. Dry axes of seeds were not examined due to problems associated with the fixation and embedding of dry tissues.

Plate 1 A & B represents cells from embryonic axes after 1 hour of imbibition at chilling temperatures and cells appear to be fully hydrated.

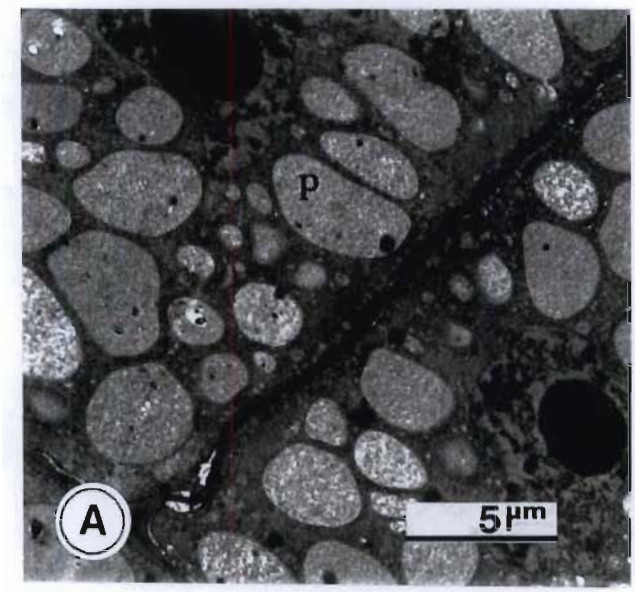
Generally, lipid bodies appeared as small, opaque spheres, whilst the protein bodies were much larger and contained finely, floccular material. On the other hand, the cytoplasm had a somewhat denser appearance. Lipid bodies were predominantly near the wall. Nuclei were irregular in shape and well developed nucleoli were evident.

After 1 hour imbibition, the ultrastructure of non-chilled cells was indistinguishable in appearance to those of chilled cells.

After 12 hours of imbibition, chilled cells (Plate 2 A & B) exhibited a very dense cytoplasm with irregular shaped nuclei and large prominent nucleoli. Nuclear membranes appeared in negative relief. Lipid bodies were predominantly aligned along the walls. Protein bodies were abundant and the constituent floccular material appeared absent in some cases, or coalesced at the vacuolar membrane.

PLATE 1 Ultrastructure of the embryonic axis of soybean seeds during imbibition

A & B) Epidermal cells after 1 hour imbibition at chilling temperatures. Note the even distribution of protein (p) and lipid (l) bodies in the cytoplasm.
Non-chilled cells were of similar appearance.


p
A
5µm

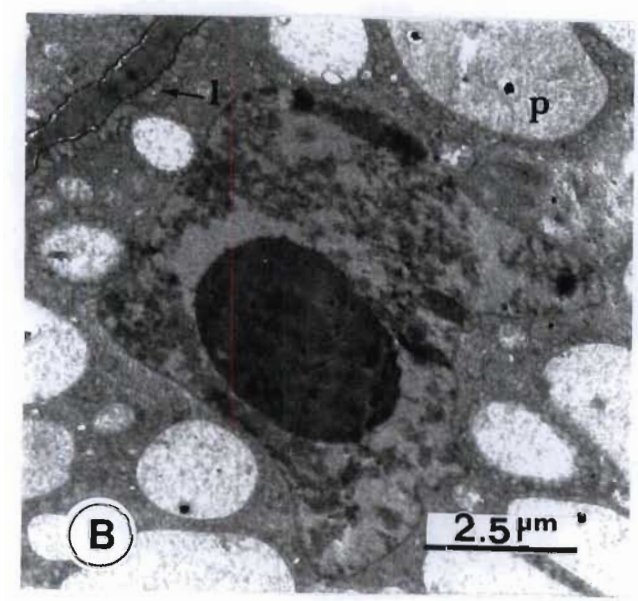
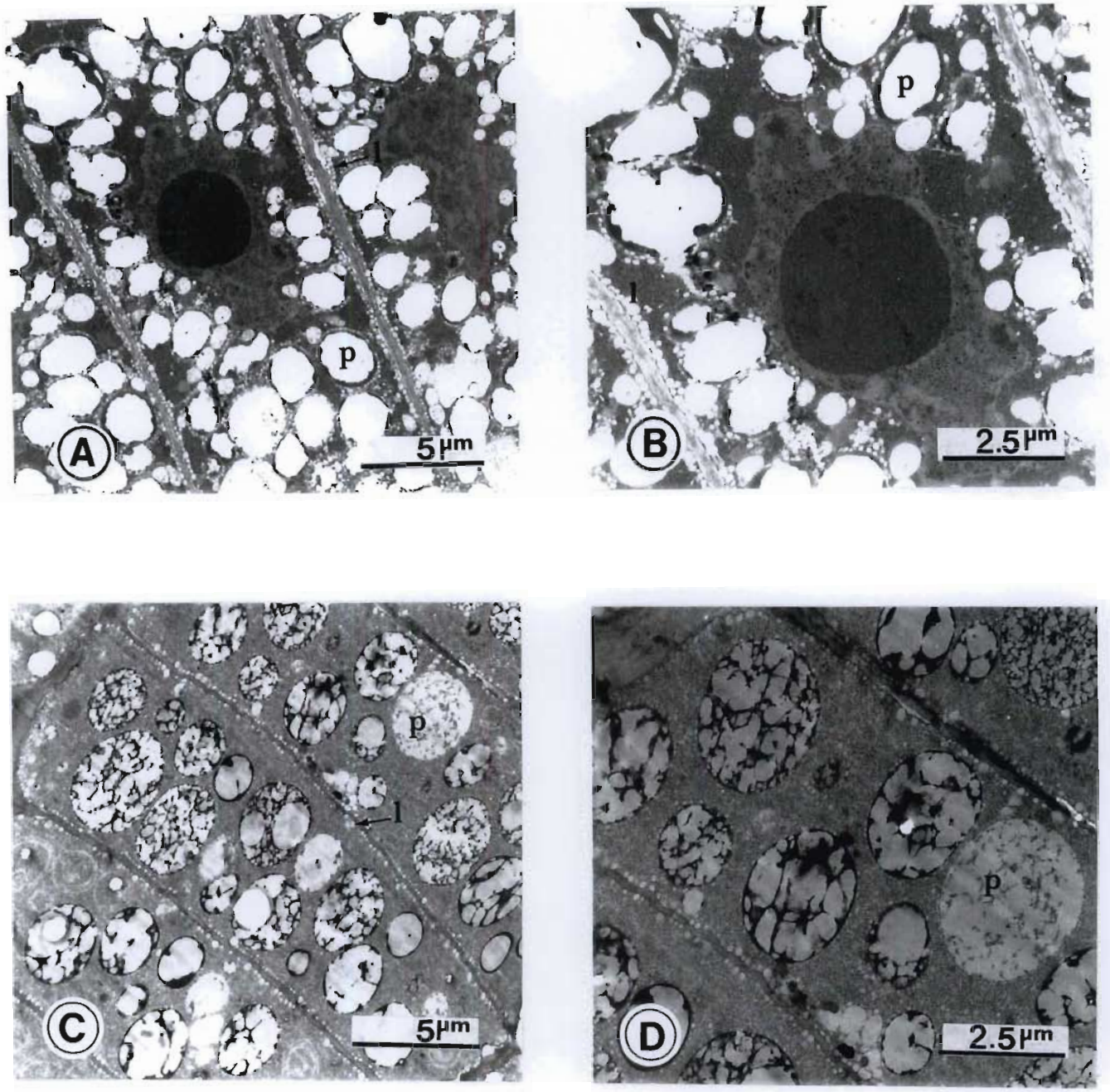

