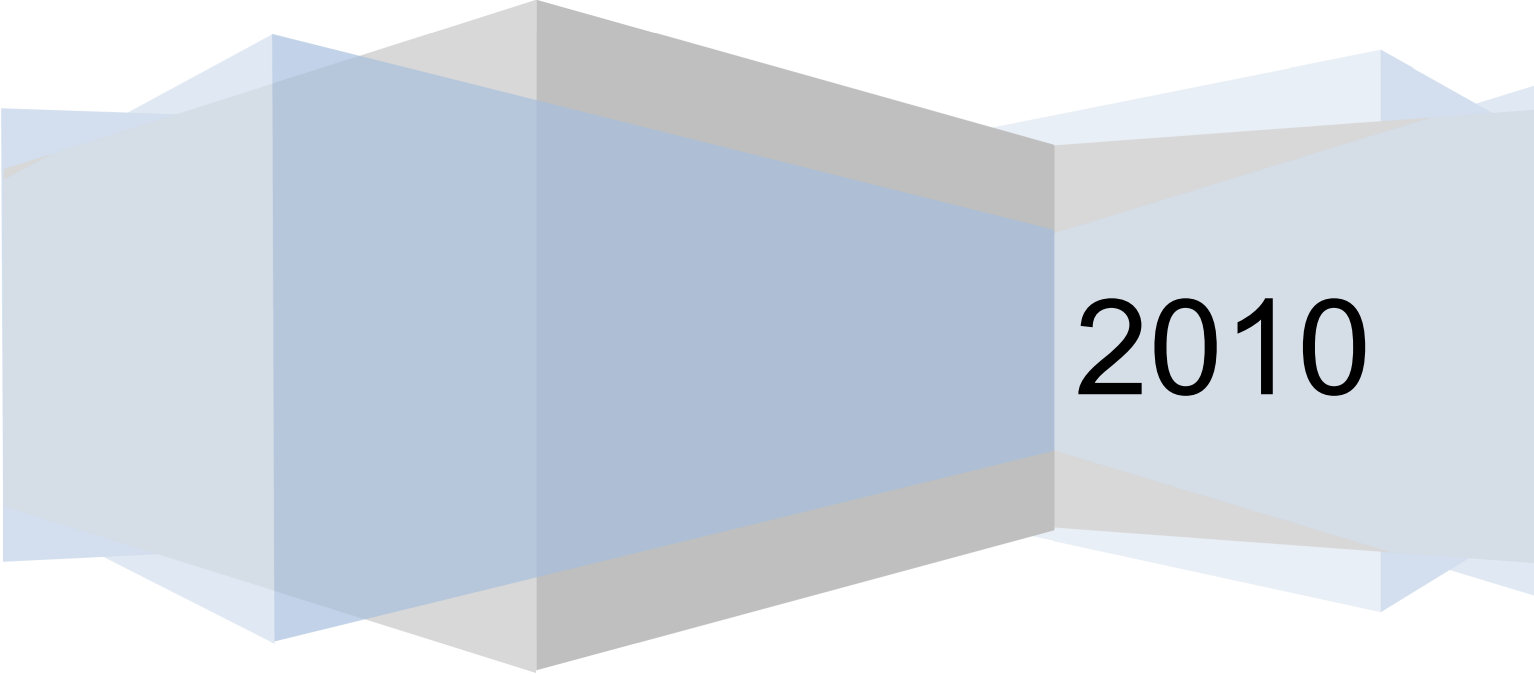


University of the Witwatersrand

***Cryopreservation of  
Zea mays L.  
embryonic axes.***

**Candace (nee Isaacs) Fowler**

**Johannesburg**



2010

**Cryopreservation of *Zea mays* L. embryonic axes.**

Candace (nee Isaacs) Fowler

A thesis submitted to the Faculty of Science, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Doctor of Philosophy in Science.

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**DECLARATION.**

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I Candace (nee Isaacs) Fowler declare that this thesis is my own, original and unaided work. It is being submitted for the Degree of Doctor of Philosophy of Science in the University of the Witwatersrand, Johannesburg. It has not been submitted for any degree or examination to any other university.



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nee

(Signature of Candidate)

08 October 2010

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(Date)

## DEDICATION

In loving memory of my Gran *Elizabeth Sarah Isaacs* who never stopped believing in me.

Words fail to adequately express the gratitude I have to all who have supported, encouraged and prayed for me through the process of completing this piece of work. My heart felt thanks to you all. I thank the Lord God Almighty for His constant presence, guidance and stubborn-love that has never and will never let go of me.

Thank you.

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Thank you.



## ABSTRACT

The successful cryopreservation of a South African maize cultivar (*Hybrid 6363*) was not possible due to the large size of the intact caryopses. To overcome the problem the embryonic axes were isolated. Isolation of the axes from dry caryopses proved challenging and resulted in the damage of 50% of all axes isolated. To ensure the successful removal of the intact axes from the caryopses, imbibition over a period of 30 minutes to 6.5 hours was employed to soften the testa and the pericarp. The effects of imbibition and subsequent cryostorage on the sample water content, vigour and viability were assessed. The water contents of excised embryos increased considerably within the first 30 minutes of imbibition without any visible differences in viability for both the control and cryostored samples. Concomitantly, so were the results for the rest of the imbibition times tested. However, a comparison of the germination indexes indicated that vigour was impeded by cryostorage. In addition to reduced vigour of cryostored material, at the higher imbibition times, morphological aberrations were observed, for example, the lack of root formation. An ultrastructural investigation of the root meristems pre- and post-cryostorage provided information of the changes associated with imbibition as well as the subsequent effects of freezing and thawing. Successful cryostorage seemed to be associated with the lack of obvious metabolic activity at the ultrastructural level (retained desiccation tolerance). The possibility of the leached solutes, out of the caryopses, performing a cryoprotective rôle was considered as a further explanation of the observed post-cryostorage successes. The results confirmed that the combined concentrations of proline,  $\gamma$ -amino butyric acid, glycine, glutamic acid and glucose (known cryoprotectants) were sufficiently high and therefore hypothesized to behave as natural cryoprotectants, but only effectively prior to the acquisition of tissue desiccation sensitivity. *Hybrid 6363* had aged in storage prior to and after its purchase, introducing cellular changes at the physiological and biochemical levels prior to any subsequent effects of imbibition and cryostorage. The onset of imbibition itself resulted in many physiological and structural changes and, subsequent cryostorage of tissue at higher water contents proved deleterious to the resulting plantlets. All of these effects were exacerbated in aged seed. It was therefore proposed that aged seed subjected to imbibition and subsequent cryostorage would sustain elevated damage and hence should not be considered for

germplasm preservation purposes. Comparative studies were done between freshly harvested seed *Hybrid 6321* and *Hybrid 6363* (fresh *Hybrid 6363* was unavailable). Marked differences were noted ultrastructurally and biochemically, but the lack of root formation was still observed in tissue hydrated for longer periods in *Hybrid 6321*. The eventuality that damage occurred at the ultrastructural level was investigated and it was found that the changes in cytoskeletal activity within *Hybrid 6321*, due to imbibition, were extensive within the first 6.5 hours. Cryostorage at specific hydration levels resulted in additional damage, however, the extent of this damage was dependent on the level of cytoskeletal organisation and is suggested to be associated with the tissue having lost or retained desiccation tolerance.

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## Chapter One                      Introduction and literature review

### ***1.1. Maize conservation***

Mexico is the centre of *Zea mays* (maize) origin and is a source of enormous genetic diversity for this cereal. There are approximately 59 races of the species, each with a large number of sub-varieties. The loss of original germplasm due to agricultural selection impacts genetic diversity of maize and effectively reduces it. Furthermore, should modern varieties crossbreed with native maize, the resulting hybrids may prove to be highly competitive and could displace some native varieties. Breeders could therefore lose access to genes from the native land races that may be important in the future. It has become imperative to conserve all varieties of original maize germplasm to ensure their availability for crop production which so many human populations depend on for survival (Bajaj 1995). In maize specifically, the broadening of the genetic pool in hybrids has been achieved by accumulating genes within separate heterotic families that maximise hybrid yield. Heterosis is the term used in genetics and selective breeding which refers to hybrid vigour or out-breeding enhancement. It involves the crossing of two inbred lines yielding progeny that are more healthy and vigorous than their parents. This strategy of conserving divergent heterotic families by inbreeding programmes within these families contributes to the maintenance of genetic diversity in maize (Mikel 2008). In addition to these *ex situ* attempts to prevent genetic erosion, efforts to preserve germplasm should continue to fulfil the main goals of genetic resource management which are, to acquire, maintain, distribute, characterize, regenerate, preserve, evaluate, and utilize the genetic diversity of crops and their wild relatives (Louette *et al.* 1997).

An international seed treaty that provides legal strength to the conservation of the world's major agricultural seed came into force on the 29<sup>th</sup> June 2004. Known as the International Treaty on Plant Genetic Resources for Food and Agriculture (2004), it was intended to ensure that plant genetic resources for food and agriculture are conserved and sustainably used.

More pertinent to the South African context, at a workshop held on Biological Resource Centres (BRCs) hosted by the Department of Arts, Culture, Science and Technology (2001), the importance of both *in situ* and *ex situ* conservation of biodiversity was outlined. Both immediate and long-term scientific, technological, social, cultural, economic and environmental importances were identified. Existing *ex situ* collections could also be viewed as BRCs. These included Herbaria, National Botanical Institutes, the ARC breeding programmes and seed collections as well as the biological resources held at a variety of institutions including universities, science councils and other laboratories. The workshop initiatives were fully supported by the government of South Africa in the development of a strategy for biotechnology, where biological resources were necessarily an important element of this strategy (State of the Nation Address, 9 February 2001). Various organizations both governmental and non-governmental were recorded as being committed to the conservation and maintenance of biological resources of which seed and tissue banks form a part. It was underneath the umbrella of conservation that the present study was conducted and still has its relevance, in particular to the South African context.

Maize is of great agricultural importance (Finch-Savage *et al.*, 2005), because it is one of South Africa's major seed producing crops and is the staple diet for a large percentage of the population. It is imperative that maize seed and its products are readily available to the South African consumer, and it is fortunate that the nature of its caryopses (its ability to be stored under controlled environments) allows it to be sourced throughout the year. In addition to year-round availability, four advantages are achieved by producing seeds namely:

- a re-sorting of genetic material
- a dispersal mechanism
- a multiplication mechanism
- a survival mechanism (Bradbeer 1988).

These make seed the conservation material of choice. In addition, angiosperm seed survival is also due to the protection provided by the pericarp and testa.

## ***1.2. Seed bearing plants***

There are two types of seed bearing plants namely the gymnosperms and the angiosperms. The angiosperms produce seed which are protected (i.e. not 'naked') by a pericarp and seed coat or testa, a feature responsible for this plant groups' relative success on land (Copeland and McDonald 2001). Seeds are important as a food source as they provide people with an important source of carbohydrates as well as some protein, and are also utilised in the manufacture of a large array of commodities, e.g. drugs and medicines (Copeland and McDonald 2001) to fibre glass (Riedel and Nickel 2000).

Seed research has resulted in the separation of seeds into two categories, namely orthodox and recalcitrant seeds. The former are more resistant, and the latter more sensitive, to chilling temperatures and desiccation (Bradbeer 1988; Farrant *et al.*, 1993) to varying degrees (depending on the species).

### **1.2.1. Orthodox seeds**

Orthodox seeds undergo a process of maturation drying (outlined below) and it is for this reason that such desiccation tolerant seeds, like those of maize can be stored for protracted periods at controlled temperatures and relative humidity's (Bewley and Black 1994). However, conventional methods of storage for orthodox seeds do not eliminate certain effects, for example, natural ageing which has been defined as a time-related decline in biological processes, exacerbated and accelerated by abiotic and biotic stress, including pathological disease (Benson 2008)(see below).

### **1.2.2. Recalcitrant seeds**

Seeds which are "shed" at relatively high water contents will not sustain any loss of water during storage. These seeds are the so-called recalcitrant seeds (Bewley and Black 1994) or desiccation-sensitive seeds (Pammenter *et al.* 2002). Unlike

orthodox seeds, the recalcitrant seeds cannot be stored for any length of time, i.e. they must maintain relatively high water contents in order to remain viable. Even when recalcitrant seeds are stored under moist conditions, their life span is often brief and only rarely exceeds a few months.

This presents a conservation problem considering that many recalcitrant seed producing species are also important crop species, e.g., coffee [*Coffea arabica*] (Dussert *et al.* 1998), kola nut [*Cola nitida*] (Bajaj 1995), cacao [*Theobroma cacao* L.] and rubber [*Hevea brasiliensis*] (Bewley and Black 1994) to name a few.

### **1.3. Seed formation and development**

#### *1.3.1. Histodifferentiation*

By definition, the sequence of events leading to the development of a multicellular embryo from a single fertilized egg by cell division and differentiation are collectively referred to as *histodifferentiation* (Bewley and Black 1994).

Morphological development of the seed occurs synchronously with cytological, chemical and weight changes (Bewley and Black 1994; Copeland and McDonald, 2001). The flower produces the reproductive parts, namely, the androecium or male whorl, and the gynoecium or female whorl. Pollen produced by the androecium of the mature maize plant can be transferred to the stigma of the same flower resulting in self-fertilization (Meng *et al.* 2005). Alternatively pollen can be transferred to the stigma of another flower of the same species by a specific vector. In maize, the vector is wind resulting in cross-fertilization. Unlike self-fertilization, where offspring are genetically similar, cross-fertilization results in dissimilar progeny (Rudall 1987; Bradbeer 1988; Meng *et al.* 2005). The process of fertilization in angiosperms is described as a double fertilization because a dual fertilization occurs. When the pollen grain lands on the stigma, it ‘germinates’ by

sending a pollen tube (containing a tube nucleus and two sperm cells) down the length of the style . It will continue to grow through the micropyle and enter into the embryo sac (Rudall 1987; Hartmann and Kester 2002). The tube nucleus disintegrates and the two sperm cells enter the embryo sac. One fuses with the diploid polar nucleus to form a triploid endosperm cell and the other sperm cell fuses with the egg cell to form a diploid zygote, or fertilized egg (Hartmann and Kester 2002). Seed formation begins with the process of fertilization or syngamy (the fusion of a male and female gamete). The zygote will start to divide giving rise to an embryo which obtains its nutrition from the endosperm, nucellus and integumentary layers directly through the wall of the embryo sac (Copeland and McDonald 2001). Detailed descriptions of seed development have been provided by Bewley and Black (1994) as well as Copeland and McDonald (2001). According to those authors, overall seed development occurs at three main levels viz. (1) morphological development, (2) changes in weight and (3) chemical changes.

After syngamy, the developing seed begins to increase in weight as a result of nutrient and water intake associated with rapidly accelerating cell division and elongation (Hartmann and Kester 2002). In monocotyledons, the developing endosperm accounts for most of the weight increase, with the testa-pericarp weighing slightly less and the embryo weight being considerably lower in comparison. This weight gain is also accounted for by the synthesis and deposition of stored reserves. Cells expand to accommodate these reserves (Copeland and McDonald 2001). The point at which the seed has achieved its maximum dry weight and seed quality and has separated from the parent plant is known as *physiological maturity*. Prior to full physiological maturity, the carbohydrate content increases rapidly as the endosperm develops. Sucrose and reducing sugar levels, initially high in the young endosperm, decrease rapidly as the starch content rises. Similarly, both sucrose and other reducing sugars increase in the testa-pericarp during early seed development and then decrease as the seed matures. The whole-seed fresh weight remains relatively stable, although the seed loses water, which is displaced with the accumulating insoluble reserves

within the cells of the storage tissues (Copeland and McDonald 2001). The decline in water content slows as the seed approaches its maximum dry weight, although this process depends on the species and the environmental conditions (Bewley and Black 1994 and Copeland and McDonald 2001).

### *1.3.2. Maturation drying and the acquisition of desiccation tolerance*

Orthodox seeds at the terminal event in their development experience a loss of cellular water and pass into a metabolically quiescent state (Kermode and Bewley 1985a; Bewley and Oliver 1992). However, it is still debatable whether desiccation tolerant organisms completely cease metabolism under the natural conditions of abated hydration (Alpert 2000).

A transition to a desiccation-tolerant state, through the programmed loss of water (Bewley and Oliver 1992; Oliver *et al.* 1997a), which occurs approximately mid-way through development, is characteristic of most orthodox seeds and occurs well before the completion of major developmental events such as reserve deposition and the commencement of normal maturation drying (Kermode and Bewley 1985a). Drying redirects metabolism from a developmental to a germinative mode and it is a prerequisite for the induction of hydrolytic enzymes essential to the post-germination (growth) phase of seedling development. Although seeds are not capable of withstanding desiccation at all stages of their development, their acquisition of tolerance is usually considerably earlier than the drying event itself (Kermode and Bewley 1985a and b; Bewley and Oliver 1992). Not only is desiccation tolerance achieved at particular stages of development, but tolerance of harsher drying regimes increases as development proceeds (Kermode and Bewley 1985a). It is also probable that seeds undergo certain metabolic adjustments associated with the onset of tolerance i.e. the synthesis of proteins, including those with hydrophylic properties (such as LEA's - see below), which have been implicated in maintaining the structural integrity of cellular membranes during dehydration (Kermode and Bewley 1985a; Koag *et al.* 2003). This has also been demonstrated in vegetative tissue (Dure *et al.* 1989; Van der Willegen *et*



*al.* 2004) and these proteins appear to be species specific (Bewley and Oliver 1992; Gallardo *et al.* 2001). The mechanisms of desiccation tolerance in seeds (although masked by a developmental programme) and vegetative tissues appear very similar (Berjak *et al.* 2007; Farrant 2000; Moore *et al.* 2008; Vitré *et al.* 2004) and it has been proposed that desiccation tolerance involves the use of the same genes that they are under developmental control in seeds but environmental control in the vegetative tissues (Farrant 2007; Illing *et al.* 2005).

Desiccation tolerance, at the whole plant level rather than just the seed level, has been defined by Alpert (2000) as the ability of an organism to equilibrate its internal water potential with that of moderately dry air, and then resume normal function after rehydration. This would require remarkable tolerance to water deficit stress. Notwithstanding the production of protective proteins during maturation drying, additional critical parameters must be met in order for plant tissues to survive severe water deficits (Bewley and Oliver, 1992). The tissue must exhibit the ability to:

- limit damage during drying to a repairable level
- maintain its physiological integrity in the dry state
- mobilise repair mechanisms upon rehydration which effect restitution of damage suffered during desiccation (Bewley 1979).

These criteria encompass a spectrum of theories on the subject of anhydrous biology (Crowe and Crowe 1986; Hoekstra 1986; Hoekstra *et al.* 1999 ; Hoekstra *et al.* 2001) but have been condensed to address two testable facets of desiccation tolerance namely *cellular protection* (against any potential damage) and *cellular repair* (of incurred damage) (Bewley and Oliver 1992). Perhaps many biological components are responsible for, or contribute to and result in, the acquisition of desiccation tolerance or the “discontinuity of life” as described by Leopold (1986). Evidence provided by molecular and biochemical studies suggest that at the whole plant level there are multiple mechanisms to slow water loss and reduce mechanical injury related to desiccation (Alpert 2000) and level of organisation at the physiological, biochemical and morphological levels to achieve tolerance to

desiccation (Gallardo *et al.* 2001). This organisation is also thought to be intricately detailed at the cellular level to ensure and maintain survival of a water deficit stress and subsequent rehydration.

The overall developmental processes within seed maturation and subsequent germination include a wet-dry-wet sequence of events (Oliver *et al.* 1997a). The first wet phase is pre-maturation drying (during seed development) phase. Post-maturation drying (non-metabolically active) is the dry phase; and the second wet phase is the onset of imbibition (becoming fully rehydrated) in preparation for germination (Bewley and Black 1994).

Membranes are composed chiefly of proteins and phospholipids and, within the first wet phase, the lipids are arranged in a fluid bi-layer which relies on hydrophobic and hydrophilic interactions to maintain equilibrium (Bryant *et al.* 2001; Crowe *et al.* 1984). Individual phospholipid molecules are in juxtaposition with their polar groups facing the aqueous phase on either side of the membrane and their hydrocarbon chains forming the hydrophobic central region. This molecular organisation is stabilised by the relationship between membrane components such as glycolipids, glycoprotein, sugars and water (Oliver *et al.* 1997). Approximately 25% hydration above fully dried weight is essential for the maintenance of this liquid-crystalline membrane configuration (Platt *et al.* 1998). The phase of the membrane lipids, either fluid or gel, is determined by the following physical parameters:

- the composition of the membrane,
- the degree of hydration
- the temperature
- the composition and interaction of chemicals
- the state of nearby solutions (causing an osmotic stress) (Bryant *et al.* 2001).

The phase behaviour of membranes and solutions in response to maturation drying are also subjected to these physical parameters including mechanical

constraints (Bryant *et al.* 2001). Hence, the ability to survive the loss of water to only 10% has become a subject of great interest and much research (Platt *et al.* 1994; Vertucci and Farrant 1995; Hoekstra *et al.* 2001). The changes introduced by dehydration include (1) cellular preparation for protection and (2) repair mechanisms in the event of any damage incurred (Oliver *et al.* 1997).

#### *1.3.2.1. Cellular protection/repair strategies employed against damage incurred during maturation drying*

Protective mechanisms employed by seeds during drying, whether through the natural process of desiccation or premature drying, vary considerably at the mechanical and developmental levels. There are those that occur as a direct result of the natural development process or as a response to an exogenous stimulus. Earlier studies of “wet-dry-wet” systems investigating cellular damage and protection were conducted on bryophytes (Bewley and Oliver 1992; O’Mahony and Oliver 2001). The principles of “dehydration-induced” protection can be seen in other desiccation tolerant tissues whereby the mechanisms of desiccation tolerance in higher plants are based on protective mechanisms as apposed to repair mechanisms (Paris *et al.* 1996; Cordova-Tellez and Burris 2002a). One mechanical means of protection is conferred by proteins, even though their primary role is not necessarily protection. These proteins, synthesised during the process of maturation drying (Bewley and Oliver 1992) are deposited into storage bodies and stably accumulate to high levels, presumably assisting in the exclusion of water during seed maturation and preventing mechanical stress during drying. Subsequently, the storage proteins are rapidly degraded to provide basic biochemicals for use by the embryo during germination (Paris *et al.* 1996; Jiang *et al.* 2001).

The occurrence of the many small vacuoles, as demonstrated in the tissue of resurrection plants, are thought to replace the volume occupied by water in the hydrated state and provide a reduction in mechanical stress (Van der Willegen *et al.* 2004). Other evidence for protection against maturation drying induced

damage is largely indirect (Bewley and Oliver 1992), for example, the production of ABA and its effects on seed tissue during maturation drying (Kermode *et al.* 1989). However, before considering the protective mechanisms seeds employ, it is necessary to describe the damage which occurs upon drying.

Upon drying, many stresses are incurred by the cellular components (Crowe and Crowe 1986). Membranes, within desiccation sensitive species, are considered one of the primary sites of lethal damage when water is lost from cells (Bryant *et al.* 2001). In desiccation tolerant species membrane integrity is severely compromised and the membrane structure is weakened, i.e. phospholipid components of the membranes undergo a phase transition to a gel like-state (Bewley and Black 1994; Hoekstra *et al.* 2001). Compromise in membrane integrity due to these phase changes are physiologically deleterious (Wolfe 1987), resulting in electrolyte leakage upon rehydration (Platt *et al.* 1994; Bajji *et al.* 2002). During membrane phase transitions, a process of “demixing” occurs resulting in the lateral phase separation of the different membrane lipids (Vertucci and Farrant 1995; May *et al.* 2000). There are two types of phase transitions associated with lipids of membranous systems. The first is a lamellar liquid crystalline to gel phase, in which the bi-layer configuration is maintained, while the second phase is the lamellar liquid crystalline to hexagonal phase in which a non-bi-layer structure is formed (Bewley and Black 1994).

Phase behaviour can also change as a consequence of chemical reactions that occur when tissues are dried (Vertucci and Farrant 1995). For instance, reactions can be peroxidative, resulting in lower levels of fatty acid unsaturation and the production of free fatty acids in cellular membranes (Vertucci and Farrant 1995). Lipid peroxidation products can be formed at low moisture levels enzymatically (Priestley and Leopold 1986; Vertucci and Leopold 1986) or non-enzymatically by free radicals (McKersie *et al.* 1988). The free radicals may be produced from the partial reduction of oxygen in active mitochondria provided of course that the mitochondria are active at low water contents. In this regard, according to Alpert (2000), all metabolic activity is not completely lost at reduced hydration levels.

Lipids within the cell membranes are thus susceptible to peroxidative attack from the by-products of these reactions which can also affect protein and nucleic acid functions (Vertucci and Farrant 1995).

During drying, some desiccation tolerant mosses have been shown to lose their ability to conduct protein synthesis (Bewley 1979; Bewley and Oliver 1992). It is reported for some desiccation tolerant species that rRNAs maintain their integrity during drying and rehydration. In non-desiccation tolerant species rRNA degradation occurs in an enthalpic fashion for both cytoplasmic and chloroplast ribosomes (O'Mahony and Oliver 2001). In the moss, *Tortula ruralis*, where rRNA integrity is maintained throughout the desiccation process, it is thought to facilitate a rapid recovery from desiccation during rehydration (O'Mahony and Oliver 2001). This has also been implied by authors investigating angiosperm resurrection plants, where proteins showing increased abundance during drying, included those binding RNA, suggesting that they play a role in the mechanisms that maintain cellular integrity during water loss (Ingle *et al.* 2007). More specifically mRNAs are stored in a stable form in the plants leaves and are available for immediate translation upon rehydration, therefore, an initial and rapid recovery is possibly completely independent of *de novo* transcription of nuclear genes (Dace *et al.* 1998).