l
p
B
2.5µm

PLATE 2 Ultrastructure of the embryonic axis of soybean seeds during imbibition

A & B) Cortical cells after 12 hours imbibition at chilling temperatures. Cytoplasm is very dense and has abundant protein (p) and lipid (l) bodies. Some protein bodies have flocculent material.

C & D) Cortical cells after 12 hours imbibition at non-chilling temperatures. Cytoplasm is less dense and some protein bodies contain flocculent material. Fewer lipid bodies are evident at the wall.


A
p
l
5μm
B
p
l
2.5μm
C
p
l
5μm
D
p
2.5μm

Non-chilled cells (Plate 2 C & D) had well preserved and contrasted membranes. The cytoplasm appeared less dense in comparison to chilled cells. Possibly as a result of this, organelles were generally more clearly visible in the cytoplasm. Protein bodies were large in size, defined, and contained a sparsely floccular material. Lipid bodies still lined the wall, but they were less abundant especially around the vacuoles.

After imbibition for 24 hours at chilling and non-chilling temperatures, the following ultrastructural changes were evident.

At 25°C, reserve mobilization had occurred to a greater extent than at 5°C (Plate 3 C & D). There was clear differentiation of internal organelles and mitochondria were numerous and visible. Nuclei were clearly evident and the nucleoli had vacuoles. The cytoplasm of meristematic cells, in particular, appeared metabolically active.

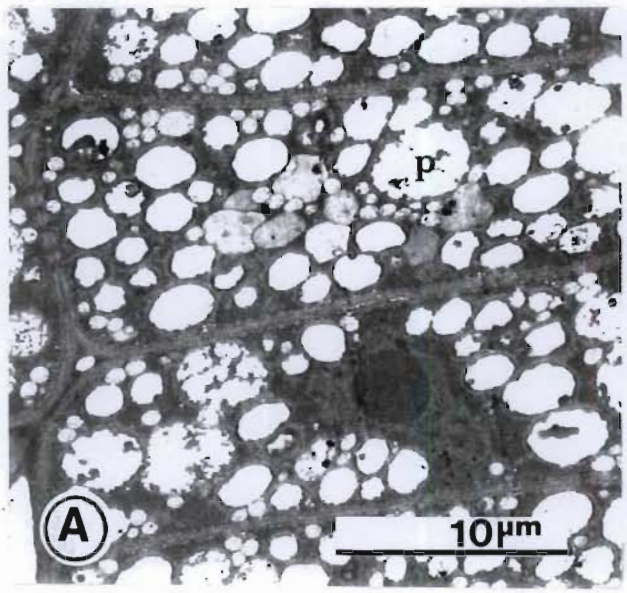
After 24 hours at chilling temperatures, the appearance of lipid and protein bodies had not altered much from the previous 12 hours (Plate 2 A & B). Protein bodies were still present and scattered throughout the cytoplasm. Lipid bodies were clustered around protein bodies and also around the periphery of the cell. Nuclei had poorly defined envelopes and nuclei were dense and without vacuoles (Plate 3 A & B).

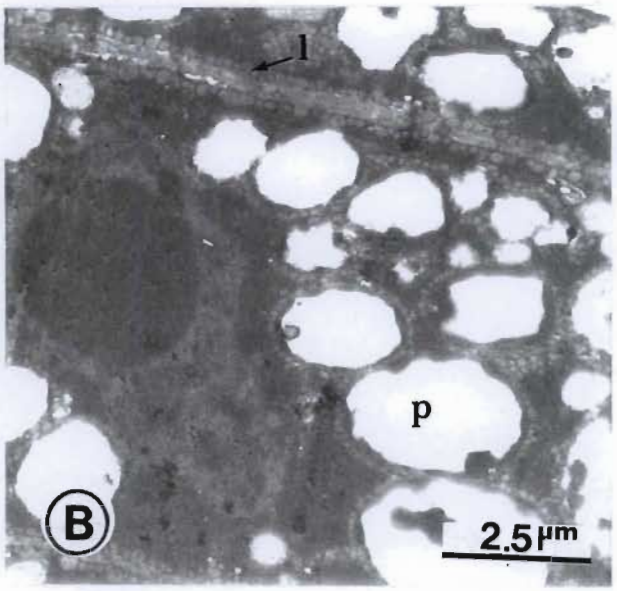
After 48 hours at 25°C, a marked change in the ultrastructure of the embryonic axis cells was indicative of a more metabolically active cytoplasm, possibly reflecting the onset of germination. The protein bodies contained less floccular

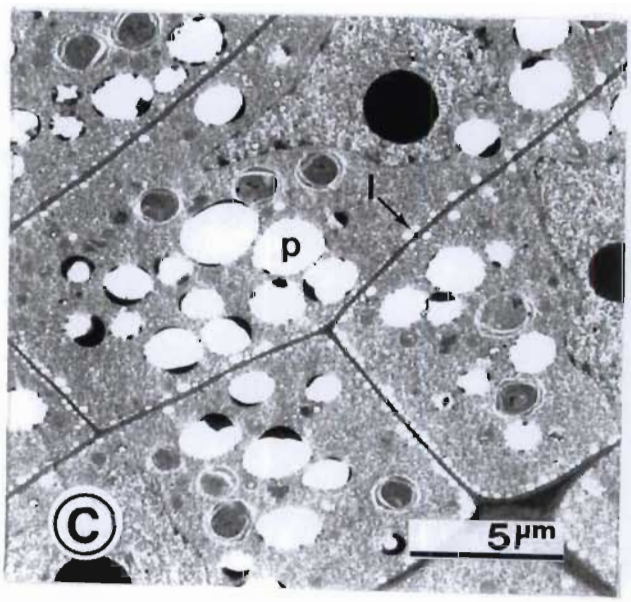
PLATE 3 Ultrastructure of the embryonic axis of soybean seeds during imbibition

A & B) Cortical cells showing undigested protein (p) bodies after 24 hours at chilling temperatures. Lipid (l) bodies are scattered throughout the cytoplasm, while some are concentrated along the wall.

C & D) Meristematic cells after 24 hours imbibition at non-chilling temperatures. Lipid and protein bodies are not abundant in the cytoplasm. Mitochondria (m) are visible.


p
A
10µm


l
p
B
2.5µm


l
p
C
5µm

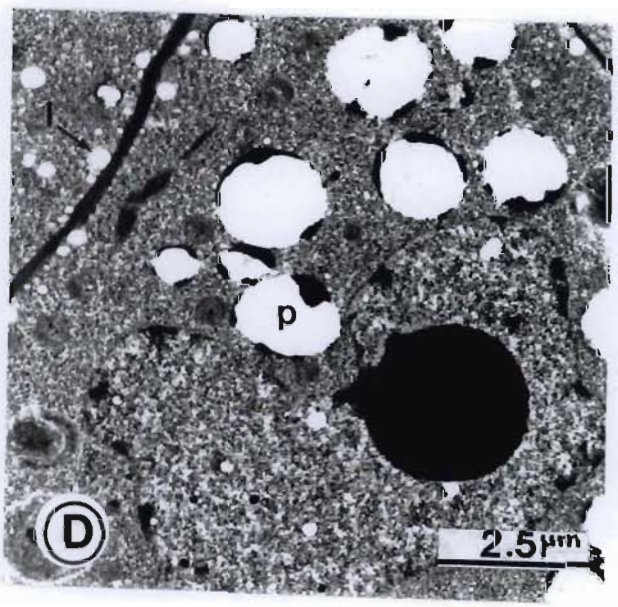
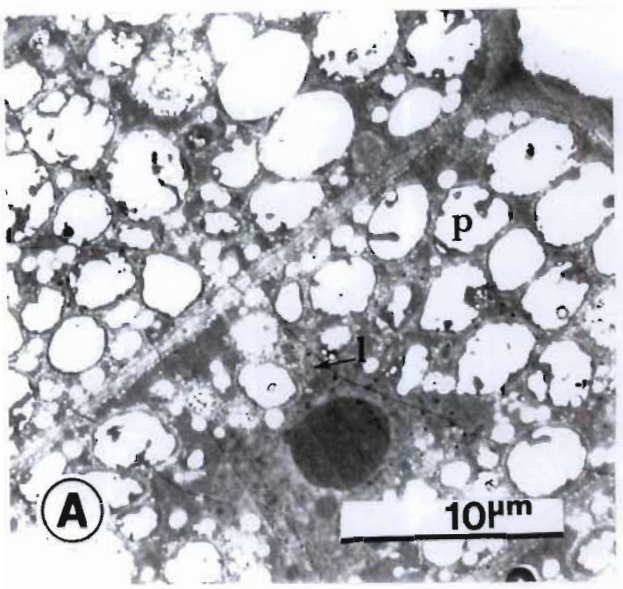
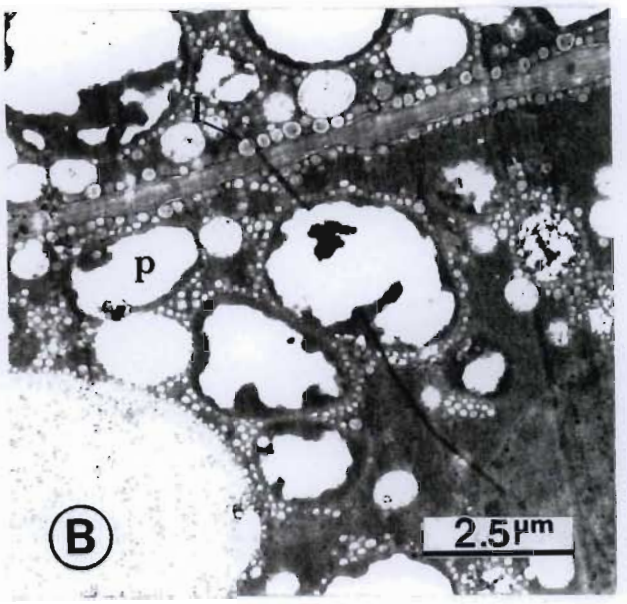

l
p
D
2.5µm

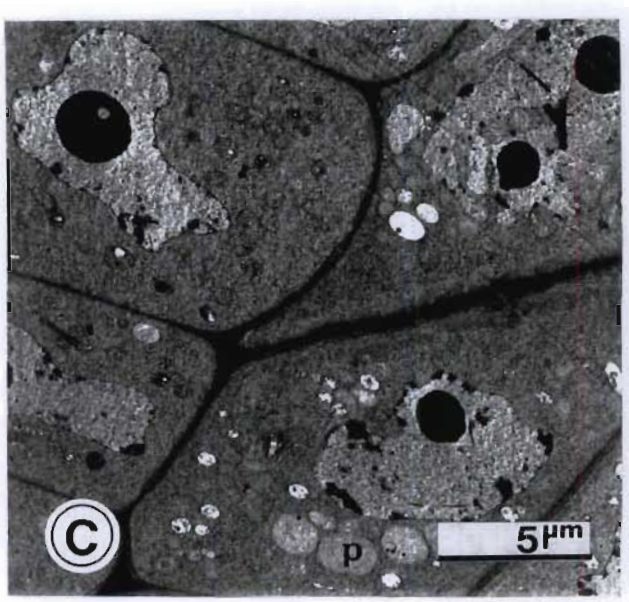
PLATE 4 Ultrastructure of the embryonic axis of soybean seeds during imbibition

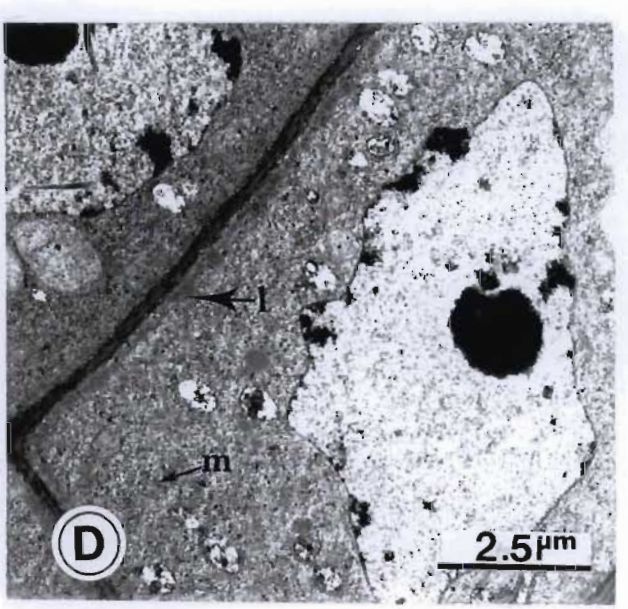
A & B) Cortical cells 48 hours after the start of imbibition at chilling temperatures. Cytoplasm is very dense with lipid (l) and protein (p) bodies. Internal organelle structure is not visible.