Cellular protection is proposed to occur at three levels, namely, at the structural, biochemical and genetic levels. Due to the varying proportions of desiccation tolerance among species (Berjak and Pammenter 2001; Sershen and Pammenter 2008) it is envisaged that protection occurs in a matrix of possible combinations of these three levels and is indicative of the organisms' possession, or lack of, desiccation tolerance. Protection of membrane components from the effects of desiccation is a likely feature of desiccation tolerance (Leopold 1986; Bewley and Oliver 1992; Oliver *et al.* 1997).

The protective effects of sugars in the specific case of anhydrobiosis (Hoekstra *et al.* 1999) are considered below. Three main hypotheses are addressed. The water-

replacement hypothesis suggests that during drying, sugars can substitute for water molecules (in particular by forming hydrogen bonds) around the polar and charged groups present in phospholipid membranes and proteins, thereby stabilizing their native structure in the absence of water (Crowe *et al.* 1984, 1992, 1997, 1998b; Carpenter *et al.* 1994; Ingram and Bartels 1996). The water entrapment hypothesis, in contrast, proposes that sugars concentrate residual water molecules close to the plasmamembrane, thereby preserving to a large extent its solvation and native properties (Belton and Gil 1994; Cottone *et al.* 2002; Lins *et al.* 2003). This hypothesis has been formulated in the context of proteins, based on thermodynamic data in solution showing that sugars are excluded from the biomolecular surface by water (Timasheff 1982; Xie and Timasheff 1997). Finally, the vitrification hypothesis suggests that sugars found in anhydrobiotic systems, known to be good vitrifying agents, protect biostructures through the formation of amorphous glasses, thereby reducing structural fluctuations and preventing denaturation or mechanical disruption (Sun and Leopold 1994, 1997; Sun *et al.* 1994, 1996; Williams and Leopold 1989). A consensus has emerged that these mechanisms are not necessarily mutually exclusive (Clegg 2001; Crowe *et al.* 1998a, 2002; Crowe 2002; Carpenter *et al.* 1994; Sun and Leopold 1997). Vitrification may occur simultaneously with a direct interaction of the biostructure with the sugar, or with an entrapment of residual water at its surface, depending on the type of the protected biostructure and on the nature of the environmental stress imposed on the organism (Pereira *et al.* 2004). Additionally, some experimental investigations have suggested that other solutes (e.g. polyfructoses, arbutin, abscisic acid (ABA), and several families of stress proteins) may play a critical role in the protection of cells, which in some cases could be interconnected with the action of the saccharides (Singer and Lindquist 1998; Oliver *et al.* 2001; Clegg 2001; Crowe *et al.* 2002).

Certain sugars play a major role in preventing membrane fusion, phase transitions and most likely also phase separation (Hoekstra *et al.* 1997). Hoekstra *et al.* (1997) also maintain that the disaccharide sugar's ability to form a stable glass and to interact directly with the phosphate of the phospholipid polar head group is

the requirement for the protection of dry liposomes and those disaccharides alone fulfill these requirements. Conversely, insufficient interaction of sucrose with the polar headgroups can occur at elevated transition temperatures in dry membranes of desiccation tolerant plants *in situ* (Hoekstra *et al.* 1997). Therefore, it is suggested that factors other than sugars suppress transition temperatures in intact cells, and that amphipathic compounds potentially have a role in this regard (Hoekstra *et al.* 1997).

Trehalose is another sugar thought to replace water during desiccation and is thought the most effective osmoprotectant sugar in terms of minimum concentration required (Crowe *et al.* 1992). Trehalose accumulation during cellular stress also protects cells and cellular proteins against damage by oxygen free radicles by acting as a free radicle scavenger (Benaroudj *et al.* 2001). However, trehalose is very rare in plants; where sucrose and other sugars are able to replace water (Ingram and Bartels 1996) the likelihood of trehalose replacing water during desiccation in plants is minimal. Other sugars that have been identified to confer a protective role during maturation drying are the raffinose series of sugars (Blackman *et al.* 1992), including stachyose (Sinniah *et al.* 1998). Furthermore, heat-stable proteins in conjunction with these sugars are equally likely to be required for complete and effective desiccation tolerance (Sinniah *et al.* 1998). Leprince *et al.* (1993) have shown the accumulation of soluble sugars during the acquisition of desiccation in developing seeds of a variety of species. More recently, Hoekstra *et al.* (2001) discussed low desiccation tolerance in the presence of ample sugars in plant somatic embryos. Sugar accumulation is therefore not the only way in which plants “manage” desiccation (Bohnert *et al.* 1995), but it is considered an important factor in tolerance. Other authors dismiss prevailing models of water replacement in plants by solutes because the majority of the studies have been conducted on tissues *in vitro* (Tunnacliffe and Lapinski 2003) and hence, do not reflect accurately what occurs in natural systems. Nevertheless, the prevailing hypotheses of membrane stabilization during maturation drying are all feasible in the light of evidence presented (Periera *et al.* 2004).

In addition to the protection offered by sugars during drying stress, various phospholipid biosynthetic pathways result in the production of other osmoprotective compounds (Bohnert *et al.* 1995). An increase in solute and salt concentrations, which occurs as a consequence of water loss within cells, initiates the production of these osmoprotective compounds (Bohnert *et al.* 1995) including some proteins.

Stress tolerance is a multigenic trait, and the biochemical pathways leading to products or processes that improve tolerance are likely to act additively and possibly synergistically (Bohnert *et al.* 1995). Protective strategies observed in the angiosperm *Craterostigma plantagineum* prior to desiccation; employ a drying induced elevation of abscisic acid (ABA) that triggers the accumulation of gene products that in turn mediate the establishment of a cellular protection system (Ingram and Bartels 1996; Oliver and Bewley 1997). Proline accumulation occurs as a consequence of ABA synthesis (Hare *et al.* 1999) and in response to many environmental stresses, including low water potentials (Yancey *et al.* 1982). Therefore, proline biosynthesis is a secondary response to drying and provides an osmoprotective effect (Hare *et al.* 1999). The expression of proline biosynthetic genes is dependant on two signal transduction cascades, both of which are induced by the presence of abscisic acid (Delauney and Verma 1990). ABA synthesis in turn occurs as a result of a water deficit stress (Oishi and Bewley 1990; Iglesias and Babiano 1996; Josè-Estanyol and Puigdomènech 1998) and as a result of the process of maturation drying (Bewley and Black 1994). Increased ABA levels have been reported to control the suppression of numerous cellular events such as embryonic DNA synthesis and the polymerisation of the microtubular network (De Castro and Hilhorst 2006).

Various proteins synthesized during maturation drying also confer protection. Genes which are expressed in mature seeds have been defined as a new class of hydrophilic proteins that are proposed to contribute to cellular protection during dehydration (Rodrigo *et al.* 2004). One subset of proteins increases in abundance



as seeds mature and may be conserved in the dry seed. However, these proteins are virtually absent in the seed tissues during subsequent germination. These hydrophilic proteins, known as Late Embryonic Abundant proteins (LEAs), are important to the final stages of maturation (Bartels *et al.* 1988; Bewley and Oliver 1992; Gilles *et al.* 2006) and have been implicated in the ability of the mature seed to withstand desiccation (Baker *et al.* 1988; Battaglia 2008), i.e. provide cellular protection (Bewley and Oliver 1992; Dure 1993; Bray 1997; Hoekstra *et al.* 2001). LEA's are expressed and accumulate to high levels in late stages of seed maturation (when acquisition of desiccation tolerance occurs in the embryo (Battaglia *et al.* 2008)) and/or upon water stress conditions in plants (Rorat 2006; Tunnacliffe and Wise 2007). They are also found in non-plant species for example, in bacteria and yeast (Stacy and Aalen 1998; Garay-Arroyo *et al.* 2000), nematodes (Solomon *et al.* 2000; Browne *et al.* 2004) and fungi (Mtwisha *et al.* 1998; Abba *et al.* 2006) and, although their precise function is unknown (Wise 2003; Wise and Tunnacliffe 2004), considerable evidence suggests that LEA proteins are involved in desiccation resistance/tolerance (Oliver *et al.* 2000; Delseny *et al.* 2001; Hoekstra *et al.* 2001; Oliver *et al.* 2001; Chen *et al.* 2002; Wise 2003; Wise and Tunnacliffe 2004; Bernacchia and Furini 2004; Rodrigo *et al.* 2004; Gilles *et al.* 2007; Battaglia *et al.* 2008). Members of the LEA protein families appear to be ubiquitous in the plant kingdom. Their presence has been confirmed not only in angiosperms and gymnosperms (Shinozaki and Yamaguchi-Shinozaki 1996; Bray 1997; Cuming 1999) but in seedless vascular plants (e.g. Selaginella; Oliver *et al.* 2000; Alpert 2005; Iturriaga *et al.* 2006) including bryophytes (e.g. *Tortula*, *physcomitrella*; Alpert and Oliver 2002; Oliver *et al.* 2004; Saavedra *et al.* 2006; Proctor *et al.* 2007), pteridophytes (e.g. ferns; Reynolds and Bewley, 1993), and algae (Honjoh *et al.* 1995; Tanaka *et al.* 2004).

Hydrophilic LEA proteins are described as intrinsically unstructured proteins due to their high degree of unordered structure in solution (McCubbin *et al.* 1985; Eom *et al.* 1996; Lisse *et al.* 1996; Russouw *et al.* 1997; Ismail *et al.* 1999b; Wolkers *et al.* 2001; Soulages *et al.* 2002, 2003; Goyal *et al.* 2003; Shih *et al.* 2004; Dyson and Wright 2005; Tompa 2005; Mouillon *et al.* 2006; Kovacs *et al.* 2008). LEA

proteins have been grouped into seven distinctive groups or families on the basis of sequence similarity (Dure *et al.* 1989; Ingram and Bartels 1996; Colmenero-Flores *et al.* 1999; Cuming 1999; Battaglia *et al.* 2008) and the first four LEA groups are considered in relation to their functions in seed desiccation tolerance.

Most LEA proteins are part of a more widespread group of proteins called “hydrophilins” and the physicochemical characteristics that define this set of proteins as hydrophilic are a Glycine content greater than 6% and a hydrophylicity index greater than 1 (Garay-Arroyo *et al.* 2000; Boudet *et al.* 2006; Battaglia *et al.* 2008). As mentioned above, there is evidence supporting hydrophilin participation in acclimation and/or the adaptive response to stress and, in plants, confer tolerance to water-deficit conditions (Imai *et al.* 1996; Xu *et al.* 1996; Swire-Clark and Marcotte 1999; Zhang *et al.* 2000). Their presence has also been associated with chilling tolerance (Ismail *et al.* 1999; Nakayama *et al.* 2007) where they confer protection via different mechanisms (Reyes *et al.* 2005). The pertinent LEA groups, to the maturation drying process in seeds, are addressed in terms of their structure and their function.

### Group 1

This set of LEA proteins, are recognized by a very large proportion of charged residues, which contributes to their high hydrophilicity (Galau *et al.* 1992; Baker *et al.* 1995) and a high content of glycine residues (approximately 18%). Group 1 LEA proteins are preferentially accumulated during embryo development, especially in orthodox seeds (Ulrich *et al.* 1990; Hollung *et al.* 1994; Williams and Tsang 1994; Prieto-Dapena *et al.* 1999; Vicient *et al.* 2001). Additionally, many of the characterised genes of this group are responsive to abscisic acid (ABA) and/or water-limiting conditions, mainly in embryos and, in a few cases, in vegetative tissues of young seedlings (Gaubier *et al.* 1993; Vicient *et al.* 2000). The function of Group 1 LEA proteins during seed development is to buffer the water loss that occurs during embryo maturation (Manfre *et al.* 2006).

## Group 2

This group of LEA proteins, also known as “dehydrins” (DHNs), are the most characterised group of LEA proteins and are typically highly hydrophilic and contain a high proportion of charged and polar amino acids (Garay-Arroyo *et al.* 2000). These proteins, like Group 1 LEA's, are also in a largely hydrated and unstructured conformation in aqueous solution (Soulages *et al.* 2003; Bokor *et al.* 2005; Lisse *et al.* 1996; Ismail *et al.* 1999b). This apparent lack of structure and a high hydrophilicity of DHNs and other proteins collectively termed ‘hydrophilins’ (Garay-Arroyo *et al.* 2000), have been hypothesised to be central to their function i.e. they essentially act as small intracellular sponges that help to retain water at low osmotic potential (McCubbin *et al.* 1995; Garay-Arroyo *et al.* 2000). An alternative hypothesis is that DHNs are similar to many other “intrinsically unstructured” proteins (Wright and Dyson 1999) in that their lack of intrinsic structure is relieved when bound to target molecules, and that their function is related to their membrane targets.

A distinctive feature of group 2 LEA proteins is a conserved Lys-rich 15-residue motif, EKKGIMDKIKEKLPG, named the K-segment (Close *et al.* 1989, 1993). The K-segment motifs of group 2 LEA proteins are predicted to form amphipathic  $\alpha$ -helical structures and are thought to protect membranes (Dure 1993b; Close 1996). Group 2 LEA proteins are also thought to be associated with the ability to withstand freezing (Muthalif and Rowland 1994; Arora *et al.* 1997). Several proteins of this group show cryoprotective activity and it is enhanced in the presence of compatible solutes (Bravo *et al.* 2003; Reyes *et al.* 2005). Group 2 LEA proteins can localize to common tissues (in root tips, root vascular systems, stems, leaves, and flowers) during development under optimal growth conditions, while other proteins of this group seem to accumulate in specific cell types (for example; root meristematic cells, plasmodesmata, pollen sacs, or guard cells; Nylander *et al.* 2001; Karlson *et al.* 2003a). Most of these proteins accumulate in all tissues upon water deficit imposed by drought, low temperature, or salinity;

although there are those that preferentially respond to particular stress conditions (Rorat *et al.* 2006). There are genes for this group of proteins whose expression during seed development or in response to stress is mediated by ABA (Nylander *et al.* 2001). Ion binding is considered one of the major biochemical functions of the acidic dehydrins, acting as calcium buffers or as calcium-dependent chaperone-like molecules (Alsheikh *et al.* 2003). Metal binding may also be related to a detoxification function needed under stress conditions, where metal toxicity is associated with the production of reactive oxygen species, commonly generated in plants exposed to water limitation (Hara *et al.* 2004).

Maize (*Zea mays* L.) DHN1 dehydrins are able to bind *in vitro* to lipid vesicles containing acidic phospholipids, and that they preferentially bind to small unilamellar vesicles (SUV's) as opposed to large unilamellar vesicles (LUV's) is likely due to the distinctive packing properties of lipids in the vesicle surface (Koag *et al.* 2003), i.e. the highly curved surface of SUV's have more lipid packing defects, through which penetration of segments in peripheral or integral proteins can be initiated (Berkhout *et al.* 1987; Riley *et al.* 1997; Johnson *et al.* 1998). DHN 1, therefore, might be adsorbed to the membrane surface or it may penetrate somewhere into the hydrophobic core. Maize DHN1 also increases its alpha( $\alpha$ )- helical structure when bound to lipid vesicles *in vitro* which suggests a possibility that the same is true *in vivo* and that the K-segment is involved in membrane binding and stabilization (Koag *et al.* 2003). Moreover, the prevalence of unordered conformations in dehydrins is consistent with a role in providing or maintaining enough water molecules in the cellular microenvironment to reserve the functionality or stability of macromolecules or cellular structures during water scarcity conditions. For instance, over-expression of multiple *Arabidopsis* group 2 LEA proteins, ERD10, RAB18, COR47, and LTI30, resulted in plants with increased freezing tolerance and improved survival under low-temperature conditions (Puhakainen *et al.* 2004a).

### Group 3

Group 3 LEA proteins are characterised by a repeating motif of 11 amino acids (Dure 1993a). Differences found in the molecular mass in this group of proteins are usually a consequence of the number of repetitions of this 11-mer motif. Soluble non-reducing sugars seem to contribute to the formation of a cytoplasmic “glass” at low water content in both mature seeds and pollen cells, which could stabilize cellular structures during this severe desiccation (Wolkers *et al.* 2001). Group 3 LEA proteins interact with membranes during dehydration as seen in pea species, *Pisum sativum*, where these proteins protect liposomes subjected to drying (Tollete *et al.* 2007). In plant embryos, these proteins are uniformly distributed in the cytosol of all cell types and contribute in counteracting the damage produced by water limitation. In addition to supporting a role as protector molecules under water limitation, these LEA proteins may function to provide a water rich environment to their target enzymes, preventing their inactivation by possibly maintaining protein integrity as long as water is restrictive (Bataglia *et al.* 2008).

### Group 4

Similar to group 3 LEA proteins, the Group 4 LEA proteins interact with sucrose and raffinose and increase the glass transition temperature of the sugar-protein matrix, which lead to the suggestion that a common role for group 3 and group 4 LEA proteins is related to the formation of tight glass matrices in dry seeds (Shih *et al.* 2004).

In summary, most LEA proteins and their mRNAs accumulate to high concentrations in plant embryo tissues during the last stages of seed development before desiccation (Baker *et al.* 1988; Hughes and Galau 1989; Ingram and Bartels 1996; Oliveira *et al.* 2007; Bies-Ethève *et al.* 2008; Hundertmark and Hinch 2008), but also in vegetative tissues exposed to dehydration, osmotic, and/or low-temperature stress (Dure *et al.* 1989; Chandler and Robertson 1994;

Robertson and Chandler 1994; Ingram and Bartels 1996; Bray 1997; Campbell and Close 1997; Thomashow 1998; Bies-Ethelve *et al.* 2008; Hundertmark and Hinch 2008). These observations have suggested their involvement in the plant response to water-limiting environments, probably by playing roles in ameliorating different stress effects. LEA protein synthesis constitutes a large proportion of the translational activity of the embryo during late maturation (up to 25%) (Bewley and Oliver 1992). The activity of particular LEA proteins can be induced by ABA-treated or water-stressed seedlings (Williams *et al.*, 1995). This has led to the suggestion that not all LEA genes are intrinsically regulated during seed development, but could be “on-call” genes whose expression is in response to water loss (Bewley and Oliver 1992), with ABA possibly acting as one of the signal transducers (Baker *et al.* 1988). Thus, they may be part of a general class of desiccation-protection proteins (Bewley and Oliver 1992).

Evidence of LEA cellular protection, specifically in maize has come from research on *Rab17* (Figueras *et al.* 2004). This protein accumulates largely during embryogenesis and also in vegetative tissue when it is subjected to stress conditions (Figueras *et al.* 2004). *Rab17* under a constitutive promoter (a gene read all the time) in vegetative tissues of transgenic *Arabidopsis thaliana* was found to induce higher sugar and proline contents and higher water loss rates under conditions of water stress (Figueras *et al.* 2004). In addition, the plants were more tolerant to osmotic stress than the non-transformed controls (Figueras *et al.* 2004).

Various RNA types are conserved during the drying process (Platt *et al.* 1994) so that upon rehydration, active metabolism commences almost immediately after imbibition (Bewley and Oliver 1992) and in this way facilitates a rapid onset of cellular repair mechanisms

#### 1.4. Priming and germination

As addressed earlier, embryonic axes of orthodox seeds undergo a transition from desiccation-sensitivity (first wet phase) to desiccation-tolerance (dry phase) in the course of their development (Dasgupta *et al.* 1982). Drying terminates the developmental processes and initiates the metabolic processes essential for the accomplishment of germination (Dasgupta *et al.* 1982; Kermode *et al.* 1985) and allows the embryo to enter into a quiescent state. A pertinent observation reported by Kermode *et al.* (1985) was that tolerance to slow desiccation was achieved well before the completion of major developmental events, such as reserve deposition and the onset of normal maturation drying, i.e. desiccation tolerance precedes the quiescent state (Kermode and Bewley 1985).

Quiescence is a resting state in which there is an absence of suitable germination conditions (Copeland and McDonald 2001) and an absence of all germination processes (Garnczarsk *et al.* 2007). Quiescence in dry seeds, such as the maize caryopses utilised in this study, is characterised by a remarkably low rate of metabolism (Gallardo *et al.* 2001) due to low moisture contents (Copeland and McDonald 2001). Water contents may be as low as 5-15% in unimbibed caryopses (Gallardo *et al.* 2001). In the natural state of quiescence, seeds are able to withstand adverse conditions (usually winter) and humans have taken advantage of this state to store these seeds. As soon as the seed begins to take up water, marked changes in the metabolism occur and the seed begins the germination process (Copeland and McDonald 2001; DeCastro and Hillhorst 2006).

Seed deterioration in storage is associated with the loss of membrane integrity, changes in enzymatic activities, a decline in protein and nucleic acid synthesis and the appearance of lesions in DNA (McDonald 1999) (see additional consequences of seed storage below). Priming treatments (i.e. pre-germination treatments) are primarily industrial seed pre-treatments (Gallardo *et al.* 2001; Cordova-Tellez and Burris 2002b) used to synchronise the germination of a batch of seeds (Haydecker

*et al.* 1973). Priming involves wetting the seeds to the point that germination-related processes are initiated (i.e. very early imbibition) and then drying them back to prevent radicle emergence (Gallardo *et al.* 2001). They are subsequently stored and marketed as treated seed (Gallardo *et al.* 2001). For germination to occur, seeds need to be hydrated under conditions favouring metabolism, e.g. a suitable temperature and in the presence of oxygen (Gallardo *et al.* 2001; Manz *et al.* 2005). This water uptake is tri-phasic, including an initial rapid period (phase I), followed by a plateau phase with little change in water content (phase II) and a subsequent increase in water content coincident with the resumption of growth and radicle emergence (phase III) (Bewley 1997; Gallardo *et al.* 2001; Manz *et al.* 2005). All biochemical processes are initiated for germination *sensu stricto* during phase I and phase II, i.e. seeds maintain their desiccation tolerance (Bradford 1990; Bewley and Black 1994; Bewley 1997), and it is at the point just prior to radicle emergence that primed seeds are dried back. Seed priming generally causes quicker germination and faster field emergence, compared to unprimed seed, which has practical agronomic implications (McDonald 2000); with maize caryopses specifically, primed maize caryopses perform better than unprimed caryopses (Chiu *et al.* 2002). One crucial determinant of primed seed performance is the post-priming storage environment (Chiu *et al.* 2002) which must be maintained at optimal humidity and temperature levels to maintain viability. In general, even under controlled conditions, primed seed (Tarquis and Bradford 1992; Nascimento and West 2000) lose viability more readily than unprimed seeds (Osborne *et al.* 2002).

The successes of seed priming are attributed to the maintenance of desiccation tolerance through the priming process (Gallardo *et al.* 2001). Field establishment for maize can be improved by priming (Finch-Savage *et al.* 2005) but the nature of the resultant changes from desiccation tolerance to intolerance, or why primed seeds do not store very well, is still not fully understood (Osborne *et al.* 2002).

There are various definitions of germination. To the seed physiologist, germination is defined as completed when the radicle emerges through the testa



(pericarp in the case of maize). According to Copeland and McDonald (2001), germination is considered to be the resumption of active growth by the embryo resulting in the rupture of the seed coat and the emergence of a young plant. This latter definition presupposes that the seed has been in a state of quiescence after its formation and development (Copeland and McDonald 2001). It is at this juncture that the effects of water uptake (imbibition) on the quiescent seed have to be considered, as it is the required seed state for the commencement of germination, whether the seed has been through a storage period or not.

### **1.5. Cellular events at rehydration**

Imbibition is the first phase of the water absorption process by dry, permeable seeds in moist surroundings (Souza and Marcos-Filho 1993) and is the second wet phase in the wet-dry-wet cycle of the seed development to germination continuum (Oliver *et al.* 1997a). The relatively water-proof phospholipid bilayer of the first wet phase dries to a gel phase and becomes porous (Bewley and Black 1994; Hoekstra and Golovina 1999) allowing for the free passage of solutes (Simon 1974). Imbibition is essentially a physical event driven by a water potential gradient established between the seed and the substratum (Woodstock 1988). Different imbibition rates are associated with different species and different physiological qualities of seed (Souza and Marcos-Filho 1993). The considerable difference in water potential between the interior of the dry embryo and the surrounding (soil) water, as well as the membrane state, i.e. gel phase when dry (Crowe *et al.* 1989), result in large amounts of water being taken up. This can result in a significant amount of imbibitional damage in a relatively short period (Leopold 1980; Duke *et al.* 1983; Powell 1988; Wolk *et al.* 1989; Garnczarska *et al.* 2007).

#### *1.5.1. Imbibitional injury*

The initial phases of rehydration occur rapidly (Vertucci and Leopold 1986; Wolk *et al.* 1989) with the majority of the water uptake occurring in the first 30 minutes

of imbibition (Platt *et al.* 1994) and with the radicle hydrating first (Platt *et al.* 1994). This has been confirmed with the aid of Nuclear Magnetic Resonance (NMR) imaging or Magnetic Resonance Imaging (MRI) (Ishida *et al.* 1987; Manz *et al.* 2005; Terskikh *et al.* 2005; Kikuchi *et al.* 2006; Garnczarska *et al.* 2007; Meyer *et al.*, 2007); a non-invasive procedure which is able to trace the dynamic movement of water in plant tissues (Callaghan 1991). Imbibitional injury/damage is characterised by oxidative stress (see later) and a rapid loss of intracellular constituents. These leached substances, or electrolytes, are mostly of low molecular weight such as ions, amino acids and sugars (Hoekstra *et al.* 1999) and their presence in the leachate, detectable by electrical conductivity readings (Rosa *et al.* 2000), is an indication of the extent of membrane damage (Williams *et al.* 1995). Most imbibitional damage is sustained during the early wetting, i.e. second wet phase, of the seed tissue (Pollock 1969), in particular within the first hour of imbibition (Rosa *et al.* 2000; Hoekstra *et al.* 2001; Osborn *et al.* 2002).