C & D) Meristematic cells after imbibition at non-chilling temperatures for 48 hours. At this stage, all pre-germinative processes were complete and radicle emergence occurred. The cells contain fewer protein and lipid bodies. Internal organelle structure is visible.


p
l
A
10µm


l
p
B
2.5µm


C
p
5µm


l
m
D
2.5µm

material and represented the early stages of the formation of vacuoles. Mitochondria and other internal membranes and organelles were differentiated and clearly visible (Plate 4 C & D). Lipid bodies were still evident.

After 48 hours of imbibition at chilling temperatures, cortical cells showed a cytoplasm filled with lipid and protein bodies. Protein bodies often contained dense material at the vacuolar membranes and nuclei had large central nucleoli with poorly defined envelopes (Plate 4 A & B).

Overall, the mobilization of reserves (lipids and proteins) appeared to be markedly impeded at 5^{o}C.

CHAPTER 5

DISCUSSION

A number of biochemical and physiological processes occur during imbibition and the initial stages of germination, such as membrane reorganization (SIMON,1974) and metabolic reactivation (BERJAK and VILLIERS, 1972) involving aspects such as increased oxygen uptake (BEWLEY and BLACK,1985), wetting of the seed coat and release of adsorbed gases (PARRISH and LEOPOLD,1977), increased mitochondrial respiratory activity supporting ATP synthesis (WEBSTER and LEOPOLD,1977), enhanced mitochondrial development (MOROHASHI,1986), rapid increases in protein-synthesizing capacity (MARCUS, FEELEY, VOLCANI,1966) and solute leakage (SENARATNA and McKERSIE,1983).

Lipid peroxidation is a fact of life during seed imbibition and more complicated processes such as hydroperoxide metabolism during seed germination has received little attention. WILSON and McDONALD(1986) revealed that during early imbibition in germinating seeds, the reaction of lipids with oxygen decreased as membranes hydrated and organized. Hydroperoxide lyase is activated and breaks down oxygenated fatty acids, causing an increase in free radicals, leading

to the formation of toxic secondary products which may inhibit respiration, protein and DNA synthesis and denature proteins. However, the capacity of germinating seeds to produce hydrogen peroxide scavenging enzymes (catalase, peroxidase and superoxide dismutases) between 24 and 48 hours of imbibition has been shown in wheat seeds (CAKMAK, STRBAC and MARSCHNER,1993). An understanding of these processes is important because they can have a major influence on seed germination and the further growth of the seedling.

5.1 Characteristics of Soybean Seed Imbibition

Many seeds are injured by low temperature exposure during imbibition such as soybean (HOBBS and OBENDORF,1972), cotton (CHRISTIANSEN,1968) and some vegetables (KOTOWSKI,1926). However, pea and radish seeds appear to be chilling resistant (KOTOWSKI,1926).

Water uptake studies revealed that soybean seeds imbibed water at a faster rate at non-chilling temperatures. This observation is in keeping with other studies which have shown that the rate of water entry into the soybean seed is enhanced by higher temperatures (LEOPOLD,1980 ; VERTUCCI and LEOPOLD, 1983). Results from this study have led to the suggestion that the slower weight increase observed in seeds that were imbibed between 6 and 12 hours at chilling temperatures is likely to be a reflection of poor turgor development, and it was probably during this time that CI damage occurs. These studies also indicated that the rate of water uptake and radicle emergence were temperature-dependent,

an observation also supported by the studies of TULLY, MUSGRAVE and LEOPOLD,1981. Using soybean seeds, they showed that imbibitional chilling is a consequence of the rate of water uptake.

At chilling temperatures, water uptake was most rapid between 6 and 12 hours of imbibition, suggesting that it was during this time that CI damage occurs. POLLOCK and TOOLE (1966) were probably the first to report that the first minutes of water entry were especially critical in terms of chilling injury to seeds. Furthermore, POWELL and MATTHEWS (1978) observed that in imbibing pea seeds, cell death occurred as a result of rapid water uptake during the first 2 minutes of imbibition. This observation led to the suggestion that the injury at low temperature results from imbibitional damage rather than the effects of low temperature *per se*. Support for this proposal was obtained by showing the effectiveness of polyethyleneglycol in slowing the initial rate of water uptake and lessening damage.

Furthermore, TULLY, MUSGRAVE and LEOPOLD (1981) also showed that slowing the rate of initial imbibition does indeed impart chilling resistance to soybean. The interference with normal membrane reorganization during imbibition (PARRISH and LEOPOLD,1977) has also been proposed as a possible basis of low temperature injury (BRAMLAGE, LEOPOLD and PARRISH,1978), and was first suggested for yeast rehydration by HERRERA, PETERSON, COOPER and PEPPLER (1956). SIMON (1974) attributed the chilling damage to an interference with a supposed transition from a hexagonal organization of phospholipids in the dry state to the lamellar organization in the aqueous state.

Thus, the fact that water uptake, membrane reorganization and metabolic activation are taking place during early imbibition and are co-incident with the induction of CI make the determination of the primary mechanism of CI rather difficult.

5.2 Initial Seed Moisture during Imbibition

Many studies have reported that initial seed moisture content is important in protecting seeds from imbibitional CI. This has been shown in cotton (CHRISTIANSEN,1969) and lima bean (POLLOCK,1969).

Results from this study showed that chilled seeds of low moisture content imbibed water at a slower rate than seeds imbibed at 25°C (Fig.5A). Furthermore at 25°C, high moisture content seeds imbibed greater amounts of water than low moisture content seeds, and this led to earlier radicle emergence and faster germination.

The water content of seeds prior to germination clearly has a marked effect on the success of germination. Seeds of low moisture content, although more stable under storage, are particularly sensitive to stress during imbibition (POLLOCK,1969). For example, HOBBS and OBENDORF (1972) found that imbibitional damage to soybeans was greatest when initial moisture contents were below 13% and ASHWORTH and OBENDORF (1980) also found that increasing moisture levels to 17% protected axes from imbibitional CI. However, results are conflicting. For example, BOCHICCHIO, CORADESCHI, ZIENNA,

BERTOLINI and VAZZANA (1991) observed that imbibing low and high moisture kernels and embryos at 5 °C and 25°C led to an inrush of water into the cells, reaching levels of injury and damage to membrane integrity which were independent of both the two moisture contents and temperatures used. This again seems to highlight difficulties in dissecting out differences between imbibition-related and symptoms unique to CI.

5.3 Leakage Studies

In this study, an attempt was made to examine leakage patterns during CI in soybean seeds as this parameter has often been used to assess membrane integrity in plant tissues.

For example, BRAMLAGE, LEOPOLD and PARRISH (1978) established that imbibition temperatures which cause chilling damage impaired germination and growth rates, and was associated with increased leakiness from soybean embryos.

Data from the present study indicated patterns of rapid and marked increases in the leakage of amino acids, potassium ions and electrolytes from chilled seeds and axes. These high leakage rates can partly be explained by the rapid uptake of water observed during early imbibition at chilling temperatures. Using pea embryos, LARSON (1968) attributed the high leakage to disruption of cell membranes as a result of the rapid inrush of water.

The role of the seed coat in retaining solute leakage from seeds during

imbibition and ameliorating imbibitional damage has been reported in several studies. TULLY, MUSGRAVE and LEOPOLD (1981) reported for soybeans and peas that the longer the period during which the seed coats remained on the seed during imbibition, the less was the leakage after seed coat removal. The seed coat has the effect of minimizing damage as indicated by the uncontrolled leakage of solutes from the isolated embryos. Thus, imbibitional chilling sensitivity may be mediated by the rapidity of imbibition, a factor which is principally controlled by the seed coat. However, the leakage of isolated axes seen at chilling and non-chilling temperatures (Fig.7) may not be an entirely accurate reflection of *in vivo* responses.

In this study, the dynamics of electrolyte leakage are likely to be complex and the levelling off which occurred in leakage from non-chilled seeds is likely to be the result of the equilibrium established between the loss, uptake, and the equilibrium with the bathing chamber of the conductivity meter (Fig.8).

Greater amounts of amino acids leached out of chilled seeds than from non-chilled seeds (Fig.9) and leakage showed a linear increase over time. Likewise, chilled axes exhibited a linear increase in the loss of amino acids, but a decline was observed after 24 hours of imbibition (Fig.10). It could be that the axes were capable of reabsorbing amino acids, but this will have to be investigated further. The appropriate membrane carriers could be fully functional by 24 hours and so able to take up amino acids from the bathing medium. This could perhaps be investigated further using radioactive amino acids. HOBBS and OBENDORF (1972) have reported that a loss of amino acids as leachate was primarily a

function of cold temperature during imbibition.

A greater leakage of solutes during imbibition may deplete the tissues of soluble food reserves and stimulate growth of pathogenic microorganisms (SCHULTZ and BATEMAN, 1968). Since most of the solutes leached are sugars and amino acids, which can be replenished during later hydrolysis (SCROTH, WEINHOLD and HAYMAN, 1966), it seems unlikely that these low molecular weight compounds are critical to the development of CI.

Loss of potassium ions during chilling has been observed in studies by LIEBERMAN, CRAFT, AUDIA and WILCOX (1958). They observed a loss of potassium ions from chilled cells and suggested that this loss may be one of the factors concerned in the loss of oxidative phosphorylation observed after chilling sweetpotato roots for 5 weeks. Earlier, LARDY (1952) also suggested that potassium ions may be involved in uncoupling oxidative phophorylation. However, observations by SHEPPARD and BEYL (1951) led to the conclusion that the release of potassium ions from chilled cells may be a non-specific effect of chilling injury. The present study has shown that leakage of potassium ions is significantly greater after 48 hours of imbibition in seeds and isolated axes (Figs.6 & 7) indicating that this is likely to be a "late event" in CI.

Amino acids and potassium ion leakage patterns clearly indicate that a variety of membrane-mediated losses are a manifestation of chilling injury. These results

are consistent with the findings of other researchers. Results from this study support the concept that upon hydration of a dry seed, some type of membrane reorganization occurs and that chilling interferes with this, as reflected by the leakage rates. This may not only reflect the more gross biophysical arrangement of membrane phospholipids as originally suggested by SIMON (1974), but rather the reconstitution of membrane functioning which may include other non-lipidic constituents, such as integral membrane proteins and carriers.

5.4 Lipid Peroxidation during Imbibition

Another objective of this study was to establish what relationship exists between peroxidation and CI. During the first 6 hours of imbibition, peroxidation levels increased greatly for both chilled seeds and axes. This peak at 6 hours and the non-significant levels of potassium ion leakage at 6 hours, indicate that only some time later is membrane leakiness impaired, even though there is evidence of early peroxidation. This suggests that peroxidation is an earlier clue to evidence for CI than ion leakage. Lipid hydroperoxide levels showed a further marked increase after 36 hours of imbibition in chilled seeds and axes, but decreased markedly in non-chilled seeds and axes after the same period of imbibition.

This increase in lipid hydroperoxide levels in chilled seeds and axes after 36 hours may be a reflection of the inability of cellular antioxidant defense mechanisms to quench free radical initiated lipid peroxidation by some

impaired aspect of membrane function occurring during chilling. The similar pattern of lipid peroxidation seen in the isolated axes of chilled and non-chilled seeds may reflect a greater susceptibility to peroxidation in axes, *per se,* irrespective of temperature, or that a rapid and uncontrolled uptake of water can initiate significant peroxidation. The data shows that there is correlation between peroxidation and the development of chilling injury, although it cannot be confirmed that peroxidation is the sole determinant of CI. Evidence for the relationship between CI and peroxidation is provided in the literature for other chill-sensitive tissues. Direct evidence was provided by PARKIN and KUO (1989) who reported that chilling stimulated lipid peroxidation in cucumber fruits prior to the development of irreversible injury. Furthermore, increases in the rate of lipid peroxidation preceded other stress indicators such as leakage. Based on results obtained in this study, and theories advanced by other researchers, we suggest that lipid peroxidation could be an early event implicated in the development of chilling injury in seeds.

5.5 Effect of Temperature Changes on Lipid Peroxidation

Seeds may be irreversibly damaged if exposed to chilling temperatures for a period at the start of imbibition before being returned to non-chilling temperatures (POLLOCK and TOOLE,1966 ; CHRISTIANSEN,1967). In order to see if CI could be ameliorated, imbibitional events were separated from chilling by allowing imbibition at non-chilling temperatures before subjecting seeds to

chilling assault. Likewise, seeds were returned to non-chilling temperatures to evaluate the extent of possible recovery. To examine this, peroxidation was monitored as it was seen to be a reasonably good marker of CI.

Lipid hydroperoxide levels were higher in seeds and axes that were imbibed for 12 hours at 25°C followed by 12 hours at 5°C than those imbibed for 12 hours at 5 °C followed by 12 hours at 25°C (Fig.12). This suggested that 12 hours at chilling temperatures was able to induce only a limited amount of lipid peroxidation or that transferring seeds to a non-chilling temperature was somewhat able to prevent or reduce increases in hydroperoxide activity. However, this hypothesis is not consistent with experiments where seeds that were imbibed for 12 hours at chilling temperature and transferred to a non-chilling temperature for a further 24 hours, as peroxide activity was greater than seeds that were imbibed for 12 hours at a non-chilling temperature before being transferred to a chilling temperature for a further 24 hours. This could indicate that 12 hours at a non-chilling temperature is sufficient enough to prevent free radical activity. Further transfer experiments and monitoring of other parameters, such as conductivity and leakage rates may help to give a better understanding of the mechanism of CI.