#### *1.5.1.1. Electrolyte leakage*

Measurement of electrolyte conductivity of seed leachate is a widely employed method of determining the level of damage within seeds (Williams *et al.*, 1995) and correlates with many responses *viz.* the state of membrane lipids (Hoekstra *et al.* 1992; Lacan and Baccou 1996; Cordova-Tellez and Burris 2002), assessment of membrane competence (Whitlow *et al.* 1992), seed vigour and field emergence (Borowski *et al.* 1995), hydration mechanisms in seeds (Konsta *et al.* 1996) and seed storability (Marks and Stroshine 1998). The conductivity value of leachate obtained from an imbibed seed therefore provides an account of the seed's biochemical and physical condition. As imbibition proceeds, the seed will reach a maximum leakage moisture level, beyond which the membrane again becomes an effective barrier against electrolyte loss and leakage rates decline (Wolk *et al.* 1989).

#### 1.5.1.2. Oxidative stress

Oxidative stress could have been included in section 1.4.2a where an account of the damaging effects of drying was given, but its occurrence during dehydration and rehydration are considered here. The formation of reactive oxygen species (ROS) occurs during drying, resulting in damage (Vertucci and Farrant 1995). ROS include hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), superoxide radicle ( $\text{O}_2^-$ ) and hydroxyl radicle ( $\text{OH}$ ), all of which are highly reactive and induce lipid peroxidation, thereby affecting the structural integrity and permeability of cellular membranes (Dai *et al.* 1997).

The metabolic conditions that dramatically increase the probability of their formation include:

- The concentration of mitochondrial electron carriers
- Depletion of the ADP pool by phosphorylation to ATP, i.e. ADP limiting condition
- The rate of  $\text{O}_2$  consumption by cytochrome oxidase (COX)
- The  $\text{O}_2$  availability (Leprince *et al.* 2000).

Oxidative stress is the result of an imbalance between the production of reactive oxygen and the tissues' ability to readily detoxify it by the production of oxygen free radicle scavengers or antioxidants (Oliver *et al.* 2001). The hydroxyl radicle formed through the iron-catalysed Fenton reaction, initiates oxidative injury (Price and Hendry 1991); and, in dehydrating organisms, the origin of oxidative stress is likely to be due to an uncontrolled formation of ROS as a result of the impairment of the electron transport chains (Oliver *et al.* 2001). This impairment is expected to occur within an interval of intermediary hydration levels where the down-regulation of metabolism becomes uncoordinated (Leprince *et al.* 1999, 2000). The accumulation of ROS has been clearly associated with the aging process in coffee seeds (Dussert *et al.* 2006).

The resulting damage from the formation of ROS during dehydration (Vertucci and Farrant 1995) occurs up to a certain level as studies using orthodox seeds suggest that a controlled down-regulation of metabolism must occur during drying to avoid over-production of ROS and free radicle damage (Leprince *et al.* 1994; Vertucci and Farrant 1995). Plants also metabolise activated oxygen species by invoking an increased activity of enzymatic antioxidant such as superoxide dismutase, catalase, peroxidase and glutathione reductase (Kondo and Kawashima 2000) in addition to producing larger amounts of non-enzymatic antioxidants such as proline, flavonoids, ascorbate and carotenoids (Arora *et al.* 2002; Matysik *et al.* 2002). An imbalance is caused by an increase in concentration of ROS without a matching counterbalance in antioxidant and lipid hydroperoxide production (Puntarulo 1994). During drying, the removal of water increases cytoplasmic viscosity which influences the diffusion of metabolites in the mitochondria and the cytoplasmic matrix, as well as the mobility of the electron carriers within the mitochondrial lipid bilayer (Fato *et al.* 1993). ROS activity, therefore, is limited by the increased viscosity caused by drying.

Upon absorption of water, an oxidative stress is initiated. The production of ROS occurs when quiescent dry seed switch their metabolism from quiescence to germination mode by resuming oxygen uptake, oxidative phosphorylation, and the metabolism of storage reserves (Tommasai *et al.* 1999); and has been regarded as a cause of stress that can ultimately affect the success of germination (Wojtyla *et al.* 2006). Therefore, antioxidant compounds and enzymes are of particular importance for successful germination (Bailly 2004). The increase in concentration of ROS is a result of the reduction of water (H<sub>2</sub>O) by mitochondrial cytochrome oxidase which requires oxygen as the final electron acceptor (Puntarulo 1994) in the electron transport chain. In addition, ROS can also evolve from oxygen which can be reduced to the free radicles, superoxide and hydrogen peroxide (Boveris *et al.* 1978; Chance *et al.* 1979; Forman and Boveris 1982). These free radicles can cause damage to developing tissue by interfering with the assimilation of various membrane components and, hence, compromise structural integrity of the cell (Puntarulo 1994). However, as seed tissues rehydrate, various

oxygen-free radicle scavengers or antioxidants are produced, as described above, and released into the cells, where they counteract the adverse effects of the ROS (Cakmak *et al.* 1993; Oliver 1996; Bailly *et al.* 2002).

The precise time, after the onset of imbibition, for the release of these enzymes appears not to have been established for maize. However, Hoekstra *et al.* (1999) have shown that there is a specific time requirement after the onset of imbibition for a transient change in plasma membrane permeability to occur in *Typha latifolia* pollen grains. Imbibitional injury has been reported in pollen, fungal conidia, yeasts, mosses and seeds and occurs within seconds of contact with liquid water (Hoekstra *et al.* 2001).

#### 1.5.2. Cellular protection and repair at rehydration

Wood and Oliver (1999) have illustrated that imbibing tissue employs one of two strategies during water uptake; namely a repair- or a protection-based strategy. Plants feasibly utilise mechanisms which include both of these strategies. However, as desiccation tolerance has evolved independently on a number of separate occasions (Oliver and Bewley 1997) it would be expected that there are examples of plants which “span the spectrum” of possible combinations of the two strategies, i.e. from plants that rely greatly on cellular protection to those that rely more on cellular repair (Oliver *et al.* 1997).

The physical protection of seeds is provided by the testa and the pericarp. The testa also acts as a barrier that regulates water movement both temporally and spatially (Osborne *et al.* 2002). It would appear that the recovery mechanisms are temporally separated with protection as the initial process and progressively a repair based strategy is employed as tissue becomes more hydrated and active. rRNA synthesis occurs within the first ten minutes of rehydration (Osborne *et al.* 2002) to facilitate the synthesis of a variety of proteins and initiate DNA repair (Osborne *et al.* 1977). The hypothesis that mRNA is sequestered into mRNA particles during desiccation and are required for recovery (Wood and Oliver 1999)

would result in the rapid repair of rehydration-induced damage, thus minimizing the period necessary to restart growth upon rehydration (Wood and Oliver 1999). For resumption of growth to be least impacted, it is necessary for the preceding dehydration process to be slow enough for protective (e.g. antioxidants) or preparative (e.g. rRNAs) molecules to be accrued in the tissue. The effects of rehydration therefore are dependent on the events at dehydration, i.e. the extent and speed of recovery is dependent on the rate at which desiccation occurred and the faster the rate of desiccation the slower the recovery (Wood and Oliver 1999).

Protection can occur at the enzyme level too (Osborne *et al.* 2002). A class of rehydration enzymes have been identified as being active during imbibition and involved in the production of antioxidants which would limit the effects of free radicals in seeds (Hoekstra *et al.* 2001) thereby conferring a protective function. The extent of imbibitional damage would therefore be prescribed by the treatments/ conditions to which these maize caryopses were subjected, for example, drying regime and subsequent storage conditions.

The results obtained by Wood and Oliver (1999) indicate that orthodox seeds require a certain amount of water loss to fully activate the protein based (i.e. repair) portion of the recovery mechanism upon re-hydration. It may also indicate that there is a mechanism or metabolism switch whereby the amount of water lost is “sensed” and “translated” into a protein synthetic response (Dasgupta *et al.* 1982; Kermode and Bewley 1985a; Kermode and Bewley 1985b; Kermode *et al.* 1985). Wood and Oliver (1999) contend that this particular repair-based strategy has evolved to parallel the amount of energy expended in repair to the amount of damage potentiated by varying extents of drying.

## **1.6. Seed storage**

Seed storage allows for the convenient retrieval of viable orthodox seeds when required for human needs and in itself is a method of conservation. Broadly, the methods of conservation can fall into two categories: *in situ* and *ex situ*

conservation.

*In situ* conservation of seeds encompasses all methods of maintenance which occur in their natural/original environment, which is critically important, as it allows the progression of evolutionary processes (Benson 2008); while *ex situ* conservation of seeds include samples in herbaria, botanical institutes, industrial seed silos, breeding programmes, and seed collections held at a variety of institutions including universities, science councils, BRCs and seed banks (Bajaj 1995; Benson 2008).

#### *1.6.1. Conventional ex situ methods of orthodox seed storage*

There are several means of seed storage, for example: simple storage methods, and advanced storage methods that are often referred to as conventional. Cryopreservation, is now also employed more frequently on a variety of orthodox and recalcitrant species (Bajaj 1995; Towill and Bajaj 2002) and this method of long term storage is recognized as an effective means of germplasm preservation.

Germplasm is the matter where one can find the principle (fundamental) genetic material which can grow and develop (Querol, 1992). The requirement for germplasm preservation has become mandatory to prevent the losses of original stock because of selection (Querol 1992; Lupotto *et al.* 1995). In third world/developing countries, although the need to conserve biological material is recognised, the facilities and finances are often unavailable and the employed methods of storage are simple and not technologically advanced.

##### *1.6.1.1. Simple storage methods*

In its most simple form, seeds may be stored using clay pots, holes dug in the ground as well as woven baskets. Seeds can be dried in the sun or close to a fire to ensure dry conditions and avoid high moisture contents, which affect seed viability. Storing the seed near a fire also reduces pest infestation. These storage

methods are commonly used throughout the third world, but are hardly optimal for storage, without financial implications (Mycock 1990).

#### *1.6.1.2. Advanced storage methods*

A good seed store is capable of regulated temperatures and relative humidity as well as protection against theft, rodents, birds, insects and micro-organisms. These factors are not always eradicated, but to some extent, at least controlled (Mycock 1990). Many of these requirements are satisfied by the use of silos, designed specifically to ensure dry, cool conditions, but unfortunately, uniformity of temperature and relative humidity are not always attainable throughout the entire storage chamber. Genetic drift is an additional factor influencing seed in conventional storage and it cannot be controlled by any means (Wolf 1993). Genetic drift can be attributed to at least two causes. Firstly, as a seed lot ages, many seeds die and the few that survive are not representative of the same average genetic composition of the original lot. Secondly, drift, manifested as mutations, occurs as storage time increases. Mutation rates are minimised by low seed moisture and cool temperatures (Smith and Berjak 1995; Walters 1998).

Many countries have developed programmes for the storage of orthodox seed. Four types of seed storage programmes are operative and applicable to the storage of maize caryopses (ISTA 1999):

- Commercial seed storage, where storage is over the same length of time as the natural quiescent period of the seed;
- Carry-over seed, which involves the storage of seeds through the first growing season after harvest until the time for the second planting, i.e. it is in storage for 12-18 months (for maize). Such stores are ventilated and fumigated;
- Foundation stock seed, which may need to be kept for a period of several years and
- Germplasm storage, where seeds are kept for many years, usually below 0



°C and in equilibrium with relative humidities between 20% and 25%. This particular type of storage is extremely expensive.

The quantity of seed stored for both foundation and germplasm is small and the costs are often borne by agricultural agencies and not individual farmers or producers (Mycock 1990), making these forms of storage practicable.

#### *1.6.2. Consequences of seed storage*

Age-induced deteriorative events, some of which are associated with ROS-induced oxidative injury (McDonald 1999; Taylor *et al.*, 1998) cannot be controlled during seed storage, except perhaps by seed priming which can prevent some of these changes (see earlier) (Taylor *et al.* 1998; Chiu *et al.* 2003), provided that the seed has not been stored for protracted periods (Walters 1998). Rate of ageing is influenced by the temperature and the moisture contents at which the seeds are stored (Walters 1998). Smith and Berjak (1995) also showed that as seeds age, their membranes become “leaky”, enzymes lose catalytic activity, and chromosomes accumulate mutations. Furthermore, embryonic axes and seedlings of imbibed aged maize caryopses have been shown to have reduced respiration rates and reduced biomass accumulation (Cruz-Pérez *et al.* 2003). At germination, the embryonic axes of aged seeds showed lower respiration rates and biomass accumulations than non-aged seed. Seed aging therefore, reduces the metabolic efficiency of embryonic axes and thus, the magnitude of damage by ageing depends greatly on how old the seed is (Cruz-Pérez *et al.* 2003).

Seeds in storage can lose vigour and eventually viability through protein degradation (Walters 1998) or can succumb to the effects of various microbes. Various ultrastructural studies conducted by Berjak and Villiers (1972a,b,c and d) on maize embryos revealed that damage occurred with increased storage time resulting in decreased rates of germination, seedling growth (Haydecker 1972), and also impacts on the integrity of DNA and chromosomes (Bewley and Black 1994). This directly results in an increased number of morphologically abnormal

seedlings post germination (Mackay 1972; Bewley and Black 1994). Membrane aberrations increase with increasing seed age (Berjak and Villiers 1972a) and the resulting increase in metabolite and ion leakage (Roberts and Ellis 1982; Ouyang *et al.* 2002) may be the underlying reason for the visible abnormalities in aged seed. Furthermore, it is hypothesised that the various events which occur with the rehydration of dry seed tissue, for example the synthesis of protective proteins and / or the initiation of repair mechanisms (Oliver *et al.* 1997), could be impaired in aged seed and thus, the deterioration which occurs in ageing seed is exacerbated by imbibition.

At the biochemical level, seed ageing is accompanied by a decline in metabolic activity upon germination (Ferguson *et al.* 1990), including changes in enzyme activity (usually a decrease) (Bernal-Lugo *et al.* 1994), and a decline in protein and nucleic acid biosynthesis (Dell'Aquila 1994; Cruz-Garcia *et al.* 1995). Nevertheless, this is simply a list of symptoms of seed ageing and does not address the biochemical and molecular mechanisms behind the loss of vigour and longevity which remain complex and beyond our current comprehension (Bernal-Lugo *et al.* 1994).

An avenue that has been extensively explored to avoid the negative consequences of lengthy seed storage, i.e. natural ageing, has been to use cryopreservation.

### **1.7. Cryopreservation**

Cryopreservation is defined as the storage of viable cells, tissues, organs and organisms at ultra low temperatures/cryogenic temperatures. Usually in liquid nitrogen (LN) at a temperature of -196°C (Benson 1994) and including -140°C the temperature of LNs vapour phase (Benson 2008). These cryogenic temperatures have also been expanded to include some of the earth's coldest environments where natural cryobanks have been established (Benson *et al.* 2007). The basic principle of cryopreserving material is "freezing biological time" (Benson 2008), i.e. cryopreserved material is presumed to remain genetically stable (Reed *et al.*

2001).

#### 1.7.1. *Advantages of cryostorage*

Cryostorage could potentially accomplish the “freezing of biological time” and then *ex situ* conservation by cryostorage (within gene banks) would not allow for evolutionary forces to continue including for instance, factors which would be important and necessary for disease resistance. However, in order to maintain diversity, cryostorage as a means of *ex situ* conservation is widely employed for many species that are both commercially important or are endangered (Towill and Bajaj 2002).

Cryopreservation, has provided an opportunity for the storage of recalcitrant seed species. For example *Quercus robur* (Sun 1999; Mukherjee *et al.* 2006), various tropical rain forest seed (Vazquez and Arechiga 1996), *Camellia sinensis*, *Theobroma cacao*, *Arrocarpus hererophyllus* (Chandel *et al.* 1995), *Azadirachta indica* (Berjak and Dumet 1996), *Garcinia mangostana*, *Lansium domesticum*, *Baccaurea polyneura* (Normah 2000), *Warbugia salutaris* (Kioko *et al.* 2003), *Trichilia dregeana* (Berjak and Mycock 2004) *Betula pendula* (Ryynanen and Aronen 2005) and *Bromus inermis* (Ishikawa *et al.* 2006) have all been subjected to cryopreservation techniques with promising results.

Various kinds of biological material have also been cryostored and include seed, pollen, shoot apices, dormant buds, excised embryonic axes and somatic embryos, of a variety of species (Towill and Bajaj, 2002; Berjak and Mycock 2004; Ryynanen and Aronen 2005; Ishikawa *et al.* 2006). Cryopreservation has important commercial applications and provides a technique of storing valuable living assets which obviates the risks of genetic change.

#### 1.7.2. *Limitations to cryostorage*

Although cryostorage appears to be a solution which obviates the problems

associated with conventional storage, i.e. the effects of relative humidity and pest invasions, there are a number of limitations to this process. The achievement of successful cryopreservation encompasses many steps, of which freezing is only one (Benson 1994). The first step is the choice and size of the sample. The sample size is important because larger samples do not freeze uniformly. For example, if one were to plunge a large seed directly into liquid nitrogen the peripheral tissues would freeze at a rate faster than the centre of the seed.

Secondly, the water content of the sample must be assessed prior to freezing to predict possible damage due to intracellular ice crystal formation (Karlsson *et al.* 1993). A mechanism to prevent ice crystal formation in tissue which is predisposed to a drying regime (Berjak 1989) could possibly be treated with a cryoprotectant (Mycock *et al.* 1995; Blakesley *et al.* 1997; Reyes-Diaz *et al.* 2005; Kioko *et al.* 2006; Nadarajan *et al.* 2006). As water in plant tissue (seeds) with moisture contents of 5-10 %, is in an unfreezable state (Burke *et al.*, 1983) exposure to extremely low temperatures does not result in freezing injury (Becwar *et al.* 1983). Samples at these water contents would therefore not require cryoprotection. The final factors that need to be considered to optimise the cryoprotocol are the rates of freezing and thawing (Bagniol and Engelmann 1992; Mycock *et al.* 1995).

#### *1.7.2.1. Freezing rate*

Two rates of freezing, slow or rapid, have been employed in the successful cryopreservation of plant material. Slow cooling methods usually involve equilibration of the material in concentrations of cryoprotective additives followed by slow cooling to -40 °C (intermediate sub-zero temperature), before submerging in liquid nitrogen (Morrisset *et al.* 1993). Rapid freezing is accomplished by direct immersion of the specimens in liquid nitrogen, thereby achieving cooling rates in the order of several hundred degrees per minute (Karthi 1987). Slow freezing was thought to be the most effective in preventing intracellular ice formation and hence, injury to the cells (Bajaj 1979; Karthi

1987). However, rapid freezing techniques have been shown to be successful (Hitmi *et al.* 2000). It has been emphasised, however, that the effects of cooling rate are dependent on the water content of the axes (Wesley-Smith *et al.* 2004).

Reduced water content increases cytoplasmic viscosity (Wesley-Smith *et al.* 2004) which in turn slows ice crystallization, making survival independent of cooling rate (Wesley-Smith *et al.* 2004). Unlike pure water, which requires an ultra rapid cooling rate to become vitrified, solutions such as those found within and surrounding cells, will vitrify more readily as their concentrations increase with decreasing hydration levels (Mycock *et al.* 1991; Wesley-Smith *et al.* 2004). Consequently, it has been suggested that, if cells can be treated to ensure vitrification (e.g. treated with a cryoprotectant) without necessarily reducing the moisture content, material could be immersed directly into liquid nitrogen and rapidly cooled (Grout 1990).

In many laboratories/institutions, cryostorage of plant material is not feasible, simply due to the financial implications. The rapid freezing of biological material for cryopreservation resulting in the required vitrification, unlike the slow freezing methods, does not require complex and expensive controlled-rate freezing equipment (Grout 1990). Hence, a concerted effort to develop protocols for cryostorage of plant cells and tissues using rapid freezing procedures could have a dramatic impact on increasing the diversity of plant genetic resources that can be conserved (Mycock *et al.* 1991).

#### *1.7.2.2. Thawing rate*

The rate of thawing is particularly critical in vitrified tissues as ice crystallization can occur during rewarming (Benson 1994). The rates at which tissues are thawed are also dependent on the freezing method. In conventional plant cryostorage methods, thawing is usually achieved by plunging the cryovials into sterile water maintained at 40-45°C until the samples have thawed and can be plated (Benson 1994).

### 1.7.2.3. *Regeneration*

Intact seeds can be germinated by “conventional” means after retrieval from cryostorage. However, isolated plant organs/tissues/organisms such as embryos require *in vitro* procedures. In such cases the correct medium for regeneration is an important factor and needs to be established for each species.

## 1.8. *Rationale*

Cryostorage/cryopreservation is employed to achieve the “freezing of biological time” (Benson 2008) and preserve tissues at sub-zero temperatures. It appears to be most effective in samples with relatively low water contents or tissues which have acquired desiccation tolerance. This freezing of biological time can be considered as occurring in two ways. Firstly, cryogenic temperatures are used to suspend biological ageing processes in active cells. The second way relates to the extent to which these temperatures can arrest molecular mobility for an undefined period (Benson 2008). Both these theories are considered in this study on imbibed tissue (post-quiescence) and at quiescence, i.e. post-maturation drying, presumed to remain in this state of arrested development during cryopreservation, until they are processed for recovery. Controlled, slow freezing methods and treatment with cryoprotectants would have considerable cost implications. Therefore, a rapid freezing method was employed commensurate with the equipment and resources available. Intact, dry (10-15% water content) maize caryopses of *Hybrid 6363* however, did not survive direct immersion into liquid nitrogen even though they had acquired desiccation tolerance through the process of maturation drying. The inability of these intact caryopses to freeze well was attributed to the size of the seed. Freezing did not occur uniformly in these relatively large caryopses and resulted in the cracking of the caryopsis, damaging the embryo. To reduce the sample size effectively for cryopreservation, embryonic axes were excised from the intact caryopses. Caryopses were imbibed to soften the pericarp and a range of imbibition times were considered to determine the optimal moisture level of the caryopsis to facilitate the removal of the axes with relative ease. Hydrating

the tissue resulted in a series of natural responses by the caryopsis and effectively started its biological clock. Subsequent cryostorage of the excised embryos at these specific hydration levels consequently gave rise to varying results. The observed differences in tissue response to cryopreservation were attributed to the loss of desiccation tolerance as imbibition time increased. Vigour, viability, ultrastructural and biochemical investigations of these maize embryos were necessary to gain an understanding of the biological processes occurring upon rehydration and the subsequent effects of cryostorage.

### **1.9. Objectives**

The key objectives of this study were to:-

- ① Determine the initial effects of imbibition and subsequent cryostorage on the ultrastructure of isolated maize root tissues.
- ② Given the successes in germinability after cryostorage of hydrated tissue, the second objective was to determine the effects of imbibition and cryostorage on the composition of the leachate and, whether electrolytes were capable of behaving as natural cryoprotectants.
- ③ To determine the effects of long term cryostorage on imbibed maize axes and whether freezing of biological time is achievable.



## Chapter Two                      Effects of cryostorage on vigour and viability after varying imbibition times

### 2.1.     Introduction

Many species produce seeds that dry to low water contents during their development (maturation drying of orthodox seeds) and in the dry quiescent/dormant state can be stored at low temperatures (Roberts 1973). Because maize produces orthodox caryopses conventional low temperature, low relative humidity methods can be used to store the caryopses of this important crop species for protracted periods (Roberts 1973; Berjak *et al.* 1986).

Mature maize caryopses have low water contents (< 10%) and thus can be stored at sub zero temperatures (-20°C) without profound losses of vigour and viability (Stanwood 1985). As a consequence, storage in liquid nitrogen (germplasm storage) has been achieved for a number of maize varieties (Stanwood 1985). However, air dried intact maize caryopses of a local South African *Hybrid 6363* cannot withstand the effects of cryostorage (direct submergence into the cryogen) and this has been attributed to the size of the caryopsis (Isaacs 1996).

The non-uniform freezing and thawing of the intact caryopses resulted in the splitting of the embryo (Fig. 2.1), thus rendering the caryopsis nonviable. It was hypothesized that if the axis was isolated, thereby reducing the size of the sample (Fig. 2.2), the material could be successfully retrieved from liquid nitrogen and regenerated *in vitro*. Isolation of the axis from the dry caryopsis was difficult, time consuming and physically damaging to the tissues, e.g. two in four axes were destroyed during isolation. Imbibition was, therefore, used as a method of softening the testa and pericarp prior to isolation of the axes. The point at which desiccation tolerance is lost in the early stages of imbibition and the effects of subsequent cryostorage on imbibed *Hybrid 6363* axes were investigated.

**Fig 2.1.** The intact maize caryopsis, when exposed directly to liquid nitrogen, splits (presumably due to the size of the seed) rendering the caryopsis non-viable. Scale Bar =3.75mm.

**Fig. 2.2.** An indication of the size of an intact caryopsis in comparison to the size of an isolated embryo axis. Scale Bar = 4.4.mm.



## 2.2. Materials and methods

### 2.2.1. Imbibition of intact caryopses

Intact caryopses of the *Zea mays Hybrid 6363* (PANNAR) were imbibed in sterile distilled water for 0, 1, 2, 3, 4, 5 and 6 hrs. Embryonic axes in each treatment were then isolated, surface-decontaminated in a 2% v/v sodium hypochlorite solution for 15 minutes and washed in sterile ultra pure water for a further 15 minutes, thereby potentially increasing each imbibition time by a further 30 minutes.