In post-harvest physiology studies, PARKIN and KUO (1989) used ethylene and ethane evolution by stored cucumber fruits to demonstrate lipid peroxidation in response to chilling. They observed that the pronounced ethylene and ethane formation during rewarming was associated with the lenghtening of the duration of chilling, up to 10 days. They also initially observed low levels of ethane in

control and chilled fruits. However, after 20 days of chilling, ethane evolution was enhanced in comparison to the controls. A visual assessment of CI showed that cucumber fruits transferred to non-chilling temperatures after 3 days of chilling showed no signs of injury, but fruits chilled for 7 days showed visible signs of injury after rewarming, leading to the conclusion that reversible injury due to chilling is possible with less than 3 days of low temperature exposure.

5.6 Fatty Acid Levels during Imbibition

A relationship between unsaturated fatty acids and chilling resistance in cotton seedlings was reported by ST JOHN and CHRISTIANSEN (1976). Likewise, studies of DOGRAS, DILLEY and HERNER (1977) have suggested that chilling resistance in seeds was dependent upon their ability to synthesize large amounts of unsaturated fatty acids during the initial stages of germination. However, we find little evidence to support this proposal as no increases in unsaturated fatty acids were observed in the axes and whole seeds over the first 36 hours of imbibition at chilling and non-chilling temperatures. Indeed, PRIESTLEY and LEOPOLD (1980) have reported that chilling sensitivity of soybean was unrelated to the properties of major membrane lipids. In contrast, STEWART and BEWLEY (1981) observed that the fatty acid composition of chill-tolerant species did not change over a 23 hour non-chilling incubation period but a marked reduction in unsaturated fatty acids was observed in the soybean axes after 12 hours at chilling temperatures. The results of the present study

shows a correlation between peroxidation and loss of fatty acid unsaturation during chilling injury in soybean axes. Increases in peroxidation in the axes during chilling (Fig.11B) were accompanied by decreases in fatty acid unsaturation and as much as an 18% reduction in linoleic acid was observed after 12 hours of imbibition at chilling temperatures (Fig.14). PARKIN and KUO (1989) have also reported increases in rates of lipid peroxidation and losses in polar and glycolipid unsaturation in cucumber fruits during chilling. In whole seeds, declines in unsaturated fatty acids with chilling were low in comparison to axes, although both showed comparable levels of lipid peroxidation. This could reflect organ differences in antioxidant capacity or reflect small changes in fatty acid unsaturation in a relatively large mass of cotyledonary lipid reserves. Experiments which attempted to separate changes associated with the imbibition process, *per se*, as against the response to chilling also highlighted differences in the response of seeds and isolated axes (Figs.13 & 14).

5.7 Effect of Temperature Changes on Total Fatty Acid Levels

Axes imbibed for 12 hours at 25°C before transfer to 5°C for a further 12 hours showed a marked loss of linoleic acid, suggesting that even after this period the tissues are susceptible to chilling injury and possible peroxidative loss of fatty acids. Axes in which the time of imbibition was increased to 24 hours at 25°C before 12 hours of chilling showed essentially no change in linoleic acid levels (Fig.16). These changes show some correlation with peroxidation levels. The

The data showed that axes imbibed for 12 at 25°C before transfer to 5°C for a further 12 hours showed an increase in peroxidation. Axes in which the time of imbibition was increased to 24 hours at 25°C before 12 hours of chilling showed a decrease in peroxidation levels. These results are in agreement with those obtained for peroxidation as a further support of CI and possible peroxidative loss of fatty acids and might also suggest that full membrane functionality as regards resistance to potential CI may be separated from earlier imbibitional-associated injury.

From this study, it is suggested that the capacity of soybean seeds to control lipid peroxidation may be an important component in CI and leads to a loss of unsaturated fatty acids.

5.8 Temperature Changes and Fatty Acid Levels of Different Lipid Fractions

As part of a further study to examine whether membranes might be a possible site for chilling injury, analyses of the fatty acids of the phospholipid fractions were made. In addition the fatty acids of the glycolipid and neutral lipid fractions were also examined. In polar lipids, a slight reduction was observed in the linoleic acid levels in response to chilling for 12 and 24 hours (Fig.17A). However, no reductions in fatty acid unsaturation was observed in the glycolipid and neutral lipid fractions. Changes only to polar lipids might suggest that not all lipid classes are equally susceptible to CI and that membranes are indeed the most susceptible site. Further studies could perhaps involve examining polar

lipid peroxidation. These results are in agreement with those obtained by PRIESTLEY and LEOPOLD (1980). They could not relate the differences in chilling sensitivity of pea and soybean to compositional differences in major fatty acid molecular species of seed membranes. In contrast, PARKIN and KUO (1989) demonstrated losses in unsaturation of phospholipids after 7 days of chilling in cucumber tissue. Despite evidence which implicates loss of fatty acid unsaturation as a central factor in CI (LYONS,1973), this has not been shown to be significant in all cases.

An analysis of the polar lipid fraction in seeds that were transferred from non-chilling to chilling temperatures, showed a reduction in linoleic acid levels (Fig.17D) and a slight decline was also observed in the glycolipids (Fig.17E) when treated similarly. No reduction was observed in seeds that were imbibed at chilling temperatures and then transferred to non-chilling temperatures. These results contrast with the experiments at constant chilling or non-chilling temperatures. The reason for this could be that hydration and pre-germinative processes were more complete and advanced in seeds that were imbibed at non-chilling temperatures, so that upon transferral to a chilling temperature, greater metabolically-derived damage occurred in comparison to seeds that were first imbibed at chilling temperatures. Similarly, PARKIN and KUO (1989) using chilled cucumber fruits, observed that a decrease in fatty acid unsaturation in the glycolipid and phospholipid fractions appeared to be reversible when the fruit was rewarmed after 3 days of chilling.

These results are in agreement with earlier results obtained for lipid peroxidation studies at transfer temperatures (Fig.12). Upon transferring seeds from a non-chilling to a chilling temperature, peroxidation increased and total fatty acid levels decreased, whereas upon transferring seeds from a chilling to a non-chilling temperature, peroxidation decreased and total fatty acid levels remained high. These results support the suggestions that following peroxidation, there is likely to be a loss in fatty acid unsaturation and that lipid peroxidation leads to a proliferation of free radicals which are capable of indiscriminately damaging membranes and proteins. PARKIN and KUO (1989) also associated losses in fatty acid unsaturation in glyco- and phospholipids with the chilling-potentiated rise in peroxidative activity.

In this study, fatty acid levels in the neutral lipid fraction were not affected at chilling, non-chilling and transfer temperatures.

Different fatty acids possess different susceptibilities to peroxidation and biomembranes represent a key site of direct injury (WILSON and McDONALD,1986). Membrane lipids have a larger surface area and are usually more unsaturated than storage lipids (MEAD,1976) thus making them potential targets of lipid peroxidation (WILSON and McDONALD,1986) which in turn could alter the liquid-crystalline state of biomembranes. Lipid peroxidation can also increase membrane permeability (SIMON,1974) and may also cause conformational changes in the organization of mitochondrial inner membrane. Since leakage of metabolites from seeds in response to chilling is

considered to reflect changes in membranes (BRAMLAGE, LEOPOLD and PARRISH,1978), it can be concluded that the high leakage rates observed in early responses to chilling could be a result of peroxidation during imbibition. However, PARKIN and KUO (1989) suggested that the use of increases in electrolyte leakage to indicate physical damage to plasmalemma resulting from low temperature stress is not an adequate assessment of CI, because while an increase in electrolyte leakage is an early response to chilling, it does not appear to be a symptom shared by all chill-sensitive plants (MURATA and TATSUMI,1979). The results of the present study lend support to the suggestion that peroxidation precedes leakage during CI, thus making it a better indicator of CI than leakage.

5.9 Sugar Levels during Imbibitional Chilling Injury

Sugar levels were monitored in response to chilling and non-chilling temperatures in order to see if these respiratory substrates are affected by CI, since mitochondrial studies in soybean showed that CI is associated with increases in electrolyte leakage and decreases in respiration (BRAMLAGE, LEOPOLD and PARRISH,1978).

Results indicated that sucrose was the dominant sugar present in axes that were imbibed at chilling and non-chilling temperatures (Figs.19,21 & 22). During the first 12 hours of imbibition, sucrose levels were high, probably due to high activity of sucrose-synthesizing enzymes and thereafter, sucrose levels

decreased slightly. This decrease could be due to a shift in carbohydrate metabolism in favour of starch synthesis or that sucrose leaked into the surrounding medium upon imbibition. These possibilities would require further experimental work for verification.

No characteristic pattern could be observed between CI and sugar levels and, overall, sugar levels were not significantly affected by either chilling or non-chilling temperatures. A decrease in respiratory activity in response to chilling observed in soybean seeds (BRAMLAGE, LEOPOLD and PARRISH,1978 ; LYONS and RAISON,1970) and ultrastructural evidence for mitochondrial damage (CHABOT and LEOPOLD,1985) might suggest possible changes in sugar levels since these serve as respiratory substrates. Several studies have examined sugars in relation to CI. For example, HOBBS and OBENDORF (1972) observed a loss of sugars as leachate and attributed this to cold temperatures during imbibition. They also observed that at 5°C, low moisture content seeds lost more sugars than high moisture content seeds. However, reports are conflicting and several studies have correlated carbohydrate levels to chilling resistance.

Cotton seedlings exposed to 5°C accumulated sugars and starch and were not injured (GUINN,1971). Post-harvest physiology studies of CI, respiration and sugar changes in sweetpotato stored at low temperatures showed enhanced sucrose synthesis and total soluble sugar content with increasing exposures to 7°C. Since sugars are primary substrates for respiration, their increase could be a contributing factor to the elevated respiratory rates (PICHA,1987).

Likewise, high levels of reducing sugars in grapefruit peel correlated positively

with CI resistance (PURVIS and GRIERSON,1982). PURVIS (1990) suggested three possible roles for soluble carbohydrates in CI. Firstly, sugars may be involved in chilling resistance by decreasing the cell water potential and reducing water loss from the tissue. Secondly, certain carbohydrates can stabilize cell membranes and enzymes by binding to constituent molecules. Thirdly, since carbohydrates are an energy source, it was suggested that the role of carbohydrates in reducing the sensitivity of plant tissues to low temperatures may operate via a metabolic mechanism.

Low temperature can also affect carbohydrate composition by initiating the hydrolysis of starch, to soluble carbohydrates (DEAR,1973). In some plant tissues, sucrose is hydrolyzed to reducing sugars by the activity of low temperature-induced invertase. Results from this study have led to the conclusion that sugar levels are not affected by chilling temperatures. The slight, but non-significant decreases observed could be an indication of hydrolysis to reducing sugars by the activity of low temperature induced invertase.

5.10 Proteins and antioxidant enzymes

Antioxidant defense enzymes (catalase, peroxidase and superoxide dismutase) play a major role in protecting cells from oxidative damage (SCANDALIOS,1990). Catalase activity was low and almost absent in dry seeds and then increased after 24 hours of imbibition at chilling and non-chilling temperatures (Fig.23),

suggesting that catalase activity was induced upon imbibition. After 24 hours of imbibition at chilling temperatures, enzyme activity was high which may indicate an adaptive response to reactive oxygen intermediates. The decrease in catalase observed after 48 hours at chilling and non-chilling temperatures could be a long-term response to chilling. FADZILLAH, GILL, FINCH and BURDON (1996) have reported a marked effect of chilling on the reduction of catalase in shoot cultures of rice.

Peroxidase activity was high in dry seeds indicating the stability of this enzyme in such seeds (Fig.24). Like catalase, a decrease in activity was observed after 48 hours at chilling and non-chilling temperatures. Superoxide dismutase activity, in particular, Mn and Cu/Zn SODs were high in dry seeds and seeds imbibed for 24 hours at chilling and non-chilling temperatures, Cu/Zn SOD levels being the greatest (Figs.25 and 26). After 24 hours, enzyme activity decreased.

These indirect measures of changes in free radical defense mechanisms may show some correlation with peroxidation levels. Thus increases in catalase and superoxide dismutase during the first 24 hours of imbibition could be an adaptive response in seeds to quench free radical damage, since a decline in peroxide levels was observed in soybean seeds and axes after 24 hours of imbibition at chilling and non-chilling temperatures (Fig.11). PUNTARULO, SANCHEZ and BOVERIS (1988) observed that H_2O_2-producing, and H_2O_2-consuming processes are active early in the germination of soybean embryonic axes. It is therefore, not surprising that catalase levels are elevated during early imbibition. SCANDALIOS (1990) reported that the combined action of catalase and superoxide dismutase

convert the superoxide and hydrogen peroxide radicals to molecular oxygen and water and, in the process, prevent the formation of the highly toxic hydroxyl radical. After 36 hours of chilling, lipid hydroperoxide activity increased and appeared to be inversely related to the declines in the antioxidant levels, indicating an inability of antioxidant enzymes to quench free radical activity.

The increases in lipid peroxidation after 36 and 48 hours of imbibition at 5°C in the axes was accompanied by a decrease in fatty acid unsaturation. These results suggest that peroxidation led to the proliferation of free radicals which may have damaged membranes and proteins. FRANK (1985) reported that the main cellular components susceptible to damage by free radicals are peroxidation of unsaturated fatty acids in membranes, denaturation of enzymes, scission of polysaccharides and DNA mutations.

Reports concerning the role of antioxidant enzymes in response to low temperature stress are conflicting. Reductions in catalase activity in response to chilling have been observed in chill-sensitive and chill-tolerant species (OMRAN,1980 ; PATTERSON, PAYNE, CHEN and GRAHAM,1984 ; MACRAE and FERGUSON,1985). A possible consequence of catalase inactivation is the accumulation of H_2O_2 (LESHEM,1981). OMRAN (1980) reported that H_2O_2 accumulation was concurrent with catalase loss in intact seedlings, whereas no accumulation was observed in experiments with excised leaves.