### 2.2.2. Water content determinations

Individual embryo (40 from each imbibition time) water contents, gravimetrically expressed on a fresh weight basis, were determined post-surface-decontamination and after drying for  $17 \pm 1$  hrs in a ventilated oven at  $80^{\circ}\text{C}$  according to the modified ISTA Low Constant Oven Temperature Method (International Seed Testing Association 1985). The following equation was used to determine the fresh weight water content values:

$$\frac{\text{fresh weight} - \text{dry weight}}{\text{fresh weight}} \times 100$$

The water content values were expressed as a percentage. Each determination was performed three times.

### 2.2.3. *Vigour and viability assessment*

#### 2.2.3.a. *Intact caryopses*

250 caryopses were placed into 90mm Petri dishes (10 caryopses per dish) and set to germinate on filter paper moistened with 10 ml of sterile distilled water. The caryopses were monitored for germination and growth every 24 hours. Emergence of the radicle served as an indicator of viability, and the length of the radicle measured over time as an indication of vigour (rate of growth). Radicles achieving lengths greater than 5mm in 48 hours were indicative of caryopses more vigorous than those producing radicles less than 5mm in length. The germination index (GI) (Czabator 1962) was determined as follows:

$$\text{GI} = \text{MDG} \times \text{PV}$$

- GI = germination index.
- MDG = mean daily germination (final percent germination divided by the length of the test period).
- PV = peak value (percentage germination on a particular day divided by the number of days taken to achieve that percentage. The highest value achieved over the 4-7 day experimental period is used as an indication of caryopsis vigour).

#### 2.2.3.b. *Isolated embryonic axes*

A further 40 isolated axes per treatment were placed into tissue culture tubes in an aseptic manner with a standard Murashige and Skoog (MS) (1962) basal medium. The MS medium was supplemented with 30 g.l<sup>-1</sup> sucrose, 2 g.l<sup>-1</sup> Gelrite and adjusted to pH 5.8 prior to autoclaving at 121°C for 20 minutes. The material was incubated at 25°C with a 16:8 hrs light : dark cycle with a light intensity of 200 μE.m<sup>2</sup>.sec<sup>-1</sup>. Developing seedlings were monitored every 24 hours and the emergence of the radicle served as an indicator of viability. Vigour was assessed

by the rate of growth. The germination index (2.2.3.a) provides an indication of the percentage germination and rate of growth, which was calculated for all treatments.

#### *2.2.4. Cryostorage of isolated embryonic axes*

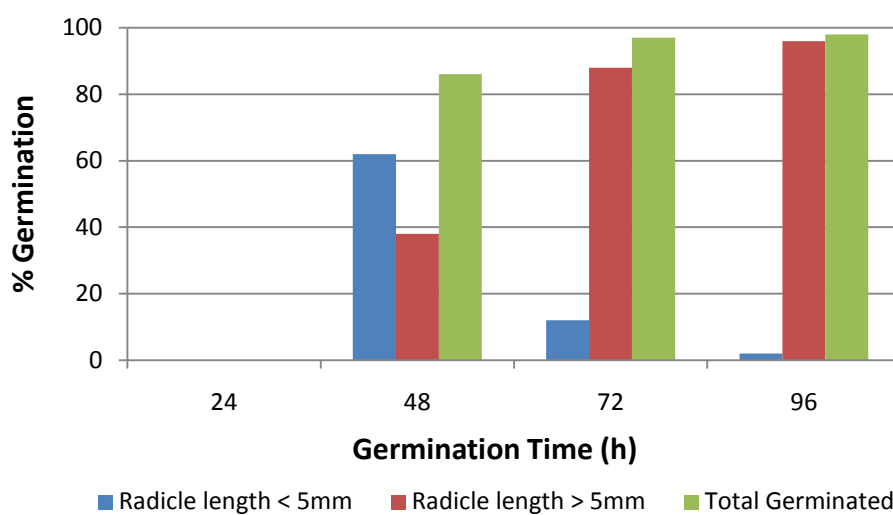
Isolated embryonic axes from each treatment (40 per treatment) were placed into cryovials on cryocanes, and plunged directly into liquid nitrogen, cooling the tissue at a rate of approximately  $-200\text{ }^{\circ}\text{C}/\text{min}$ . The samples were held in liquid nitrogen for 15 minutes, after which, the cryocanes were immersed into a  $30\text{ }^{\circ}\text{C}$  water bath to thaw the embryonic axes. The axes were placed into tubes with the MS basal medium (2.2.3. b) and tested for viability and vigour. Each treatment was repeated at least three times.

#### *2.2.5. Data analysis*

Water content data sets were arcsine transformed to approximate normality and subjected to a one-way ANOVA using Statistica 6.0 (Statsoft 2001). Tukey post hoc comparisons were used to establish significant differences between each imbibition time. Viability data were analysed using contingency tables and Chi square tests using Instat version 3.0 (GraphPad software 2003). Germination indexes and percentage root formation data were analysed using Fisher's exact tests using Instat version 3.0.

### 2.3. Results and discussion

98.4% of the caryopses germinated within five days and 97% had obtained radicle lengths greater than 5mm in that time. These results were indicative of the quality of the caryopses, i.e. this hybrid was viable as well as vigorous in growth.



**Figure 2.3.** Percentage germination and size classes of radicle length of caryopses over time (96 hrs).

**Table 2.1. Water contents of isolated maize embryonic axes (n=40) imbibed for 0 - 6 hrs.** Water contents with the same superscript are not significantly different ( $P < 0.001$ , Tukey post hoc test).

| Imbibition Time (hrs) | Water Content % (FWB) $\pm$ Standard Error |
|-----------------------|--------------------------------------------|
| Intact Caryopses      | $7.4 \pm 1.3^a$                            |
| 0 (dry excised axis)  | $12.36 \pm 0.8^b$                          |
| 0.5                   | $52.58 \pm 2.1^c$                          |
| 1.5                   | $54.17 \pm 4.7^c$                          |
| 2.5                   | $57.73 \pm 1.8^{cd}$                       |
| 3.5                   | $57.93 \pm 3.5^{cd}$                       |
| 4.5                   | $60.9 \pm 6.2^d$                           |
| 5.5                   | $59.31 \pm 5.0^d$                          |
| 6.5                   | $61.36 \pm 10.2^d$                         |

Imbibition is the first phase of the water absorption process by dry, permeable seeds in moist surroundings (Woodstock 1988). With an increase in imbibition time there was a corresponding increase in water content (Table 2.1). Isolated dry embryonic axes were at higher water contents than the whole caryopsis (Table 2.1) indicating that a greater proportion of the total caryopsis water is found in the embryo. This is expected as the embryo radicle is the primary site for water uptake in seeds (McDonald *et al.* 1994; Platt *et al.* 1994; Ishida *et al.* 1987; Manz *et al.* 2005; Terskikh *et al.* 2005; Kikuchi *et al.* 2006; Garnczarska *et al.* 2007;



Meyer *et al.* 2007).

Imbibition not only results in an increase in water content but in several membrane and biochemical changes (Bewley and Black 1994). The uptake of water by a physiologically mature seed, i.e. seed that has completed maturation drying (see Chapter One) occurs in three phases (Bewley 1997). Within the first two hours of imbibition (Table 2.1) a rapid initial uptake of water occurs (coinciding with phase I of water uptake described by Bewley (1997)) followed by a plateau phase between 3.5 and 6.5 hours of imbibition (possibly phase II of water uptake). The third phase of water uptake only occurs after germination is completed, as the embryonic axes elongate leading to radicle emergence, and this was not investigated in this study. McDonald *et al.* (1994) indicated that water uptake, specifically in maize, occurs largely within the first six hours of imbibition. Additionally, water uptake was found to be greater in the embryo of maize caryopses than in the endosperm (Mc Donald *et al.* 1994) and differences in viability and vigour would be anticipated post-cryostorage due to increased hydration levels of the embryo.

Although the effects of the surface decontaminant on the membranes are poorly understood it is thought to intensify water uptake within the initial hour of imbibition but not necessarily result in excessive electrolyte leakage (Pammenter pers. comm.)<sup>1</sup>. Further investigation is necessary to determine the effect of the surface decontaminant on water uptake.

Vigour and viability of the 0.5 hrs (surface decontamination) 3.5 hrs and 6.5 hrs imbibed, isolated axes (Fig 2.3), remained comparable with the intact caryopses (Fig 2.4). All embryos in all treatments germinated within 96 hours (Fig. 2.4). Similarly, cryostored axes were equally viable (Fig. 2.5) despite the increasing water contents with each imbibition time ( $\chi^2=0.598$ ,  $P=0.9964$ ). This would

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<sup>1</sup> Pammenter, N (2001) School of Biological and Conservation Studies. University of Kwa Zulu Natal University Republic of South Africa.

indicate that the cryoprocurement did not reduce the viability of tissue at higher water contents. A comparison of the germination indices (Fig. 2.6) however, showed that although cryostorage did not significantly affect vigour in the 3.5 hrs sample ( $\chi^2_1 = 2.016$ ,  $P=0.05$ ) it did affect vigour significantly for the 0.5 hrs ( $\chi^2_1 = 4.934$ ,  $P=0.05$ ) and 6.5 hrs ( $\chi^2_1 = 5.114$ ,  $P=0.05$ ) imbibition times respectively. This was clearly seen by the reduced germination index value for each imbibition time. In addition to the reduced germination index, cryostored samples in the hydrated tissue post-cryostorage (i.e. 6.5 hrs) exhibited some changes/abnormalities such as the lack of root formation (Table 2.2) and inverted shoots, which appeared to be negatively gravitropic (Fig 2.7). Reduced growth rates were also observed (Fig. 2.6).

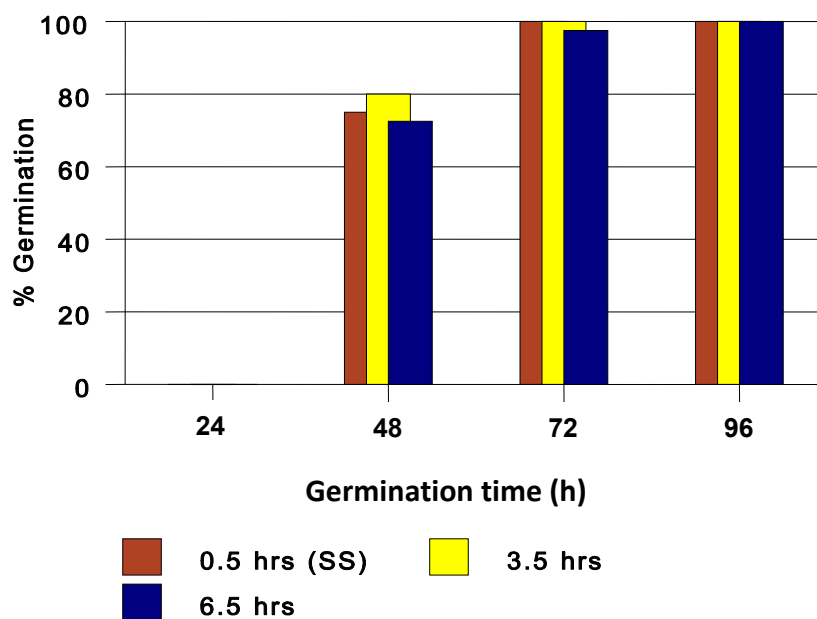


Figure 2.4. % Germination of axes isolated from dry, intact caryopses and from caryopses hydrated for 0, 3 and 6 hrs respectively (+ additional 30 min surface sterilization step).

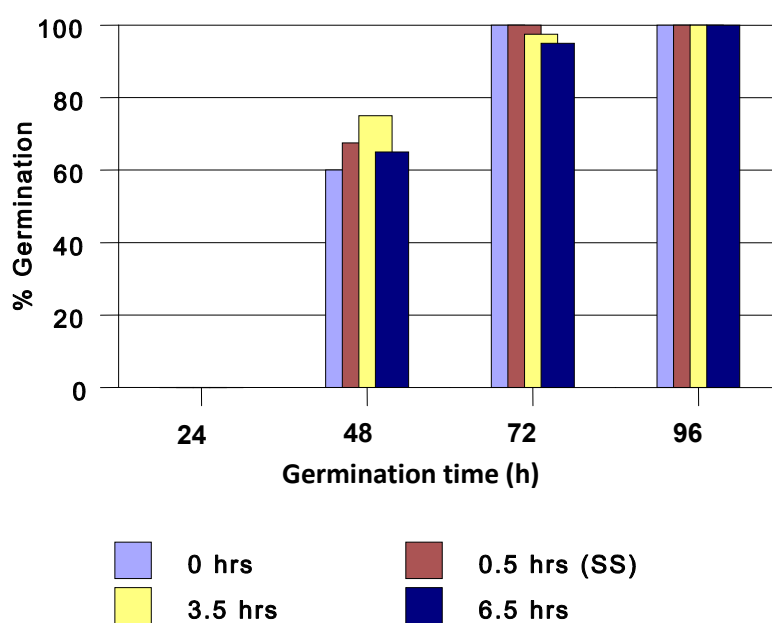
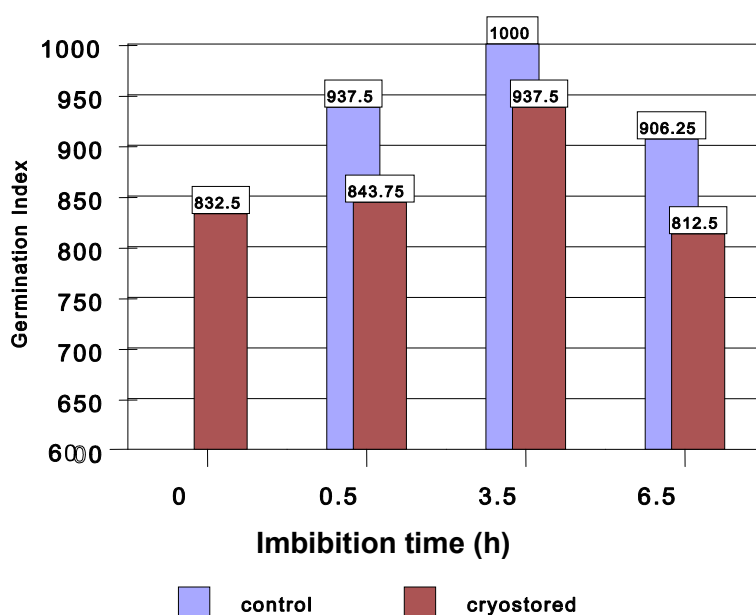
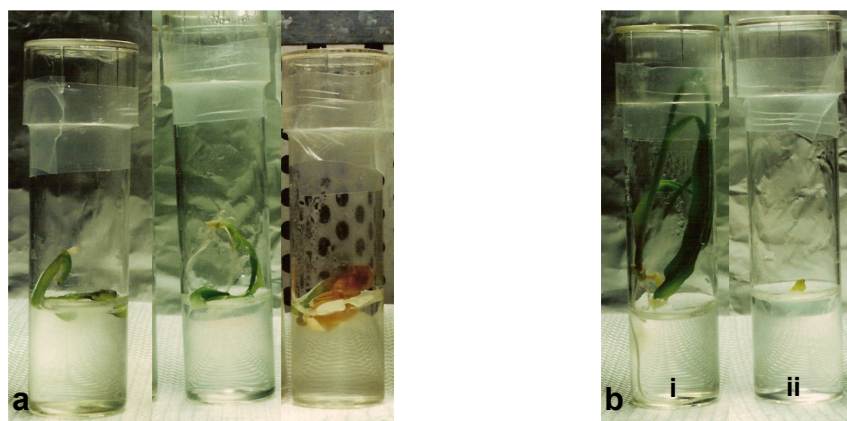


Figure 2.5. % Germination of axes isolated from intact caryopses, from caryopses hydrated for 0, 3 and 6 hrs respectively, subsequently surface sterilized for 30 min and cryostored.



**Figure 2.6.** Germination indices for isolated axes post-imbibition (control) and post-cryostorage. Results reveal that cryostorage does impact viability and vigour significantly for 0.5 and 6.5 hrs samples ( $\chi^2_1=3.84$ ,  $P=1.00$ ).



**Figure 2.7.** a) Abnormally developing plantlets from caryopses imbibed for 6.5 hrs and subsequently cryostored. Tissue exhibited inverted roots and shoot and some completely necrotic. b) A dry, isolated, cryostored axis (i) and a 6.5 hrs imbibed post-cryostorage axis (ii) where plated and in culture for one week.

**Table 2.2.** Table indicating the germination index (GI) values after Czabator (1962) for control and cryostored isolated axes. Values with the same superscript are not significantly different ( $P < 0.050$ ).

| Imbibition time (h) | GI                      |                     | % Root formed |                  |
|---------------------|-------------------------|---------------------|---------------|------------------|
|                     | Control                 | Cryostored          | Control       | Cryostored       |
| 0                   | 6642 (intact caryopses) | 832.5               | 100           | 100 <sup>e</sup> |
| 0.5                 | 937.5 <sup>ac</sup>     | 843.75 <sup>d</sup> | 100           | 100 <sup>e</sup> |
| 3.5                 | 1000 <sup>ac</sup>      | 9.37.5 <sup>c</sup> | 100           | 100 <sup>e</sup> |
| 6.5                 | 906.25 <sup>abc</sup>   | 812.5 <sup>d</sup>  | 100           | 37 <sup>f</sup>  |

The germination index value at the 3.5 hrs imbibition time was higher than any other values including caryopses not subjected to imbibition and cryostored. Stanwood (1980) has shown that orthodox seeds, including maize, are known to survive cryostorage (rapid freezing in liquid nitrogen at -196 °C) in the dry state. However, hydrated pea seeds (also orthodox) did not have a good survival rate post-cryostorage (Mycock *et al.* 1991). A low survival rate after cryostorage has also been observed in seeds of recalcitrant species and is presumably due to their high moisture contents (Boucard *et al.* 1991; Berjak *et al.* 1995; Berjak 2005). Imbibing the caryopsis prior to isolation of the axes effectively increased the water content in the axis and, therefore, increased the probability of cellular damage upon freezing due to ice crystal formation. The removal of the axis from the caryopsis imposed further stresses on the tissue and the cell membranes (Kermode 1995) and this was further exacerbated by the surface decontamination process. Collectively, these stresses could result in cryodamage and, although viability and vigour (even if somewhat reduced) were still observed in axes subjected to all the hydration levels tested, a lack of root development was significant in the developing plantlets at 6.5 hrs imbibitions, with less than half the plantlets producing normal roots (Fisher's exact:  $P < 0.001$ ).

Membrane phase transitions are associated with a transient loss in membrane integrity during imbibition and result in the leakage of solutes from the cells (Bewley and Black 1994). Biological membranes also undergo phase changes during the freezing and thawing processes associated with cryopreservation (Bryant *et al.* 2001). The successful cryostorage of root meristem tissue previously subjected to 3.5 hrs imbibition could have been due to a number of reasons. Firstly, membrane repair mechanisms could have been initiated and the tissue was therefore able to withstand the effects of cryostorage, despite increased hydration levels. Secondly, the water may have been in a different state at this time, when the solute concentration would be expected to be high and therefore imbuing it with cryoprotective properties.

Samples subjected to the longest imbibition time, however, may have been at a stage more susceptible to cryo-injury even though water contents were not significantly different from the 3.5 hrs sample (Table 2.1). For example, desiccation tolerance may have been lost at this hydration level. Root meristem tissue could have commenced metabolic activity and become more susceptible to the effects of cryostorage. This could explain the observed abnormalities/morphological changes noted in the recovered plantlets. These observations led to the decision to investigate root meristem ultrastructure (Chapter Three) and the biological processes initiated by imbibition and the subsequent effects of cryostorage (Chapter Four).

## Chapter Three      Ultrastructural Effects of Early Imbibition and Cryostorage on *Zea mays* L. Root Meristems

Aspects of this chapter were published in *Cryo-Letters* **20**, 183-192 (1999).

Poster Presentation at: M.S.S.A. (1996). "Cryopreservation of Isolated *Zea mays* Embryonic Axes."

SAAB (1997). "Cryopreservation of Isolated *Zea mays* Embryonic Axes."

ISTA (1998). "*In Vitro* Storage of *Zea mays*"

### 3.1. Introduction

Maize (*Zea mays* L.) is an extremely diverse species, exhibiting many morphological and biological differences (Neuffer *et al.* 1997). On the basis of variation in inbred maize lines, modern lines have distinctly diverged from their ancestral race through to the loss of rare alleles, either due to genetic drift or selection (Ho *et al.* 2005). Similar trends have been reported for wheat, where domestication and breeding from 1950-1989 has resulted in a reduction in genetic variation (Reif *et al.* 2005). Although a reduction in genetic variation due to genetic drift (Ho *et al.* 2005), selection (Halward *et al.* 1993; Reif *et al.* 2005; Mikel 2008), self pollination (Herselman 2003), natural disasters, deforestation, wetland destruction and the physical development of cities (UNEP 2002) is expected, breeders have been able to avert genetic narrowing. For example, the wheat germplasm base, through the introduction of novel genes increased genetic diversity (Reif *et al.* 2005). The self-pollinating nature of the cultivated peanut could easily have resulted in genetic isolation, but breeding programs have used a few elite breeding lines to develop new cultivars and enhance the narrow germplasm base (Herselman 2003). In maize specifically, the broadening of the genetic pool in hybrids has been achieved by accumulating genes within separate heterotic families that maximise hybrid yield. This strategy of conserving divergent heterotic families by inbreeding programmes within these families contributes to the maintenance of genetic diversity in maize (Mikel 2008).

Efforts to preserve germplasm and *ex situ* attempts to prevent genetic erosion should continue to fulfil the main goals of genetic resource management which are; to acquire, maintain, distribute, characterize, regenerate, preserve, evaluate,

and utilize the genetic diversity of crops and their wild relatives (Louette *et al.* 1997). *In situ* long term storage of maize by cryopreservation is presently being considered for the purposes of conservation rather than development (Brush 2005).

Cryopreservation of intact, orthodox maize caryopses (*Hybrid 6363*) was unsuccessful (see Chapter Two), presumable due to the size of the caryopsis. However, material could be successfully retrieved from liquid nitrogen and regenerated *in vitro* if the axes were isolated, thereby reducing the size of the sample. Isolation of the axes from the dry caryopses resulted in damage to the axes; and imbibition was employed as the method to soften the testas and the pericarps to successfully remove the axes.

When an orthodox seed is provided with water, the water is taken up by imbibitional forces which result in the expansion of both the embryo and the endosperm (if present). Water uptake initiates a sequence of biochemical and cellular developmental events which ultimately leads to germination. Hydration transforms a dehydrated, resting embryo by initiating all the processes necessary for growth and development. During this transition, desiccation tolerance and the ability to be cryostored, which characterizes the resting embryo, is lost (Vertucci and Farrant 1995). The precise time that desiccation tolerance is acquired during maturation and/or point active metabolism is arrested, depends on the species, the rate of water loss and the final water content after drying (Hong and Ellis 1992; Ellis and Hong 1994; Wechsberg *et al.* 1994; Hay and Probert 1995). This critical water content is, however, difficult to determine (Sargent *et al.* 1981; Vertucci and Farrant 1995). Studies on maize (Cordova-Tellez and Burris 2002a) revealed that the acquisition of desiccation tolerance is dependent on the pre-conditioning protocols employed by seed producers. This includes the effects of drying regimes and the seed maturity levels which directly affect initial moisture contents of the seed at harvest (Cordova-Tellez and Burris 2002a). Other authors (Pammenter and Berjak 1999; Cordova-Tellez and Burris 2002) view the concept of critical water content, on a species basis, as inappropriate and should be viewed in terms of a



continuum of behaviour rather than attempting precise categorisation.

Drying regimes employed on the hybrid utilised in this study were not disclosed, as well as the seed maturity levels at harvest. Isolated immature maize (*Zea mays* L.) embryos have been shown to acquire tolerance to rapid drying between 22 and 25 days after pollination (DAP) and to slow drying from 18 DAP onward. The slow drying of excised, immature embryos leads to an increased proportion of  $\alpha$ -helical protein structures in their axes, which coincides with additional tolerance of desiccation stress (Wolkers *et al.* 1998). The pre-conditioning treatments that *Hybrid 6363* caryopses were subjected to, had they been disclosed, would have been useful in our understanding of these caryopses responses to rehydration and the ability to withstand the effects of subsequent cryostorage. These effects appear to be correlated to the point where desiccation sensitivity is reached and or desiccation tolerance is lost.

The present study was an investigation into the effects of early imbibition and subsequent cryostorage on the ultrastructure of root meristems of isolated maize axes. A range of imbibition times were tested so that the ultrastructure of roots successfully recovered from cryostorage could be described.

### **3.2. Materials and methods**

#### *3.2.1. Imbibition and cryostorage*

Whole maize caryopses obtained from PANNAR, Olifantsfontein, South Africa (*Hybrid 6363*), were hermetically sealed in plastic buckets and stored at 4°C. When required, the caryopses were removed from the bucket and imbibed for a range of periods (0-6 hrs). In all treatments, the embryonic axes of 40 caryopses were isolated directly after imbibition. Once isolated, the axes were surface-decontaminated in 2% v/v sodium hypochlorite for 15 minutes and washed for a further 15 minutes (three washes) in sterile water, which increased each imbibition time by 30 minutes.

After surface-decontamination, the isolated axes were placed in cryovials and plunged directly into liquid nitrogen resulting in a freezing rate greater than  $200^{\circ}\text{C. min}^{-1}$ . The samples were then left submerged in the liquid nitrogen for 15 minutes and subsequently thawed in a water bath set at  $30^{\circ}\text{C}$ .

### 3.2.2. *Water content determinations*

The water contents of all 40 replicate embryos in each treatment were determined and expressed as a percentage of the fresh weight. This was achieved by weighing the axes directly after the surface-decontamination step and again after drying them for approximately 18 hours in a ventilated oven at  $80^{\circ}\text{C}$  according to the Modified ISTA Low Constant Oven Temperature Method (International Seed Testing Association 1985).

### 3.2.3. *Viability assessments*

Viability and vigour were determined on axes from each imbibition time pre- and post-cryostorage. The tissue was placed aseptically onto plates containing Murishige and Skoog (1962) nutrient medium (pH 5.8),  $30\text{ g.l}^{-1}$  sucrose and  $2\text{ g.l}^{-1}$  gelrite. The material was incubated at  $25^{\circ}\text{C}$  in a 16:8 hrs light:dark cycle, with a light intensity of  $200\mu\text{E.m}^{-2}.\text{sec}^{-1}$ . The number of developing axes (considered viable when the radicle extended or shoot emerged) was recorded daily for five days and vigour was calculated using the Czabator (1962) index calculated as follows:

$$\text{GI} = \text{MDV} \times \text{PV}$$

GI = germination index

MDG = mean daily germination (final percent germination divided by the length of the test period).

PV = peak value (percentage germination on a particular day divided by the number of days taken to achieve that percentage. The highest value achieved over the 4-7 day experimental period is used as an indication of seed vigour.

#### 3.2.4. Ultrastructural studies using Transmission Electron Microscopy (TEM)

The ultrastructure of the root meristem of 10 replicate axes in each treatment before and immediately after cryostorage was investigated. The terminal 3mm of the radicle was excised and prepared for Transmission Electron Microscopy using the following method:

##### ***Sample Embedding Schedule***

(Berjak *et al.* 1986)

1. The root tips were excised and placed into 3% v/v gluteraldehyde diluted in a 0.1N sodium cacodylate buffer overnight.
2. The samples were then washed with 0.1 N sodium cacodylate buffer (pH 7.2) six times over one hour.
3. The material was then post fixed in 4% v/v osmium tetroxide diluted in a 0.1N sodium cacodylate buffer for one hour.
4. After washing with buffer, the samples were dehydrated through an ethanol series, 10 to 15 minutes each, of 10%, 25%, and 50% v/v ethanol.
5. Samples were then placed for one hour in a saturated uranyl acetate solution (diluted in 75% v/v ethanol).
6. After two changes of 75% ethanol, the samples were transferred to absolute ethanol (2 x 10 min) and then to propylene oxide (2 x 10 min).
7. The samples were then submerged in a 1:1 mixture of Spurr's (1962) epoxy resin and propylene oxide for five hours. The 1:1 solution was replaced with pure resin and the material was allowed to infiltrate overnight.

8. The samples were then transferred to moulds with fresh resin and polymerised at 70°C for eight hours.

Ultra-thin sections were cut using a Reichert UltracutE microtome and glass knives, picked up on grids and allowed to dry overnight. The grids were then stained with uranyl acetate for seven minutes (in the dark) and left to dry. They were then further stained with lead citrate for 15 minutes and allowed to dry. The sections were then viewed and photographed using a JEOL - 100 S transmission electron microscope.

### 3.3. Results

As imbibition times increased, axis water content rose (Table 3.1). The isolation of these axes and the subsequent surface-decontamination process was associated with a particularly rapid water uptake. For instance, the water content of intact caryopses after one hour imbibition was  $12.7\% \pm 2.37$ , whereas, the water content of the isolated axes, from the 1 hrs imbibition and 30 min surface decontamination, was  $54.2\% \pm 4.7$ . This rapid water uptake was presumably a result of the axes being isolated and placed in direct contact with the water. Nevertheless, the isolation of the axes from the intact caryopses had no effect on their viability (Table 3.1 & Fig.3.1).

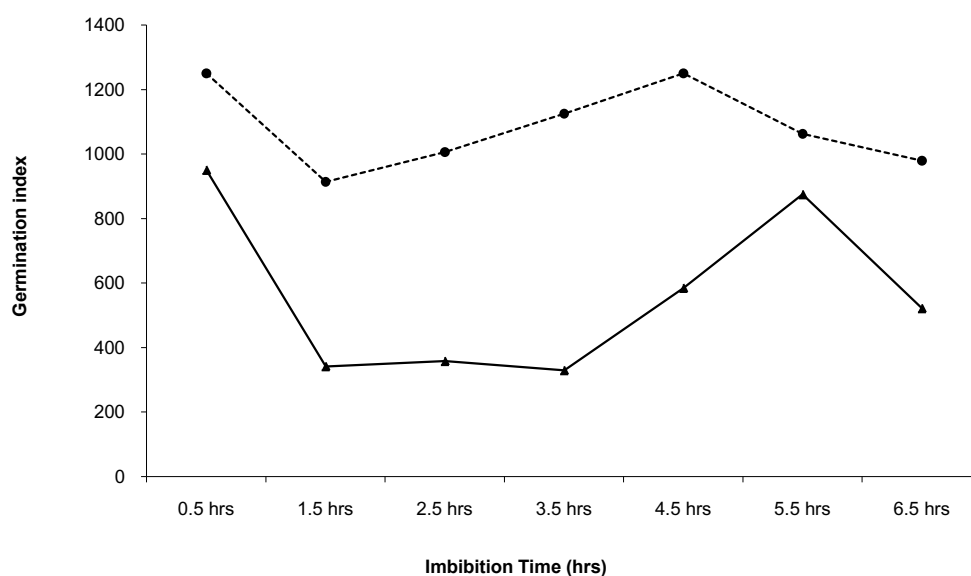
Axes were successfully recovered from the frozen state despite having high water contents. The Czabator germination index (1962) takes into account both the rate and the total number of axes germinated; for the cryostored material this index was consistently lower than the non-frozen control (Fig. 3.1). Since cryostorage had little or no effect on germination totality (Table 3.1) the lower germination index shown by the cryostored material could be directly attributed to a slowing in the rate of growth of the axes. This was presumably due to the necessity for repair processes to be completed before germination *per se* could be initiated (see later).

The pre-cryostored control (unimbibed) axes (Fig. 3.2) exhibited an ultrastructural profile similar to that reported in other dry orthodox seed tissues (Berjak and Villiers 1972b; Smith 1991) and vegetative tissue in the desiccated state (Farrant *et al.* 1999) i.e. withdrawn plasmamembranes from cell walls and condensed cytoplasms. However, the impact of the aqueous phase of the surface decontaminant could result in the expansion of plasmamembrane to the cell wall, viz. the withdrawal of the plasma membrane from the cell wall, cannot be discounted as an artifact on the ultrastructural appearance of the dry tissue as reported by Wesley-Smith (2001). Smith (1991), however, did show that the anhydrous fixation of dry lettuce seed tissues were similar in ultrastructural appearance to these maize hybrids.

The plasma membranes of the cells were withdrawn from the cell wall but remained attached at the plasmodesmata (Fig. 3.3). The cell walls were slightly buckled, a feature exacerbated by liquid nitrogen storage (Fig. 3.4). Although not closely associated with the cell wall, the plasmalemma was intact and enclosed a densely packed cytoplasm (Fig. 3.2). A layer of lipid droplets was situated immediately below the plasmalemma (Fig. 3.2) and these were not affected by cryostorage (Fig. 3.4). Although mitochondria were discernable in the compact cytoplasm, these organelles were generally electron translucent, indicating a lack of metabolic activity (Fig. 3.2) and did not appear to be any different post-cryostorage at this water content.

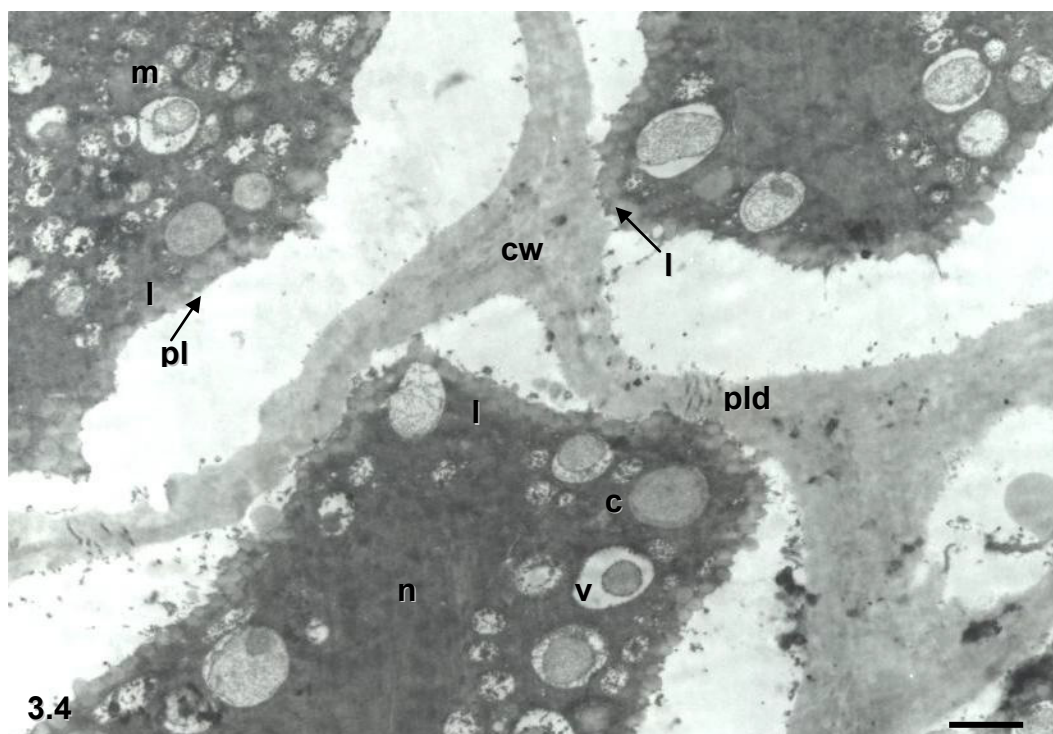
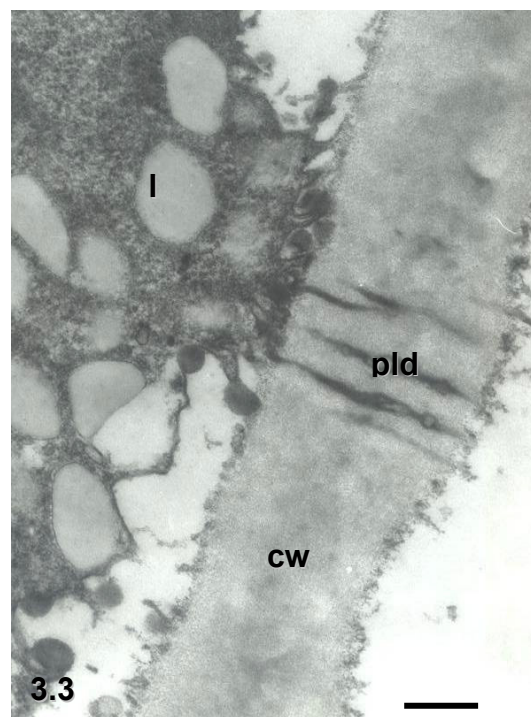
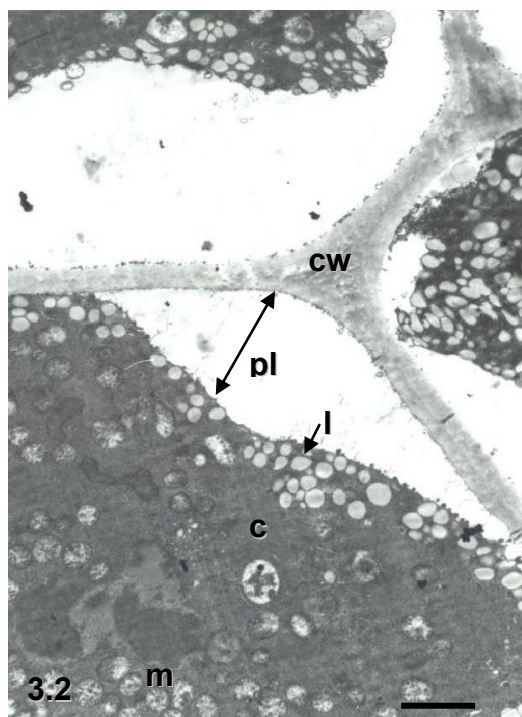
**Table 3.1.** Viability of the isolated axes after various imbibition times (inclusive of surface decontamination treatment) before and after cryostorage (n=40). The water content of the axes immediately before cryostorage is also presented (standard deviation in parentheses). The number of abnormal seedlings was also recorded after cryostorage. NT- not tested.

|                                 | Imbibition time (h) |              |              |              |              |              |               |
|---------------------------------|---------------------|--------------|--------------|--------------|--------------|--------------|---------------|
|                                 | 0.5                 | 1.5          | 2.5          | 3.5          | 4.5          | 5.5          | 6.5           |
| Water Content                   | 51.6<br>±12.1       | 54.2<br>±4.7 | 57.7<br>±8.1 | 57.9<br>±3.5 | 60.9<br>±6.2 | 59.3<br>±5.0 | 61.4<br>±10.2 |
| Viability (%)                   | 100                 | 100          | 100          | 100          | 100          | 100          | 100           |
| Viability (%) after cryostorage | 97.5<br>±2.5        | 92.5<br>{NT} | 82.5<br>{NT} | 92.5<br>±2.5 | 97.5<br>{NT} | 100<br>{NT}  | 97.2<br>±27.5 |



**Figure 3.1.** Germination indices of maize embryonic axes isolated from intact caryopses that had been imbibed for various periods. After isolation, the axes were surface-decontaminated then immediately tested for viability (•---•) or cryostored in liquid nitrogen and viability assessed after retrieval from the frozen state (▲—▲).

- Figure 3.2      Root meristematic tissue in dry (pre-cryostored) axis. Cells have a plasma membrane (pl) withdrawn from the cell wall (cw). Lipid molecules (l) directly underlie the plasma membrane. The densely packed cytoplasm (c) (as a result of desiccation) and mitochondria (m) are visible.
- Figure 3.3      Root meristematic tissue in dry (pre-cryostored) axis - the plasma membrane remains attached to the cell wall (cw) at the plasmodesmata (pld).
- Figure. 3.4      Post-cryostored unimbibed axes. Cells demonstrate a slight buckling in the cell wall (cw). However, in all other respects cryostorage had no effect relative to the pre-cryostored tissue, that is, lipids (l) remained peripheral, the plasma membrane (pl) withdrawn, except when attached at the plasmodesmata (pld).





After the 30 minute surface-decontamination period, the plasmalemma was intact and the cytoplasm had expanded and pushed against the cell wall; lipid droplets lined the inner surface of this membrane (Figs. 3.5 & 3.6). Although the nuclear membranes were more clearly definable than in the dehydrated state, the organelles themselves were lobed and contained clumped chromatin (Fig. 3.6). Numerous small vesicles containing electron-dense material were scattered throughout the cytoplasm. Cryostorage of this higher water content material had little or no effect on viability of the embryos (Table 3.1) but did reduce the vigour of the axes (Fig. 3.1). After retrieval from the frozen state, the plasmalemma was generally withdrawn from the cell wall (Fig. 3.7), but the characteristic cortical layer of lipid droplets did not appear to be effected (Fig. 3.8) and the nuclei remained similar to the pre-cryostored tissue (Fig. 3.6).

At the 1.5 hrs imbibition time, prior to cryostorage (Fig. 3.9), the dense cytoplasm filled the cell volume and mitochondria were clearly visible, but their internal membrane development was minimal. The few small membrane bound vesicles were interspersed with sheets of endoplasmic reticulum (Fig. 3.10). After cryostorage there was a mosaic of different effects on cells, ranging from those which were apparently unaffected (Fig. 3.11), those exhibiting lipid coalescence and those exhibiting various levels of damage (Fig. 3.12). In general, the cell walls were buckled, and the characteristic peripheral lipid found in the unfrozen material had congregated into groups of droplets after retrieval from liquid nitrogen (Fig. 3.11), and coalescence was also observed (Fig. 3.12). In some cells the plasmalemma was ruptured and these cells were presumed to be dead. This damage could be attributed to the high water content at which the material was frozen (Table 3.1). Nevertheless, the vast majority of the frozen embryos were still able to germinate into plantlets although the rate at which they developed was retarded compared with that of control embryos (Fig. 3.1). This implies that the repair mechanisms necessary for the recovery of the damaged cells had not been totally compromised by the freezing and thawing processes.

Figure. 3.5

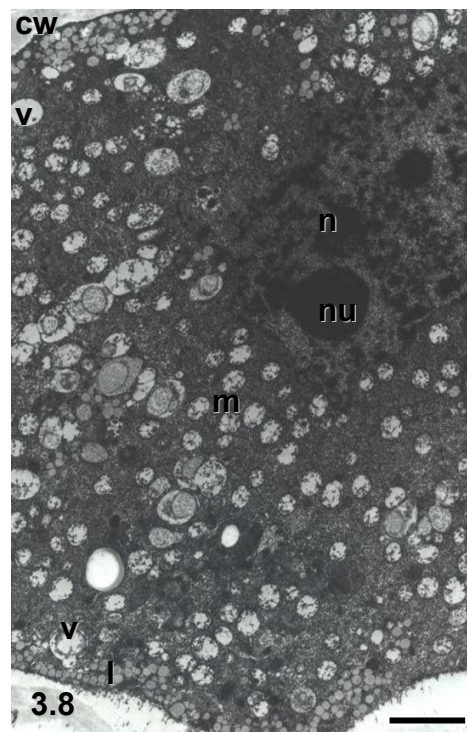
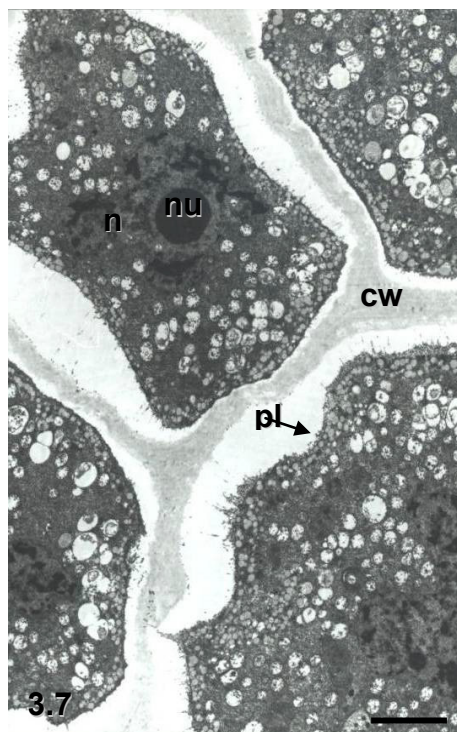
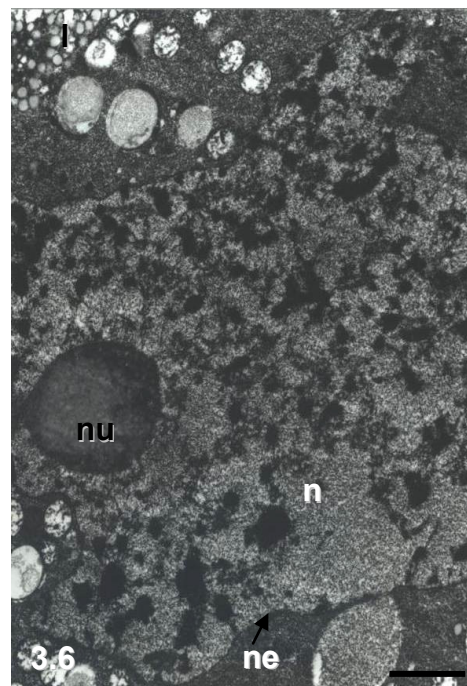
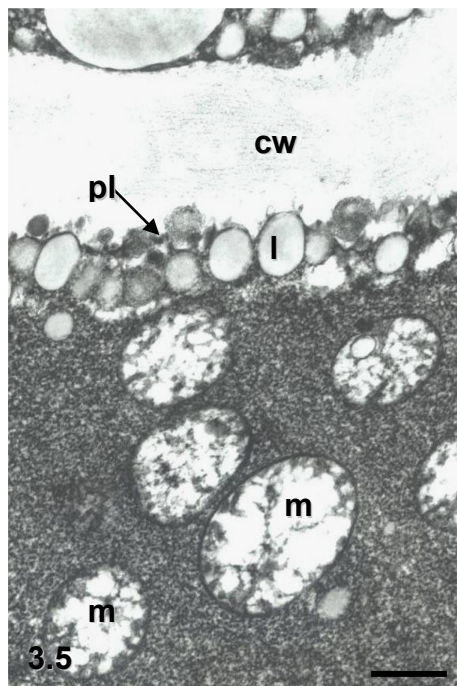
Root tissue of excised axes exposed to a 30 minute surface decontamination procedure. Cells exhibited an increased hydrated cytoplasm (c) which expanded to the full volume of the cell plasma membrane (pl) underlying the cell wall (cw), and the lipid droplets (l) remained peripheral. Numerous small mitochondria (m) were scattered throughout the cytoplasm containing electron dense material.

Figure. 3.6

Root tissue of the excised embryo allowed to imbibe for 30 minutes. The nuclear envelope (ne) is more clearly definable than that of the dehydrated state, was lobed, and contained clumped chromatin (n) with a clearly defined nucleolus (nu).

Figure. 3.7 &amp; Figure. 3.8

Excised embryonic tissue hydrated for 30 minutes and then cryostored, exhibited the same withdrawn plasma membrane (pl) as seen in the dry tissue. Numerous vesicles (v) were visible and the cortical layer of lipid droplets (l) did not appear to be affected (Fig 3.8).



After 4.5 hours, the axes were at a water content of approximately 60% (Table 3.1). The dense cytoplasm of unfrozen material contained numerous small vesicles, the nuclei were lobed (Fig. 3.13) and the mitochondria did not appear to have developed cristae (Figs. 3.13 & 3.14). The level of vesiculation was enhanced after cryostorage and these structures contained electron dense material, possibly precipitated biomolecules (Fig. 3.15) due to the freezing and thawing processes. As was observed at the earlier imbibition times, freezing results in the peripheral lipid coalescing into larger droplets (Fig. 3.16). Despite the observed changes, these axes remained viable and it is suggested that damage was confined to the membrane bound vesicles.

Cells of tissue allowed to imbibe for 6.5 hours, but unfrozen, were characterised by dense cytoplasm, peripheral lipid droplets and lobed heterochromatic nuclei (Fig. 3.17). The mitochondria had evidence of some cristae membranes, indicating that respiration may have resumed (Fig. 3.18). Freezing after this imbibition time resulted in extensive cryodamage, and again there was a range in response of the cells from those that remained intact (Fig. 3.19) but which exhibited apparent mitochondria enclosed in vacuoles/vesicles or whose mitochondria had membrane swelling, to those that exhibited ruptured plasma membranes (Fig. 3.20) and broken cell walls (Fig. 3.21). In less damaged cells, the endoplasmic reticulum was dilated. This was associated with an increase in the number of electron-translucent, membrane-bound vesicles (Fig. 3.22), the latter possibly the extreme forms of the former.

### **3.4. Discussion**

Two types of ultrastructural changes were observed in this investigation, those associated with the imbibition process and those due to the freezing and thawing procedures.

- Figure 3.9 & Figure. 3.10      Root meristematic tissue of pre- cryostored axes imbibed for a total of 1.5 hours. Cells had a more hydrated cytoplasm (c) which filled the entire cell volume. Mitochondria (m) were clearly visible with very little internal development (Fig. 3.9). Small membrane bound vesicles (v) interspersed with sheets of endoplasmic reticulum (er). Lipids (l) remained peripheral at this hydration level (Fig 3.10).
- Figure 3.11 & Figure 3.12      The effect of cryostorage on tissue hydrated for 1.5 hours. The characteristic peripheral lipid layer seen in the unfrozen material had coalesced into larger droplets (l) after retrieval from the cryogen (Fig 3.11). Scale Bar = 0.27  $\mu\text{m}$ . Buckled cells walls (cw) together with lipid coalescence (l) were observed due to cryostorage (Fig 3.12).