In this study, the decrease in catalase activity observed after 48 hours at chilling

and non-chilling temperatures cannot be linked with certainty to H_2O_2 accumulation, since H_2O_2 levels were not assayed for. However, it can be concluded that if catalase has a protective role, its activity should be high in response to free radical production. In this study, this is the case, since a response to chilling appeared to involve peroxidative activity.

The response of peroxidase to chilling has not been studied extensively. OMRAN (1980) showed that although catalase activity decreased in cucumber seedlings in response to chilling, peroxidase activity was not affected, suggesting that the accumulation of H_2O_2 in the tissue was due to the lack of increase in peroxidase activity to compensate for the slow removal of H_2O_2 by catalase, since catalase and peroxidase function to remove H_2O_2 (SCANDALIOS,1990). In contrast, CAKMAK, STRBAC and MARSCHNER (1993) showed the capacity of seeds to increase enzyme activity for H_2O_2 detoxification during imbibition and germination. Peroxidase was shown to increase 12-fold between 24 and 48 hours after imbibition and this increase was thought to be a reflection of enhancement of growth and development of the embryo and seedlings. Evidence implicating the involvement of plant peroxidases in cell wall metabolism and development has been provided by GASPER, PENEL, CASTILLO and GREPPIN (1985) and these have no clear relationship to the lipid peroxidation measured in this study. Superoxide dismutases are a family of metalloenzymes that have Cu/Zn, Fe and Mn cofactors (ALLEN,1995).

The high levels of Mn SODs in dry seeds and seeds imbibed for 24 hours at

chilling and non-chilling temperatures are similar to results obtained by CAKMAK, STRBAC and MARSCHNER (1993). They showed significant increases in Mn SOD activity in mitochondria during enhanced respiration induced by various stress factors. Cu/Zn and Mn SOD isozymes have also been reported to increase in activity in response to stress (MATTERS and SCANDALIOS,1986). Furthermore, TROLINDER and ALLEN (1994) showed that expression of the chloroplastic Mn SOD gene in cotton may provide increased chilling tolerance and CI resistance has also been related to high SOD activity in strains of *Chlorella ellipsoidea* (CLAIRE, RABINOWITCH and FRIDOVICH,1984). While high SOD levels observed in dry seeds and seeds imbibed for 24 hours at non-chilling temperatures may be linked to the activity of mitochondria, the decrease in activity observed after 48 hours at chilling could be related to reduced respiratory rates, induced by CI.

5.11 Transmission Electron Microscopy

The ultrastructural aspects of CI were investigated in axes isolated from chilled and non-chilled seeds in order to localize possible cellular sites of damage. Results from this study showed no ultrastructural differences between chilled and non-chilled cells, except that reserve (protein and lipid bodies) mobilization was markedly impeded in chilled cells. The high leakage rates observed in these studies in response to imbibitional CI was used to assess plasma membrane integrity. Despite the rapid leakage rates, no changes in membranes at the ultrastructural level were observed.

Several researchers have shown a response to low temperature injury at the ultrastructural level such as an increase in lipid granules (PLATT-ALOIA and THOMSON,1976), swelling of mitochondria and disorganization of plastids (NIKI, YOSHIDA and SAKAI,1978) and the formation of dense deposits (RISTIC and ASHWORTH,1993) amongst others.

Changes in plastids were also found in several plant species, including swelling of plastids and disorganization lamella in leaf cells of cucumber (KISLYUK,1963). Using tomato seedling cotyledons, ILKER, BREIDENBACH and LYONS (1979) observed discontinuities in the mitochondrial envelope, followed by swelling of the cristae after short chilling exposure. Cell death occurred in a ratio of 1:10 cells and damage to the mitochondria preceded symptoms of plasmalemma damage.

Recently, ISHIKAWA (1996) observed three kinds of cell injury during chilling of mung bean suspension cultures. During early imbibition at chilling, swelling occurred, followed by disruption of the vacuolar membrane and shrinking of the plasma membrane.

A classical example for the effect of CI on ultrastructure in soybean seeds was demonstrated by CHABOT and LEOPOLD (1985). Mitochondria of chilled seeds showed weakly defined envelopes and cristae, in contrast to the well-formed internal structure in the controls. Nuclei of chilled cells were irregular in shape, a feature which was also observed in the present study. CHABOT and LEOPOLD (1985) also used tannic acid as a probe to study plasmalemma integrity and showed that chilled and non-chilled cells were able to exclude tannin, indicating

that CI effects did not damage the plasma membrane.

In the soybean, CI is associated with an increase in leakage rates (BRAMLAGE, LEOPOLD and PARRISH,1978) and a decrease in respiration (LYONS and RAISON,1970). In this study, leakage rates could not be correlated with evidence of plasma membrane changes and mitochondria were poorly resolved in the early stages of imbibition, being only clearly discernible between 24 and 48 hours. The reason for differences between previous studies and the present one is not easily explained. However, mitochondrial changes have not been extensively studied in seed stress because normal fixation of seed tissue often yields poor structural detail in mitochondria (WEBSTER and LEOPOLD,1977).

5.12 Conclusions

Results from this study have led to the following conclusions :

Water uptake occurred at a faster rate in seeds imbibed at non-chilling (25°C) than at chilling (5°C) temperatures, facilitating radicle emergence and germination;

Moisture content played a major role in water uptake. High moisture content seeds imbibed water at a faster rate and had a higher percentage germination than low moisture content seeds;

Leakage rates were greater in chilled seeds, indicating that membrane integrity in the tissues is impaired at chilling. These results support the concept that upon water entry into dry tissues, some type of membrane reorganization occurs and

chilling interferes with this. Leakage is also likely to be a late event in chilling injury;

Lipid hydroperoxide levels increased in response to chilling suggesting that the capacity of seeds to control lipid peroxidation may be an important component in CI. Peroxidation appeared to be a better clue to CI than leakage;

The decrease in fatty acid unsaturation observed in chilled axes implies that a consequence of peroxidation is likely to be a loss of fatty acid unsaturation;

Transferring seeds from a warm to a cold temperature, increased peroxidation and reduced fatty acid unsaturation, while transferring seeds from a cold to a warm temperature, reduced peroxidation and unsaturated fatty acid levels remained high. These results indicate that at low temperatures, peroxidation leads to a proliferation of free radicals which are capable of damaging membrane lipids;

Sugar levels were not affected by chilling and non-chilling temperatures and no relationship could be established with CI;

Antioxidant defense enzymes were expressed at chilling and non-chilling temperatures. The increases observed in catalase and superoxide dismutase after 24 hours of imbibition could be seen as an adaptive response to quench peroxide activity as levels were low during this period.

Peroxide activity increased after 36 hours and subsequently, a decrease in catalase, peroxidase and superoxide dismutase was observed, suggesting the inability of antioxidant defense mechanisms to quench free radicals during late imbibition;

CHAPTER 6

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