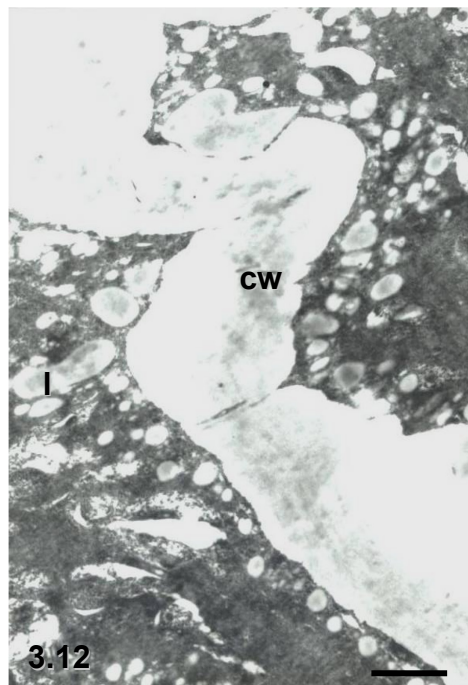
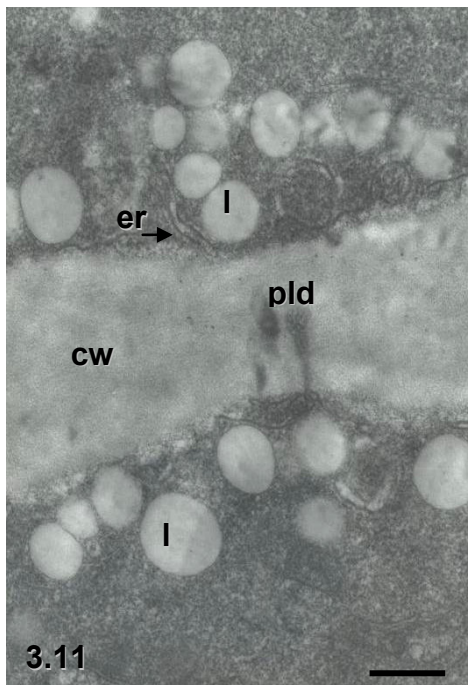
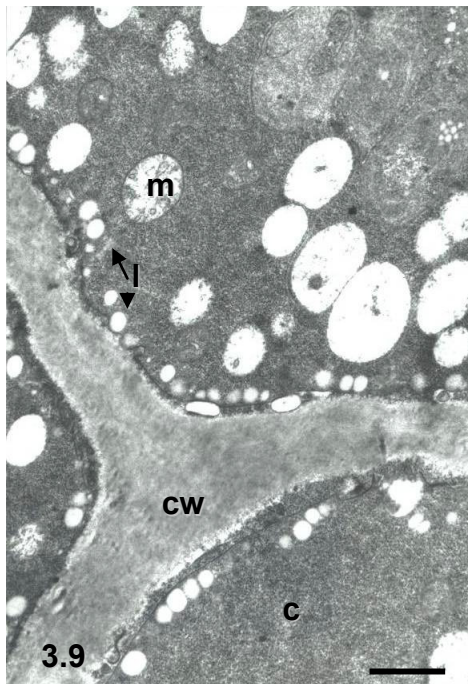


Figure. 3.13 & Figure. 3.14 Embryonic tissue imbibed for 4.5 hours ( $\pm$  60 % w/c) pre-cryostorage. Cells contained numerous small vesicles (v), lobed nuclei (n) and mitochondria (m). Lipid droplets (l) remained characteristically peripheral. The mitochondria (m) (Fig. 3.14) did not appear to have developed cristae at this hydration level. The vacuole (v) in this micrograph contains electron dense material.

Figure. 3.15 & Figure 3.16 Embryonic tissue imbibed for 4.5 hours and cryostored. The level of vesiculation (v) in cells appeared to be enhanced. Lipid aggregation/coalescence formed larger lipid droplets (l) (Fig 3.16).

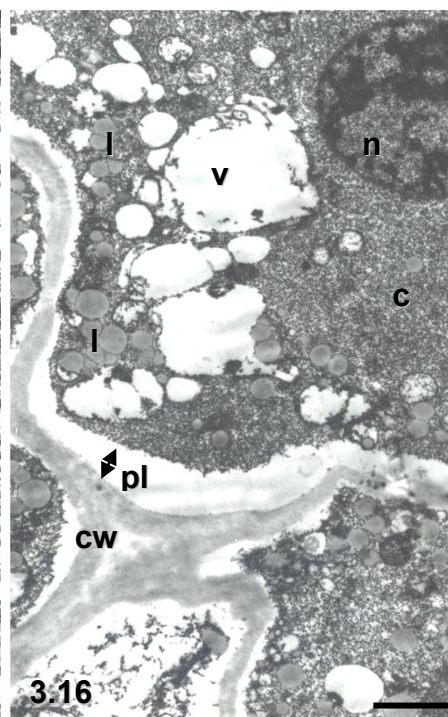
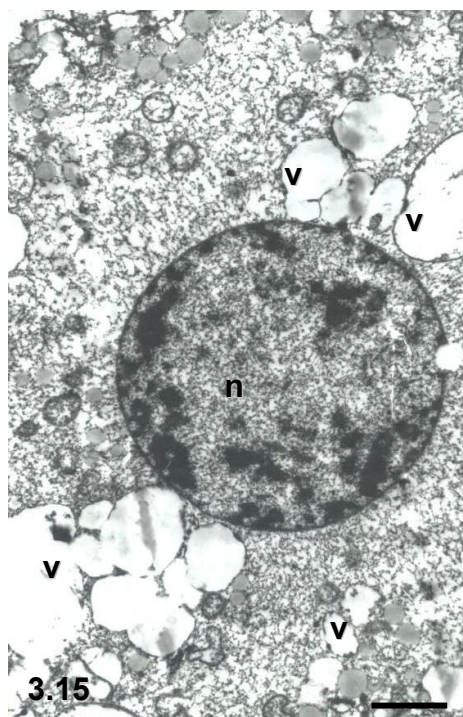
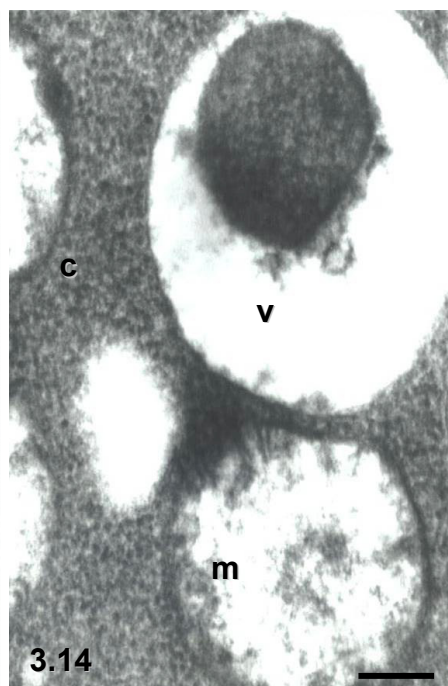
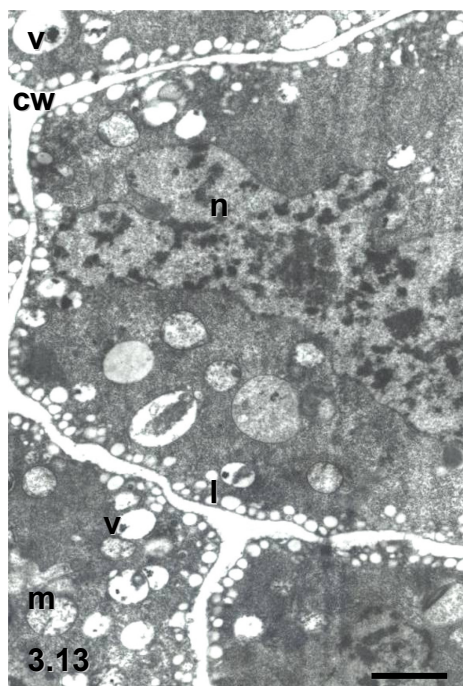




Figure 3.17 & Figure 3.18    Embryonic tissue imbibed for 6.5 hours pre-cryostorage. Cells do not appear very different to other hydrated tissues. The cytoplasm(c) contained heterochromatic and lobed nuclei (n) and the lipid droplets (l) remained peripheral. (Fig. 3.17). Mitochondria (m) appeared to show some cristal membrane development indicating that respiration may have resumed at this imbibition time (Fig. 3.18).

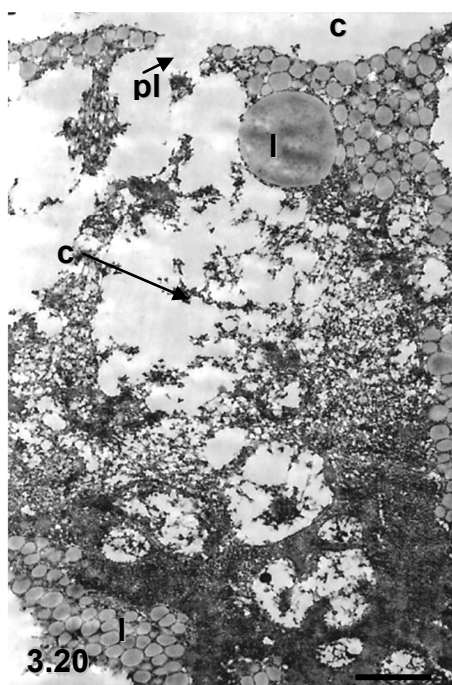
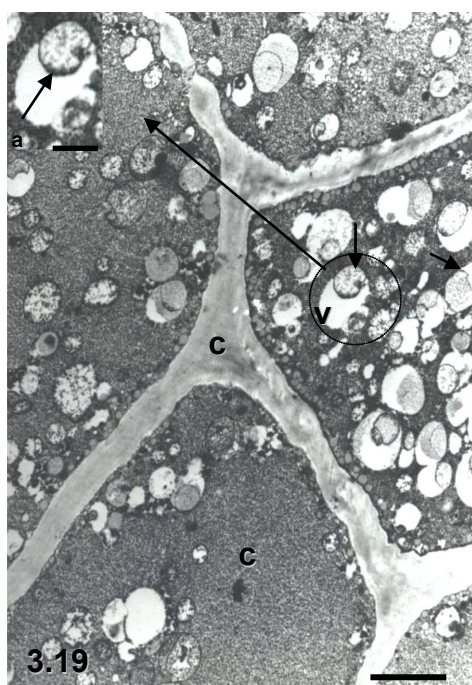
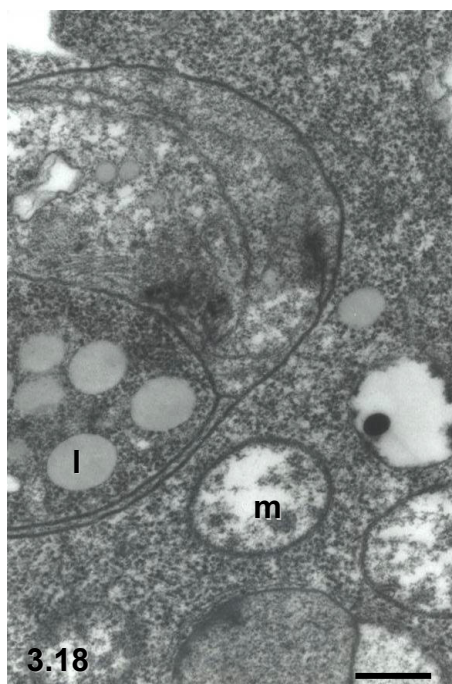
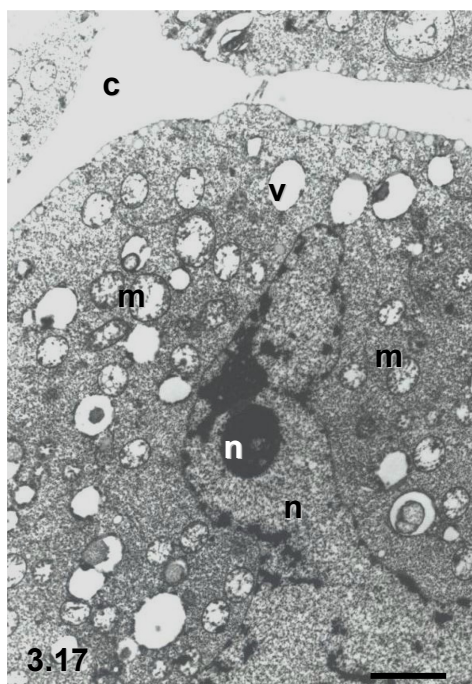
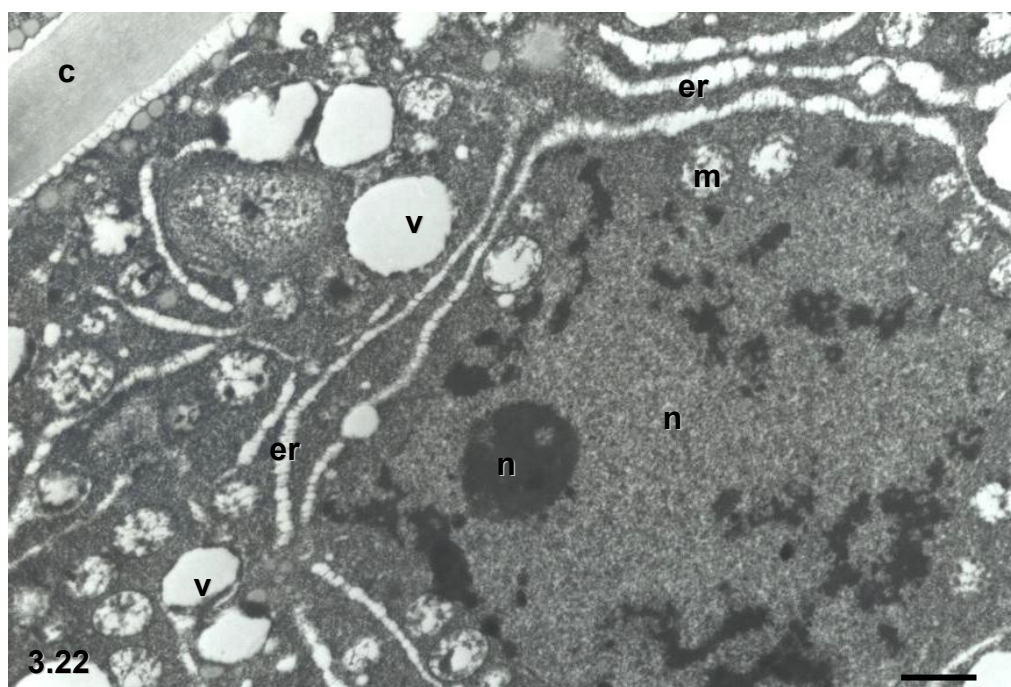
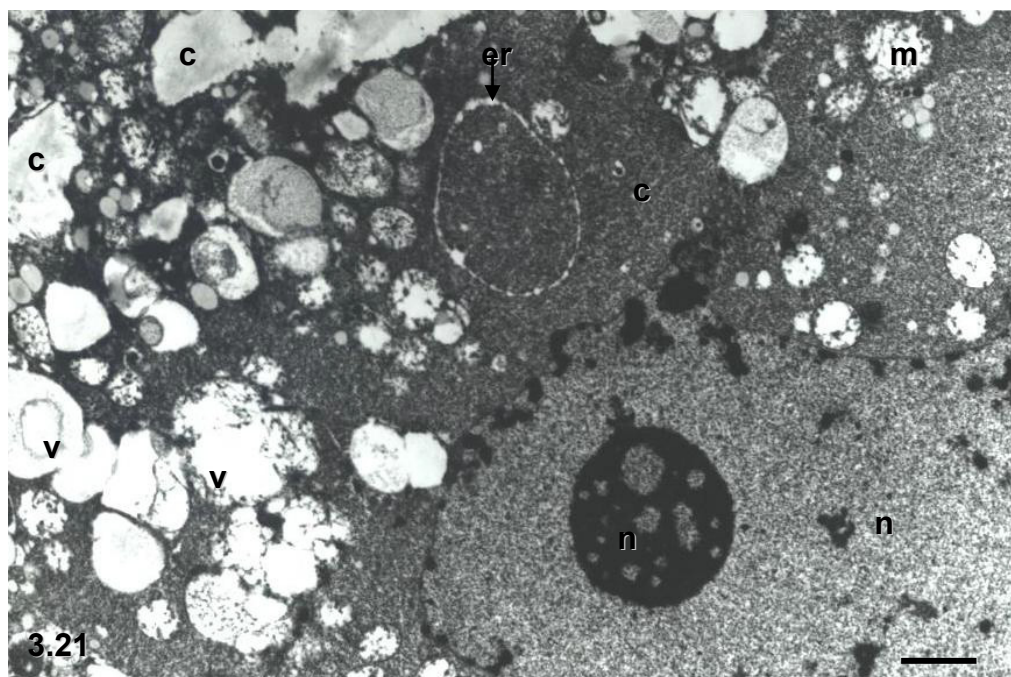


Figure. 3.19 - Figure. 3.22      Root meristematic tissue of embryonic axes subjected to 6.5 hours of imbibition followed by cryostorage. This tissue included cells relatively intact with the inclusion of mitochondria within vesicles ( $\rightarrow$ ) (Fig 3.19). Scale bar = 1.5  $\mu$ m (Fig. 3.19a demonstrates the possibility of a double membraned mitochondrion encapsulated within a vacuole) to ruptured plasma membranes (pls), damaged cytoplasms (Fig. 3.20) and broken cell walls (cw) — (cw) where the cytoplasm, i.e. compartments of two adjacent cells have fused (Fig. 3.21). Less damaged cells exhibited dilated endoplasmic reticulum (er) associated with an increase in the number of electron translucent membrane bound vesicles (v) (Fig. 3.22).





During maturation drying in normal seed development there is a general degeneration of subcellular organelles and there appears to be a correlation between the loss of internal membrane structure (e.g. in mitochondria) and the attainment of desiccation tolerance (Vertucci and Farrant 1995). Lipid bodies are deposited on the inner surface of the plasmalemma and these are proposed to act as a reserve for membrane expansion during imbibition (Vertucci and Farrant 1995). It is also proposed that the phospholipid component of the cell membranes undergo a phase transition from liquid crystalline to a gel-like state (Bewley and Black 1994). When the seeds imbibe, the membrane lipids change back to the liquid crystalline phase (Crowe *et al.* 1992). These phase transitions are associated with transient loss in membrane integrity and result in the leakage of solutes from the cells (Bewley and Black 1994). Biological membranes are also known to undergo phase changes during the freezing and thawing processes associated with cryopreservation. The control unimbibed axes exhibited an ultrastructural profile commensurate with these observations, in that lipid droplets were in juxtaposition to the plasmalemma which was withdrawn from the cell wall, and, the subcellular organelles (when visible in the dense cytoplasm) appeared dedifferentiated (Fig. 3.2). See later for further discussion.

The uptake of water by the tissues resulted in the expansion of the cytoplasm to fill the entire cell volume and this was presumably due to hydration of the cellular components (e.g. proteins) and the filling of subcellular spaces (Bewley and Black, 1994). As a result of this expansion, subcellular detail that was obscured by the compaction of the cytoplasm in the dry state became increasingly clear. The numerous small vesicles observed in juxtaposition to the plasmalemma during imbibition (Fig. 3.9) could be a result of the inability of the plasmalemma to reconstitute properly causing the lipid bilayer to vesiculate (Smith and Berjak 1995). This effect may have been more profound due to the isolation of the axes after the various imbibition times and the surface-decontamination step, which was associated with the uptake of large amounts of water. Alternatively, the close association of the vesicles with the plasmalemma and the lipid droplets could represent transitory membrane derived from the lipid droplets and destined for

incorporation into the expanding cell membrane. This suggestion obviously requires methodical investigation into both the composition of the lipid droplets and the mechanism of lipid insertion into the membrane bilayer. The normal pattern of membrane synthesis occurs in the endoplasmic reticulum which then vesiculates and reaches the cell membrane via endomembrane flow. However, a fully functional cytoskeletal system must be present for this to occur at this hydration level and is investigated further in Chapter Six.

For all the imbibition times tested, the nucleus was one of the most prominent organelles. In newly harvested maize, the nuclear profile of cells from embryos is spherical and the matrix homogeneous (Berjak *et al.* 1986). In the present investigation the nuclear profiles were always highly lobed and the chromatin was clumped. Berjak *et al.* (1986) have associated nuclear lobing and chromatin clumping with protracted storage and have attributed the changes to natural ageing. Since the caryopses used in these experiments have only been in cold storage (4°C) for 18 months post harvest, it is possible that the observed nuclear morphology was an early indication of ageing. Alternatively, these symptoms may have resulted from the rapid imbibition associated with axis isolation and the subsequent surface-decontamination step. For example, the double membrane of the nucleus is supported by an internal meshwork of lamins (intermediate filaments) (Minquez *et al.* 1993) and it is possible that the lobed state was a result of alterations in the structure of this supporting-interlaced system. Intermediate filament polymerisation is controlled by phosphorylation, which in turn is regulated by intracellular calcium levels (Wolfe 1993). Changes in membrane properties during imbibition could have resulted in fluctuations of intracellular ion concentrations and these could have triggered the cascades associated with intermediate filament dynamics (Minquez *et al.* 1993; Wolfe 1993).

Lipid congregation and coalescence just inside the plasmalemma in cells of cryostored tissue was observed for all imbibition times. Lipid bodies found in seeds are reported to be surrounded by a monolayer of phospholipids embedded in hydrophobic oleosin proteins and this complex prevents the coalescence of the

droplets (Huang 1992; Murphy 1990). Cytoplasmic acidification or an increased influx of calcium into the cell as a result of altered plasmalemmal properties is suggested to allow for lipid droplet fusion (Smith and Berjak 1995). The freezing process could also facilitate changes in intracellular solute concentrations and result in lipid droplet coalescence. Alternatively, the rapid shrinkage of the cytoplasm during the freezing process could have brought these hydrophobic structures into close physical contact and in this way facilitated their fusion. The fusion of the lipid into larger structures would reduce their surface area, and, should they be acting as a source of membrane lipid, then this may slow the rate of incorporation of lipid from the droplets into the cell membranes during recovery growth. This could be a contributing factor in the slowing of the rate of growth post-cryostorage which was observed in this investigation (Fig. 3.1) and which is so often reported for species after cryopreservation (Bajaj 1995).

Membrane vesiculation during cryostorage has been attributed to both the dilation of the endoplasmic reticulum (Takashi *et al.* 1997) and to plasmalemma budding (Steponkus 1985; Wesley-Smith *et al.* 1995). Wang *et al.* (1998) have suggested that the larger vesicles may be derived from endoplasmic reticulum and the smaller vesicles from the plasmalemma. These ultrastructurally-detectable changes presumably reflect alterations in membrane composition due to lipid phase changes during freezing and thawing (Webb and Steponkus 1993). The current findings support these observations in that varying degrees of endoplasmic reticulum dilation and numerous membrane-bound vesicles were observed, scattered in the cytoplasm and close to the cell membrane. Although the tissues used by Takashi *et al.* (1997) and Wesley-Smith *et al.* (1995) were hydrated, metabolically-active systems, the tissues were still in the process of imbibition and may well not have been fully metabolically-active, but were viable. The observed vesiculation was a physical response to cryostorage, but was observed more frequently after 6.5 hrs imbibitions (Fig. 3.22), suggesting that metabolic activity (in the endoplasmic reticulum) could have been adversely affected by the cryoprotocol and thus further causing vesiculation (Fig. 3.19). Mitochondria were observed post-cryostorage within what appeared to be a vesicle, but it is not

clear if autophagy (Liu *et al.* 2005) had occurred or if the mitochondrial inter-membrane space had swelled/expanded (Balk *et al.* 2003) in response to cryostorage. Either of these possibilities would indicate very definite cellular responses to cryostorage at this hydration level (6.5 hrs imbibition). If the mitochondrial inter-membrane space was expanding, it is assumed that DNase activity would be increasing in preparation for a programmed cell death (Balk *et al.* 2003). Alternatively, if the mitochondrion was encapsulated within a vesicle, which is one of the mechanisms employed by a plant's resistance to damage and seen as a hypersensitive response (Liu *et al.* 2005), this could demonstrate the cell's attempt to localise damage and regulate programmed cell death. Micrographs at higher magnification (Fig. 3.19a) demonstrate it is the latter rather than the former suggestion.

Post-cryostorage, at all imbibition times a range of damage was observed but generally, with increasing time, there was an increase in the severity of injury. The damage, however, was not extensive enough to compromise viability but did have an effect on vigour. The slowing in the rate of growth can be attributed to the requirement for a refractive period during which repair processes are initiated prior to the onset of germination. It also appears that there was a threshold in the extent of damage, above which the quality of regenerative growth was affected. Material subjected to imbibition times of more than 3.5 hours (in particular the 6.5 hrs sample) demonstrated increasing amounts of cellular damage which was associated with a higher percentage of abnormal development. Such damage and resulting anomalous growth was not evident in material subjected to the shorter imbibition times. At the higher imbibition times there was evidence for some metabolic activity e.g. there was obvious cristae formation within the mitochondria, and associated with this may have been the loss of desiccation tolerance. Cryostorage at these times would therefore have an adverse influence on the functioning of these newly-initiated biochemical activities and thus would intensify the cryo-damage. Cryo-damage of the root/radical meristem is therefore the cause of poor root formation (Table 2.2).



The successful cryostorage of isolated maize axes appears to be associated with desiccation tolerant material, the ultrastructure of which is indicative of low metabolic activity. Thus, if the tissues are not metabolically-active they can be cryostored. There is also the possibility that solutes, such as amino acids with known cryo-protective abilities e.g. proline (Kantha 1985; Benson 2008), that leach out of the caryopses during imbibition, may perform a cryoprotective role. The ability of the frozen material to repair the cryodamage after thawing is also an important factor. If the repair mechanisms are compromised by the cryoprocure, vigour and ultimately viability would be lost. Maintenance of the subcellular matrix in a viable state is thus an important aspect in the development of any cryostorage protocol. Investigations into some of the effects of cryostorage on selected biochemical activities, as well as the cytoskeleton of cells of maize embryos, are discussed in the following chapters. In particular, the possible role of cryoprotectants being present in the leachate, and their contribution to observed successes after cryostorage, are considered in the next chapter.

## Chapter Four      Biochemical Changes Associated with the Onset of Imbibition and Subsequent Cryostorage

Poster presentation ISSS: Merida (1999): Biochemical and ultrastructural Studies on Cryostored Maize Embryonic Axes.

### 4.1. Introduction

The dehydration process during seed maturation (see Chapter 1.2.2) results in physiological and biochemical changes, e.g., proline accumulation. Proline, an amino acid naturally occurring in plants and known to behave as a cryoprotectant for maize tissue (Withers and King 1979) seems to have diverse roles under osmotic stress conditions. In plants, proline is synthesised and accumulates in response to various stresses and stress adaptation (Lalk and Dorffling 1985), for example, high temperatures (Change and Lee 1999), osmotic, ionic stresses (Krackhardt and Guerrier 1995) light transitions, cold adaptation (Helliot and Mortain-Bertrand 1999), drought stress (Rampino 2006) and various other abiotic stresses (Kavi Kishor 2005). Some of the diverse roles include stabilisation of proteins, membranes and subcellular structures (Vanrensburg *et al.* 1993) and protecting cellular functions by scavenging reactive oxygen species (Bohnert and Shen 1999). Increased abscisic acid (ABA) levels are a natural response to desiccation or water loss (Copeland and McDonald 2001). Proline also accumulates within tissues in response to increased ABA levels (Nieves *et al.* 2001) and can play an integral role in dehydrating seed tissue.

The prerequisite of rehydration for seed germination (Kermode and Bewley 1985) also results in physiological and biochemical changes which occur at differing seed water contents (Powell and Matthews 1978; Cordovo-Tellez and Burris 2002a; Kikuchi *et al.* 2006; Wojtyla *et al.* 2006 and Meyer *et al.* 2007). Both have been investigated extensively for recalcitrant and orthodox seeds (Koster and Leopold 1988; Berjak 1989; Oishi and Bewley 1990; Bewley 1995; McKersie *et al.* 1995; Smith and Berjak 1995; Iglesias and Babiano 1996; Bewley 1997; Gawronska *et al.* 2000; Cordovo-Tellez and Burris 2002b; Gilles *et al.* 2007; Garnczarska *et al.* 2007 and Liu *et al.* 2007). Crowe and Crowe (1986) reported that solute leakage rate is reduced at higher seed water contents. This has also

been observed in soybean seeds where a hydration plateau is reached after six hours of imbibition (Krackhardt and Guerrier 1995). Magnetic resonance imaging reveals that hydration plateaus vary depending on the species as observed in western white pine seeds (Terskikh *et al.* 2005), tobacco seeds (Manz *et al.* 2005) and bean seeds (Kikuchi *et al.* 2006). Imbibition of mature air-dried orthodox caryopses resulted in an overall increase in water content and cellular injury (see Chapters Two & Three). This injury attributed, at least in part, to the disruption of cell membranes during the initial phases of rehydration, is characterised by a rapid loss of intracellular constituents (Leopold 1980; Bewley 1997). The rate at which these intracellular constituents were lost during imbibition suggests that this injury is sustained during the early wetting of seed tissue (Pollock 1969; Bewley 1997). Bewley & Black (1994) have documented the physical and structural changes that occur in viable seeds during the initial phases of imbibition. As seeds start to take up water, there is a release from colloids of adsorbed gases and a rapid leakage into the surrounding medium of solutes such as sugars, organic acids, ions, amino acids and proteins (Bewley and Black 1994). At all imbibition times a range of damage was observed at the ultrastructural level (Isaacs & Mycock 1999; Chapter Three); but generally, with increasing time, there was an increase in the severity of injury. The damage, however, was not extensive enough to compromise viability but did have an effect on vigour. The observed damage is proposed to be a result of soaking injury, which was due to the loss of integrity of the cellular membranes during the process of maturation drying. The membranes did not act as effective retentive barriers during the initial stages of imbibition (Bewley and Black 1994) and hence, the release of electrolytes occurred. The biochemical changes, which occur specifically in maize embryos at the onset of imbibition (within the first six hours), were investigated in this chapter.

The previous chapter addressed the observed imbibitional damage and cellular repair at the ultrastructural level pre- and post-cryostorage (Isaacs and Mycock 1999). Embryonic tissue is progressively rehydrated during imbibition and presumed not to survive subsequent cryostorage. The success in plantlet recovery post-cryostorage at relatively high water contents (Fig. 2.3) was postulated to be, at least in part, attributed to the endogenously produced solutes leached out upon

initial imbibition and these solutes resulted in measurable electrolyte leakage. Some or all of the electrolytes in the leachate could have behaved as natural cryoprotectants. This chapter considers the possibility that various solutes such as proline, glucose and other amino acids [natural cryoprotectants (Withers 1985)] released from the caryopsis tissues upon imbibition, contribute to cryo-successes in plantlet recovery. To provide germplasm relevance, this chapter also considers the above hypothesis on material in short term and long term cryostorage.

## **4.2. Materials and methods**

### *4.2.1. Water content determinations*

The water contents of individual embryos (40 from each imbibition time 0, 1, 2, 3, 4, 5 and 6 hrs), gravimetrically expressed on a fresh weight basis, were determined post-surface decontamination and after drying for  $17 \pm 1$  hrs in a ventilated oven at  $80^{\circ}\text{C}$  according to the modified ISTA Low Constant Oven Temperature Method (International Seed Testing Association, 1985).

### *4.2.2. Vigour and viability assessments*

40 isolated axes per treatment were placed into tissue culture tubes in an aseptic manner with a standard Murashige and Skoog (MS) (1962) basal medium (Appendix 1). The material was incubated at  $25^{\circ}\text{C}$  with a 16 hrs photoperiod and light intensity of  $200 \mu\text{E.m}^2.\text{sec}^{-1}$  and in the dark for eight hours. Developing seedlings were monitored every 24 hours for viability (the number of axes germinated was recorded). Germination was determined by the emergence of either the root or shoot, or both. Vigour was assessed by the rate of growth. The germination index devised by Czabator (1962) (Chapter 2.2), which provides an indication of the percentage germination and rate of growth, was calculated for all treatments.

#### *4.2.3. Cryostorage of isolated embryonic Axes*

Isolated embryonic axes from each treatment (40 per treatment) were placed into cryovials on cryocanes and plunged directly into liquid nitrogen, cooling the tissue at a rate of approximately 200°C/min. The samples were held in liquid nitrogen for 15 minutes, after which the cryocanes were immersed into a 30°C water bath thereby thawing the embryonic axes. Each treatment was repeated at least three times.

#### *4.2.4. Conductivity tests*

Conductivity tests were completed using a CM 100 conductivity meter (original caryopses). The instrument consisted of 100 electrodes which were submerged into wells each containing 3 ml of ultra-pure water, seventy five wells contained an intact caryopsis (stored at 4°C in a hermetically sealed container prior to experimentation). The remaining twenty five wells, containing only ultra-pure water, served as the control. The instrument then recorded the conductivity measurements for each imbibition time. After the designated time (0-6 hrs) the caryopses were removed from the wells and the embryos excised. The conductivity values were calculated per gram of tissue.

#### *4.2.5. Biochemical analysis*

Biochemical assays were conducted on the excised embryos and on the leachate (pooled together for 25 caryopses i.e. 3 ml from each well concentrated together X 25 totalling three replicates of 75 ml) and analysed for amino acids and glucose. Twenty five embryonic axes (to compare directly to the leachate results) from each imbibition time were isolated, immediately covered in liquid nitrogen in a pestle and mortar, and finely ground. The ground tissue was then divided into two roughly-equal amounts and suspended in either a Tris-phosphate-glycerol buffer (for glucose) or an acetic acid buffer (for amino acids).

Once the samples were suspended in their respective buffers for 15 minutes they were vigorously stirred with a vortex for 15 seconds, and the extracts were centrifuged at 10 000 (x) for 15 minutes. The supernatant was isolated and used to test for amino acids and glucose in the embryos. The collected and pooled leachate was also tested for the presence of the selected biomolecules.

#### *Quantitative determination of amino acids*

The analysis was performed by the addition of 0.2 ml of ninhydrin reagent (2 mg.ml<sup>-1</sup> of ninhydrin (Merck) in 20 mM acetic acid acetate buffer, pH 5) to 1.0 ml of the test solution. The solution was heated for 10 minutes at 100°C and cooled, and the absorbance measured at 440 nm for proline and at 570 nm for the other amino acids i.e.  $\gamma$ -amino butyric acid, glutamic acid and glycine. Standard curves were prepared for each amino acid using serial dilutions. The total amino acid standard curve was prepared using serial dilutions of caseine hydrolysate and the absorbance at 440 nm for proline and 570 nm for all the other amino acids was noted (Holme and Peck 1983). The limit of sensitivity was 0.1 $\mu$ mol. Leachate values were expressed as  $\mu$ mol/ml to facilitate direct comparisons with the levels of amino acids recovered from the embryo tissues, which were expressed as  $\mu$ mol/g<sup>-1</sup> FW.

#### *Quantitative determination of glucose*

The amount of glucose in isolated embryonic axes was determined using a glucose oxidase reagent. The glucose oxidase reagent consisted of 30mg of glucose oxidase (Merck), 3mg of horseradish peroxidase (Merck) and 10mg of orthodianisidine (Merck) hydrochloride dissolved in 100ml of Tris-phosphate-glycerol buffer, pH 7.0 (Holme and Peck, 1983). This reagent was stable for one week provided it was stored in a brown bottle at 5°C.

The procedure involved the addition of 1.0ml of sample to 2.0ml of the glucose oxidase reagent. The mixture was incubated at 37°C for 30 minutes and then 4.0ml of 5 N hydrochloric acid was added and the absorbance measured at 525

nm. A standard curve was prepared using 1-100 $\mu$ g/ml of glucose (Holme & Peck 1983) and expressed in these units for direct comparison with the leachate values. Glucose from the embryo tissues were expressed as  $\mu$ mol/g<sup>-1</sup>. FW.

#### 4.2.6. *Cryostorage of isolated embryonic axes for 12 months*

The procedures described in 4.2.1, 4.2.2, 4.2.3 and 4.2.5 were also repeated on maize embryonic tissue which had been in cold storage ( $\pm 4^{\circ}\text{C}$  and hermetically sealed) for 12 months, hereafter referred to as the stored caryopses. This tissue was then subjected to cryostorage for 15 minutes (as a direct comparison to the original caryopsis tissue) and 12 months respectively (to determine the effects of long term cryostorage).

#### 4.2.7. *Data analysis*

Each treatment was repeated three times. Water content data sets were Arcsine transformed to approximate normality and subjected to a one way ANOVA using Statistica 6.0 (Statsoft 2001). Tukey post hoc comparisons were used to establish significant differences between imbibition times. Viability data were analysed using contingency tables and Chi square tests using Instat version 3.0. (Graphpad software 2003). Germination indexes and percent root formation data were analysed using Fisher's exact tests using Instat version 3.0. Conductivity data sets were subjected to a one way ANOVA using Statistica 6.0 (Statsoft 2001) and Tukey post hoc comparisons were used to establish significant differences between conductivity values for each imbibition time. All biochemical data on amino acids and glucose were subjected to one way ANOVAs using Statistica 6.0 (Statsoft 2001). Tukey post hoc comparisons were used to establish significant differences between imbibition times, and the presence of these biochemical molecules in the leachate as well as the embryos. Biochemical data obtained over three different time periods (the original caryopses and the stored caryopses pre- and post-cryostorage) were subjected to a two way ANOVA using Statistica 6.0 (Statsoft 2001) and Tukey post hoc comparisons were used to establish significant differences between imbibition times and the effects of long term cryostorage.

The dry tissue values were not included in the statistical analysis in order to prevent skewing statistical groups.

### 4.3. Results and discussion

#### 4.3.1. Imbibition

Water content appeared to increase with each imbibition time (Table 4.1). The unimbibed dry caryopses were at average water contents of 7.4% and after a 30 min decontamination step the largest percentage of water was absorbed relative to imbibition time. Statistical analysis showed that water content remained the same for the next three hours of imbibition and the only significant difference in water content occurred between the 1.5 hrs and 4.5 hrs imbibition times. Water contents between 4.5 and 6.5 hrs are statistically the same. Analysis of the conductivity data revealed that the values obtained between 0.5 hrs - 3.5 hrs are significantly different from the 4.5 hrs - 5.5 hrs values and 6.5 hrs imbibition times ( $F_{6, 524} = 34.307$ ,  $P < 0.001$ ). These results demonstrated that conductivity at these early imbibition times were not directly correlated to water content, even though conductivity increased with each imbibition time (Table 4.1), but rather provided an indication of membrane status.

**Table 4.1.** Water contents in percentage (%) and conductivity ( $\mu\text{S}$ ) [sd]  
Values with the same superscript alphabet are not significantly different ( $P < 0.001$ , Tukey post hoc test).

| Imbibition Time (hrs)          | 0                  | 0.5 surf decont.      | 1.5                  | 2.5                     | 3.5                     | 4.5                   | 5.5                   | 6.5                    |
|--------------------------------|--------------------|-----------------------|----------------------|-------------------------|-------------------------|-----------------------|-----------------------|------------------------|
| W/C (%)                        | 7.4<br>$\pm 1.3^a$ | 51.58<br>$\pm 12.1^b$ | 54.17<br>$\pm 4.7^b$ | 57.73<br>$\pm 8.1^{bc}$ | 57.93<br>$\pm 3.5^{bc}$ | 60.9<br>$\pm 6.2^c$   | 59.31<br>$\pm 5.0^c$  | 61.36<br>$\pm 10.2^c$  |
| Conductivity ( $\mu\text{S}$ ) | -                  | 35.23<br>$\pm 8.75^x$ | 40.4<br>$\pm 6.49^x$ | 39<br>$\pm 5.76^x$      | 42.44<br>$\pm 8.42^x$   | 54.81<br>$\pm 8.65^y$ | 53.35<br>$\pm 9.25^y$ | 67.64<br>$\pm 11.19^z$ |



#### 4.3.2. Conductivity

Conductivity tests indirectly determine the integrity of seed membrane systems (Bewley and Black 1994) and are used for the assessment of seed vigour, because this test was initially thought to detect the seed deterioration process at its earliest phase (Fessel *et al.* 2006). Conductivity is based on the fact that seeds, when soaked in water, exude ions, sugars and other metabolites from commencement of soaking, due to changes in the integrity of the cell membranes, as a function of the quantity of water and the level of seed deterioration (Fessel *et al.* 2006). Conductivity values for *Hybrid 6363* maize caryopses appeared to increase with imbibition time (Table 4.1). Dry orthodox seed membranes, in a liquid crystalline phase (Belwey and Black 1994) would be most leaky at the onset of imbibition. Conductivity values remained constant between 0.5 hrs - 3.5 hrs of imbibition demonstrating that the majority of the solutes were leached out of the embryonic tissue within the first 30 minutes of imbibition. This observation supports that of Iglesias and Babiano (1996), who noted that when dry seeds (i.e. with a 5 - 10% water content) plunged directly into water, lost solutes into the external medium, the electrolyte loss was predominantly within the first hour. This result suggests that membrane status at 3.5 hours is similar to that in the dry tissue, implying that membranes remain in the liquid crystalline phase despite the increase in water content. The ultrastructural data, however, do not support this (Chapter Three) as the membranes underwent a measure of reorganisation after 3.5 hrs imbibition which should result in a reduction in electrolyte loss or re-absorption of electrolytes. There was a significant increase in conductivity between 3.5 and 4.5 hrs imbibition ( $F_{6,524}=34.307$ ,  $P<0.001$ ) as well as between 5.5 and 6.5 hrs imbibition ( $F_{6,524}=34.307$ ,  $P<0.001$ ).

Different seeds reach different hydration plateaus (Crowe and Crowe 1986: Krackhardt and Guerrier 1995: Terskikh *et al.* 2005: Manz *et al.* 2005: Kikuchi *et al.* 2006). For these maize caryopses it reached its hydration plateau after 4.5 hours of imbibition, but electrolyte leakage had not slowed after 5.5 hours. Recent studies on maize reveal that conductivity, as a vigour test, does not account for seed deterioration in seed lots stored at 10°C (Fessel *et al.* 2006) and is

insufficient evidence of compromised membrane integrity (Panobianco *et al.* 2007). The conductivity values alone, therefore, cannot solely be considered as an indication of seed deterioration and loss of membrane integrity at these imbibition times.

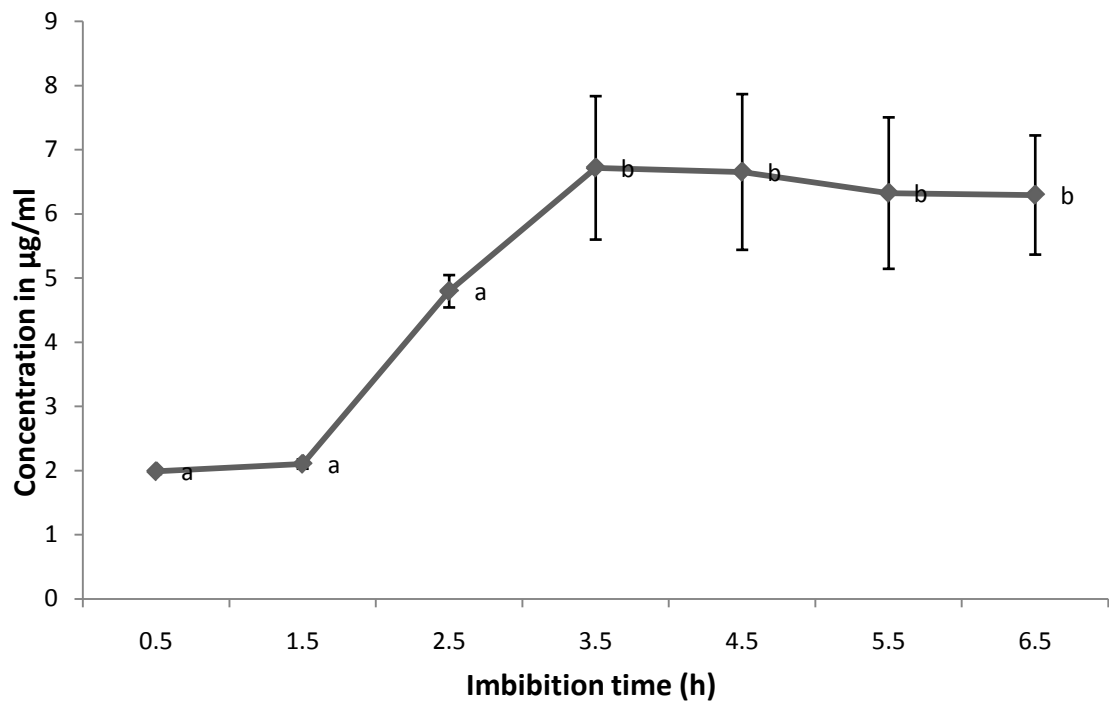
The availability of mineral nutrients and organic compounds (such as proteins, lipids and carbohydrates) in seed tissues is important for germination and seedling vigour (Cordova-Tellez and Burris 2002a; Cordova-Tellez and Burris 2002b; Volk *et al.* 2006) i.e. for incorporation and formation of developing tissue. The variety in uptake, transport and distribution of mineral nutrients exists among genotypes of a given plant species (Brown and Ambler 1973; Clark and Brown 1974; Clark 1983; Graham *et al.* 1992), in that the highest concentration of nutrients per unit of weight is generally found in the embryo (O'Dell *et al.* 1972; Mazzolini *et al.* 1985; Pearson and Rengel 1994; Pearson *et al.* 1996) and accumulates during the process of maturation drying (Yancey *et al.* 1982). These large reserves of nutrients in the cells of the embryo ensure that the embryo can grow during the initial stages of development and be self-sustaining.

The extent of nutrient mobility within plant tissues depends on the nutrient itself and its physiological function (Gallego *et al.* 1991). The observed successes post-cryostorage in the current study, are a result of the changes in nutrient mobility status (i.e. either in a free or bound state) caused by soaking. Embryos cryostored after 3.5 hours imbibition produced the largest percentage of normal plantlets (see Table 2.2) and it is proposed that at this imbibition time, leachate and available biomolecules are at concentrations sufficient enough to confer cryo-protection.

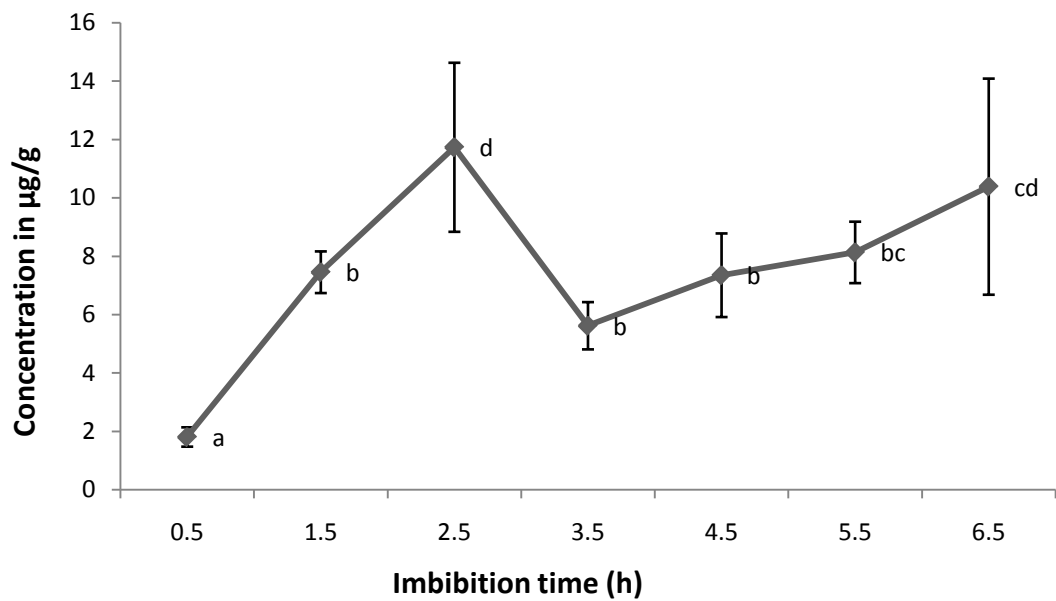
### 4.3.3. Biochemical analysis - imbibition

#### *Glucose*

Glucose levels in the leachate, after 3.5 hours of imbibition, occurred at a very low level (Fig. 4.1) but had reached a maximum and then remained at a similar concentration for all the subsequent imbibition times. Measurable quantities of glucose post-imbibition (Figs. 4.1 and 4.2) were marginal within both the leachate and the isolated axis, but Tukey post hoc tests revealed significant differences for the various imbibition times (see below). Soluble carbohydrates, monosaccharides are usually found in trace amounts in mature orthodox seeds (Vertucci and Farrant 1995; Dussert *et al.* 2006). Initial imbibition demonstrated glucose levels at low concentrations in the leachate and, as water uptake continued, an increase in glucose quantities within the leachate was observed between the 0.5 and 2.5 hrs imbibition times ( $F_{6,14}=4.6164$ ,  $P<0.001$ ). Glucose concentrations in the embryo samples, between 2.5 and 3.5 hours, decreased by approximately half ( $F_{6,14}=39.754$ ,  $P<0.001$ ) Fig 4.2. During dehydration, glucose aids in reducing the fluid-gel transition temperature  $T_m$  and provides stability to membrane lipids (Crowe and Crowe 1986; Bryant *et al.* 2001; Hinch and Hagemann 2004). If the glucose, between these imbibition times, was being utilised to minimise the gel-fluid transition temperature then it is probable that the transition of membranes from gel to fluid phase occurred between these imbibition times. By 3.5 hours, membranes had restructured, after which, glucose loss was restricted by the re-functional plasmamembrane and, hence, glucose remained at similar concentrations after 3.5 hrs imbibition in the leachate (Fig. 4.1).



**Figure 4.1.** Glucose in µg/ml present in the leachate after each imbibition time. Values with the same alphabet are not significantly different ( $P < 0.001$ , Tukey post hoc test)

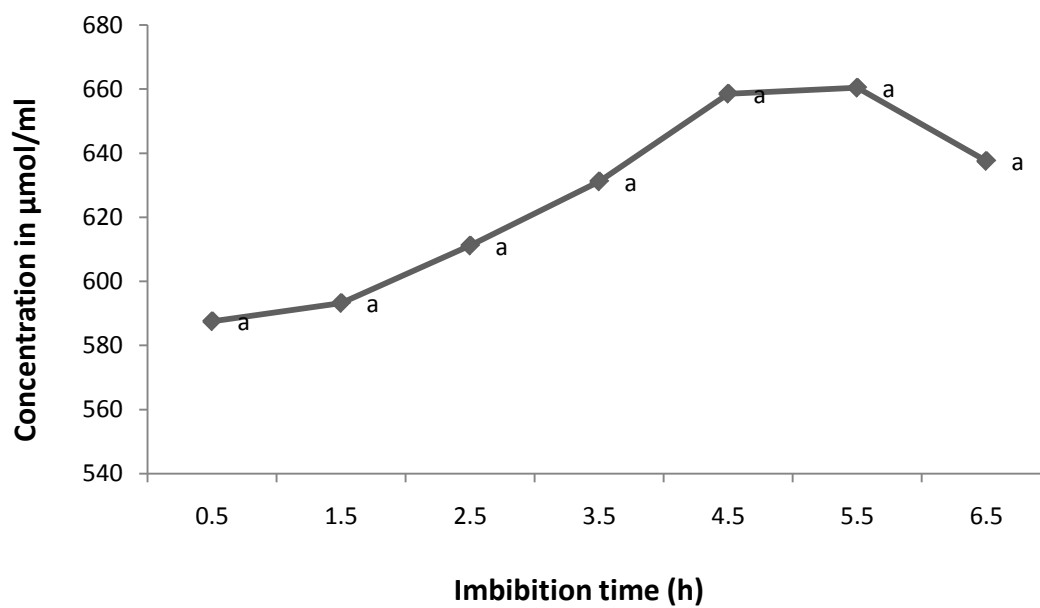


**Figure 4.2.** Glucose present, per gram of embryonic tissue after each imbibition time. Values with the same alphabet are not significantly different ( $P < 0.001$ , Tukey post hoc test)

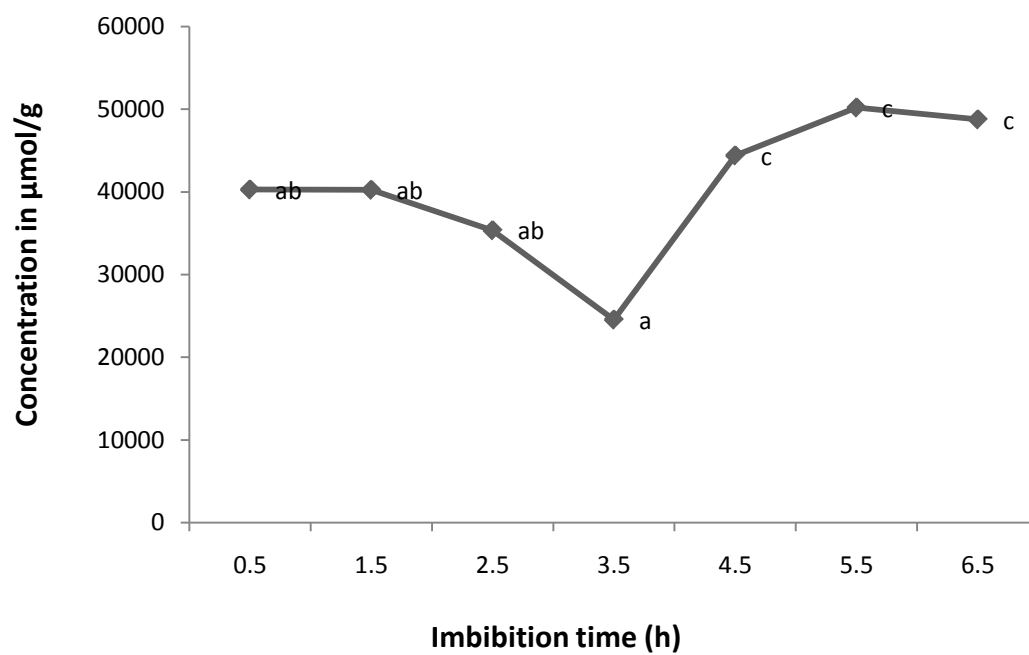
### *Amino acids*

Total amino acids (in the leachate) were measured at the onset of imbibition and remained at similar concentrations throughout the 6.5 hrs imbibition (Fig. 4.3). Although it appears that the amino acids had been reabsorbed by the caryopsis tissues from the leachate (Gallego *et al.* 1991) the values in the present study were not statistically different at the  $P=0.05$  level between 5.5 and 6.5 hours. The overall level of amino acids in the embryonic tissue decreased 3.5 hours after the initiation of imbibition (a common trend in the amino acids tested) and, it is possible that the amino acids were reabsorbed and utilised in the process of protein synthesis (if operational at this point). However, polysomes (indicative of protein synthesis) were never detected in sectioned material (see Chapter Three), possibly because the material was fixed too soon after the commencement of imbibition, even though all the components necessary for the resumption of protein synthesis upon imbibition are present within the cells of mature orthodox embryos (Bewley 1997). Within minutes of rehydration there is an observed decline in the number of single ribosomes as they become recruited into polysomal protein-synthesising complexes (Bewley 1997). It is possible that the observed drop in amino acid levels at 3.5 hrs imbibition indicates the redirection of amino acids for protein synthesis to commence. These trends were reflected in the profiles of all the individual amino acids (Figs. 4.5 - 4.10) except proline (Fig. 4.12).

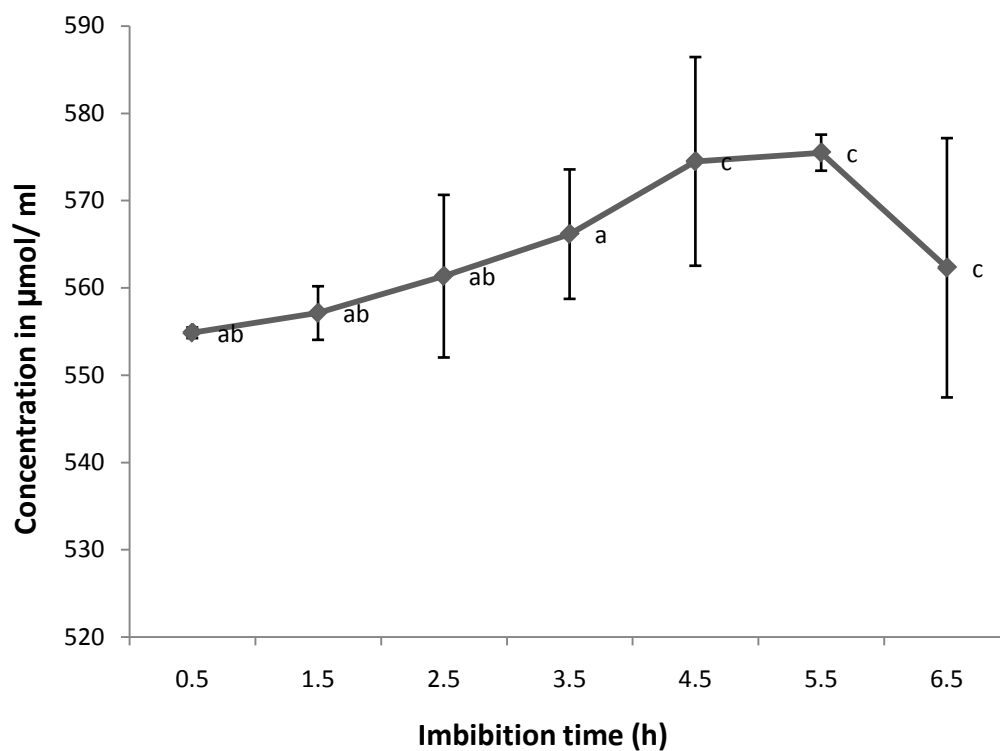
The total amino acid concentrations were much greater in the embryo (Fig 4.4) than in the leachate (Fig. 4.3) indicating that the bulk of the detectable amino acid was retained within the embryo. After 3.5 hours imbibition, the total amino acids began to increase significantly in the embryos, indicating that amino acid synthesis had resumed or that they were being mobilised from a bound state, as tissue became more hydrated. If the glycine values are considered as an example, we know that Group 1 LEA proteins (Chapter One) preferentially accumulate to high levels during maturation drying. As the tissue is imbibed, glycine values decline during the 3.5 hrs of imbibition (Fig.4.9), indicating that these LEAs could become bound for membrane stabilisation.



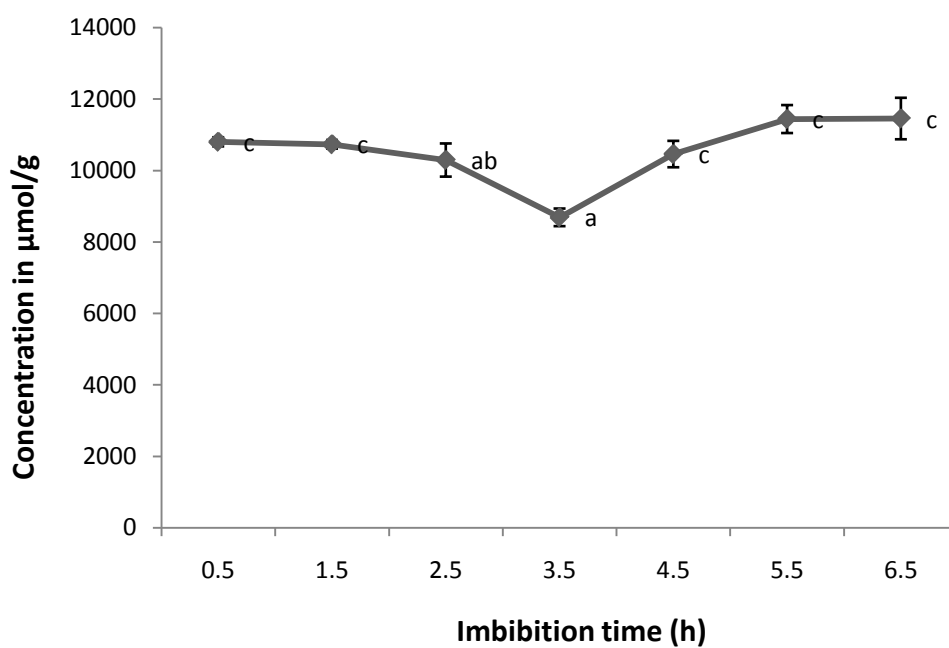
**Figure 4.3.** Total amino acids present in the leachate for each imbibition time. Values with the same alphabet are not significantly different ( $P < 0.001$ , Tukey post hoc test).



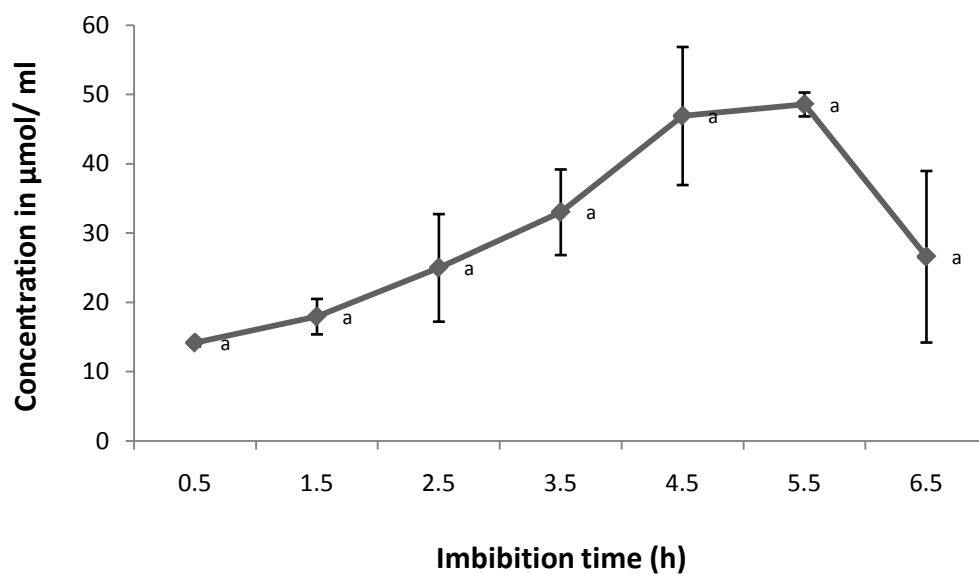
**Figure 4.4.** Total amino acids present per gram of embryonic tissue for each imbibition time. Values with the same alphabet are not significantly different ( $P < 0.001$ , Tukey post hoc test).



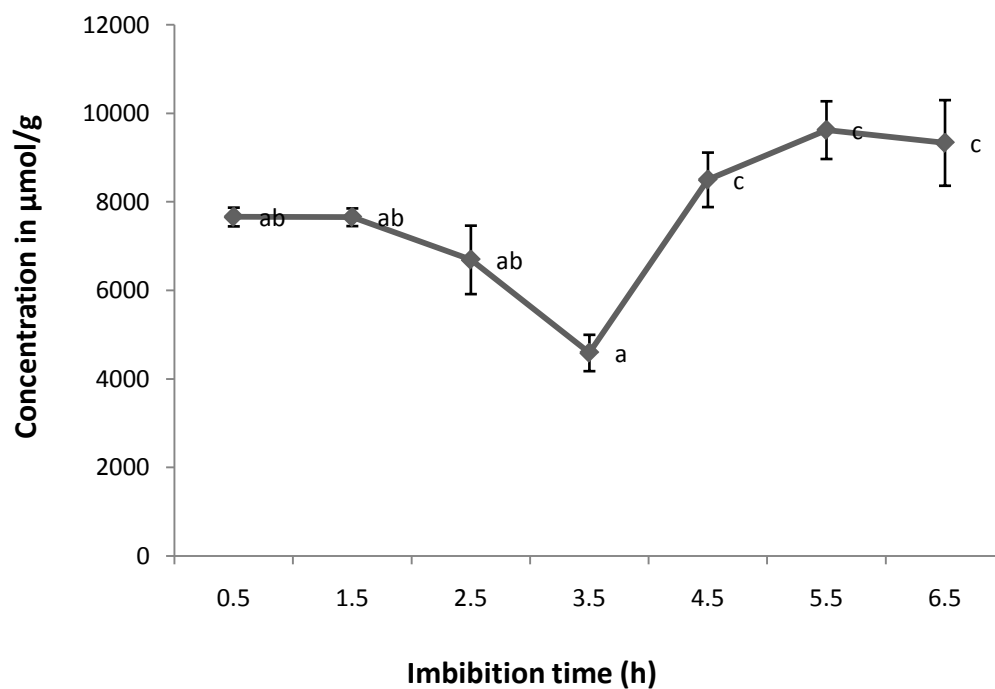
**Figure 4.5.**  $\gamma$ -Amino butyric acid present in the leachate for all imbibition times. Values with the same alphabet are not significantly different ( $P < 0.001$ , Tukey post hoc test).



**Figure 4.6.**  $\gamma$ -Amino butyric acid present per gram of embryonic tissue for each imbibition time. Values with the same alphabet are not significantly different ( $P < 0.001$ , Tukey post hoc test).

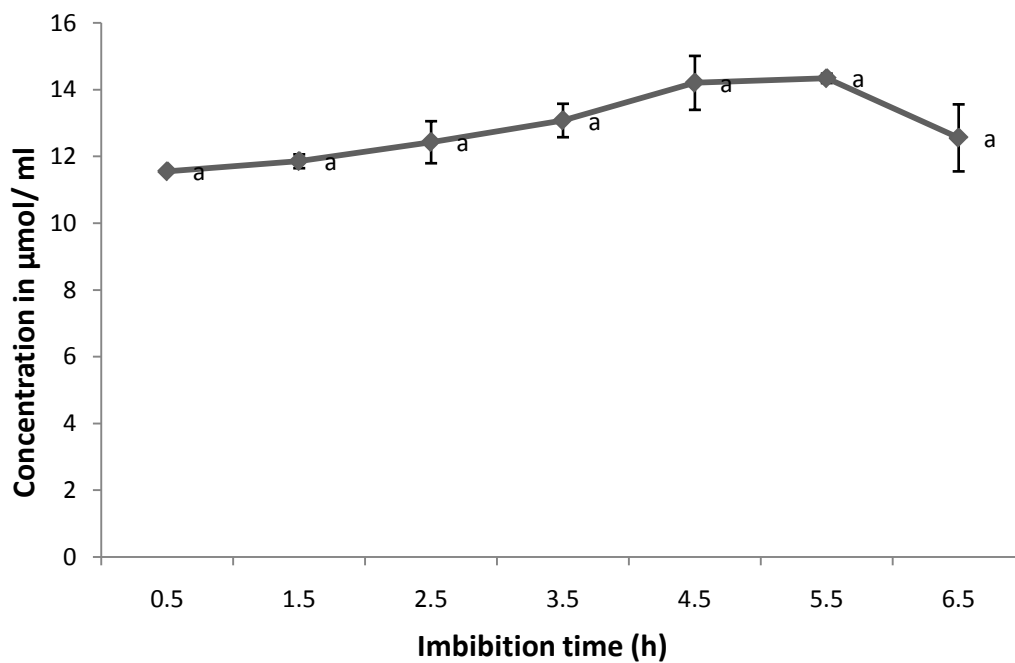


**Figure 4.7.** Glutamic acid present in the leachate for all imbibition times. Values with the same alphabet are not significantly different ( $P < 0.001$ , Tukey post hoc test).

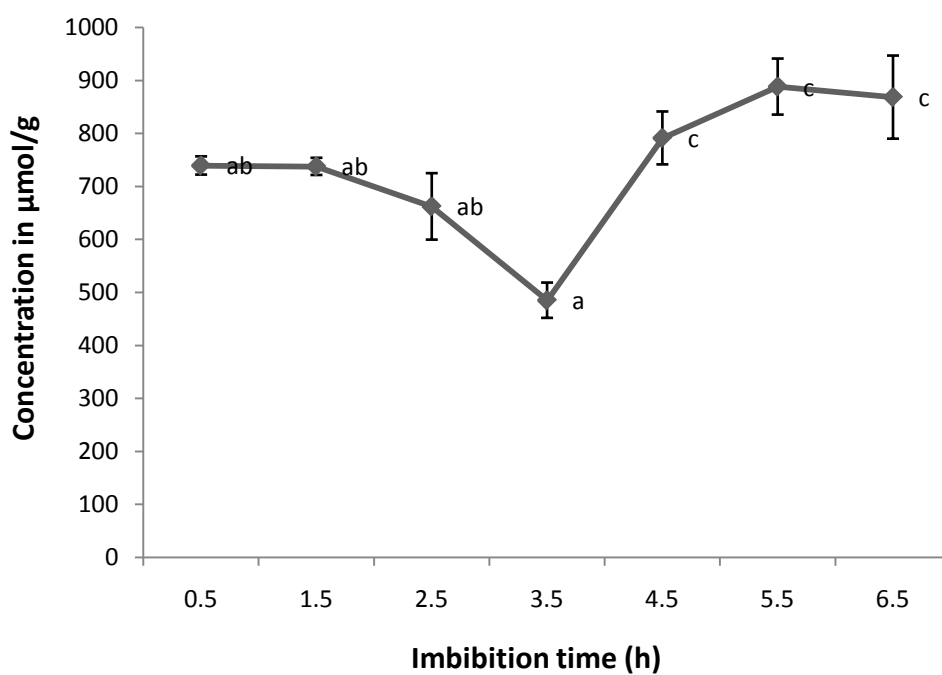


**Figure 4.8.** Glutamic acid present per gram of embryonic tissue for each imbibition time. Values with the same alphabet are not significantly different ( $P < 0.001$ ).

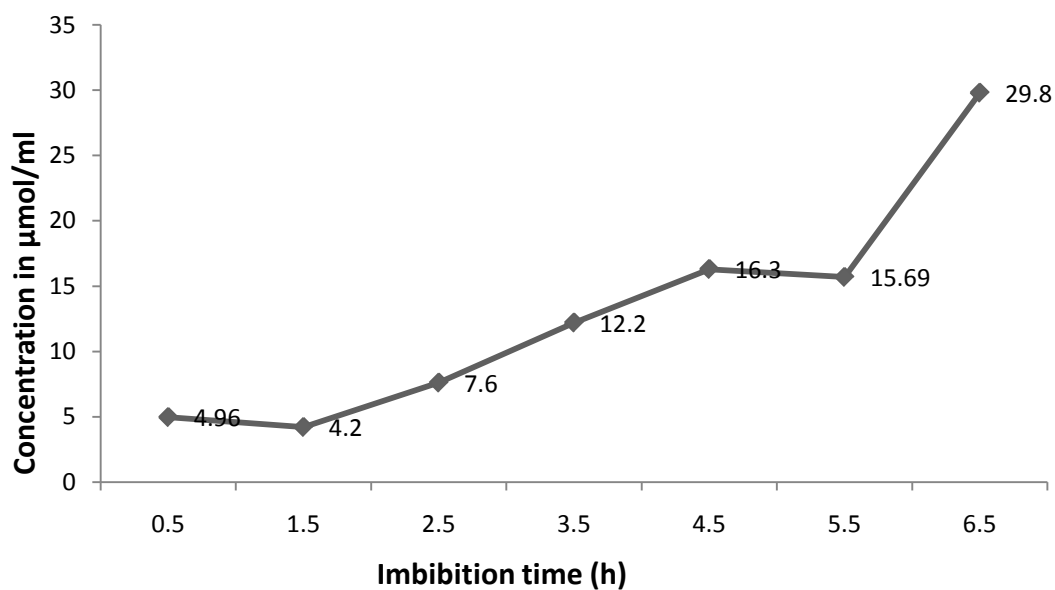




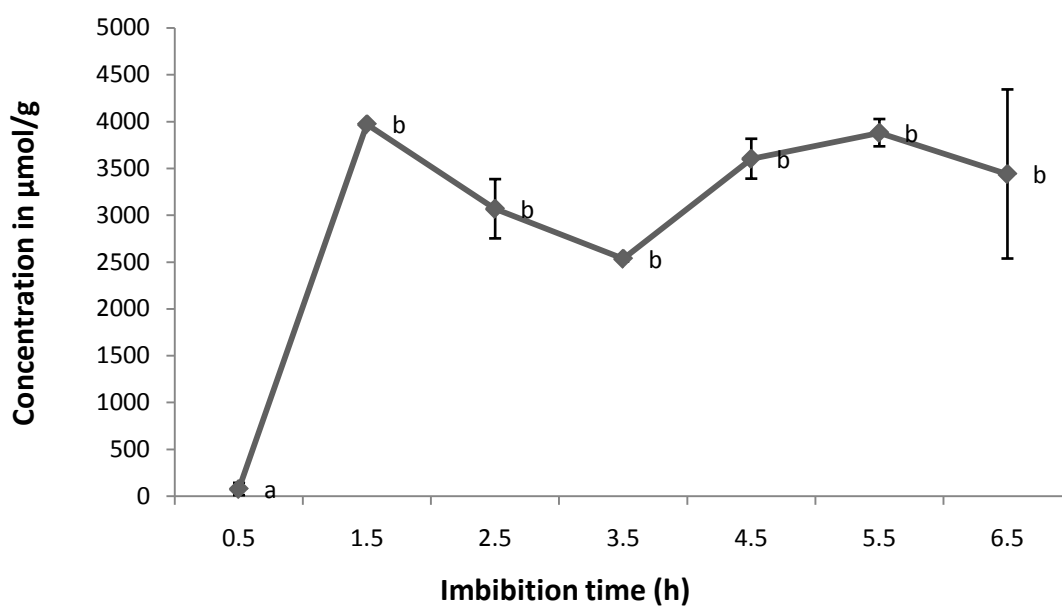
**Figure 4.9.** Glycine present in the leachate for all imbibition times. Values with the same alphabet are not significantly different ( $P < 0.001$ , Tukey post hoc test)



**Figure 4.10.** Glycine present per gram of embryonic tissue for each imbibition time. Values with the same alphabet are not significantly different ( $P < 0.001$ , Tukey post hoc test).



**Figure 4. 11.** Proline present in the leachate for all imbibition times. Only one value was obtained for each of the imbibition times as only one of the three samples tested positive for proline.



**Figure 4. 12.** Proline present per gram of embryonic tissue for each imbibition time. Values with the same alphabet are not significantly different ( $P < 0.001$ , Tukey post hoc test).

Between 4.5 - 6.5 hrs imbibition, glycine values increased significantly indicating that they could become unbound from the LEA protein as tissue was hydrated. Further investigation would be necessary to definitively conclude that Group 1 LEAs are responsible for membrane stabilisation at 3.5 hours but, given the tissues response to cryostorage at this hydration level, it does seem likely that LEAs were playing a role in membrane stabilisation and providing chilling tolerance (see Chapter One and below).

### ***Proline***

An increase in ABA concentrations as a result of maturation drying indirectly results in an increase in proline concentrations. Maturation drying, however, is a stress in itself (Verslues 2006) and could directly result in the accumulation of proline. At the onset of imbibition, therefore, it is expected that the stored proline would be released into the leachate owing to membrane permeability. However, proline was only found to leach into the surrounding water in very small quantities and even after 6.5 hours imbibition, was only at  $\pm 30 \mu\text{mol/ml}$  (Fig. 4.11). These concentrations are much lower than those exogenously applied to tissue as routine cryoprotectants i.e. 5.5M (Stanwood 1986) and for this reason it is highly probable that the proline in the solution in the leachate was not performing a cryoprotective role. However, free/soluble proline detected within the embryo was at a high concentration relative to that in the leachate throughout imbibition, indicating that it was not leached into the surrounding water but was still soluble, and therefore, may well have acted as an endogenous cryoprotectant.

An alternative to the mobilisation of proline at the onset of imbibition is that proline is synthesised. It has been established that the flooding of water into the dry embryonic axis at the onset of imbibition can be considered an osmotic stress (Chapter One) and, therefore, it would be expected that this process would initiate proline synthesis, showing a significant difference in the concentration of proline at 0.5 hrs to 1.5 hrs imbibition ( $F_{6,14}=9.7642$ ,  $P<0.00025$ ). This could only occur provided the biosynthetic pathway was active. The embryonic proline concentration,

after 1.5 hours imbibition, had increased from practically zero to almost 4000  $\mu\text{mol/g}$  (Fig. 4.10).

There was a slight decrease in soluble proline between 1.5 and 3.5 hrs of imbibition, but the values at these points did not differ statistically. If the decrease in value was legitimate it would be due to proline binding to (Fig. 4.12), or becoming incorporated in membranes during cell restructuring/repair mechanisms (Gallego *et al.* 1991; Hare *et al.* 1999). After the 3.5 hrs imbibition, if proline levels increased significantly, it would indicate that proline synthesis had commenced at this point, due to the tissues increasing their metabolic rate.

Proline is synthesised from either the glutamate or the ornithine pathway (Delauney and Verma 1993) depending on the plant systems involved or the nature of the stress sustained. Under osmotic stress, the glutamate pathway is generally considered the primary route for proline synthesis (Delauney and Verma 1993). The glutamate pathway itself is initiated by the phosphorylation of L-glutamate to L-glutamyl-g-phosphate by g-glutamyl kinase (g-GK). Then, g-GK is converted to glutamate-g-semi-aldehyde (GSA) by GSA dehydrogenase. Finally, GSA spontaneously forms D-pyrroline-5-carboxylate (P5C), and P5C is reduced to proline by P5C reductases (Change and Lee 1999). This process is possible given that glutamic acid (Fig. 4.9) occurred in almost double the concentrations of proline. However, Verslues and Sharp (1999) have shown that proline accumulation in the cytoplasm is not primarily due to synthesis from glutamic acid but from glucose along the ornithine pathway. Proline accumulation in both of these pathways is dependent on osmotic stress (Delauney and Verma 1993). Venekamp (1989) does however state that proline synthesis consumes ATP, reductants and carbon skeletons, so, if the tissue is not metabolically active, the synthesis of proline seems unlikely.

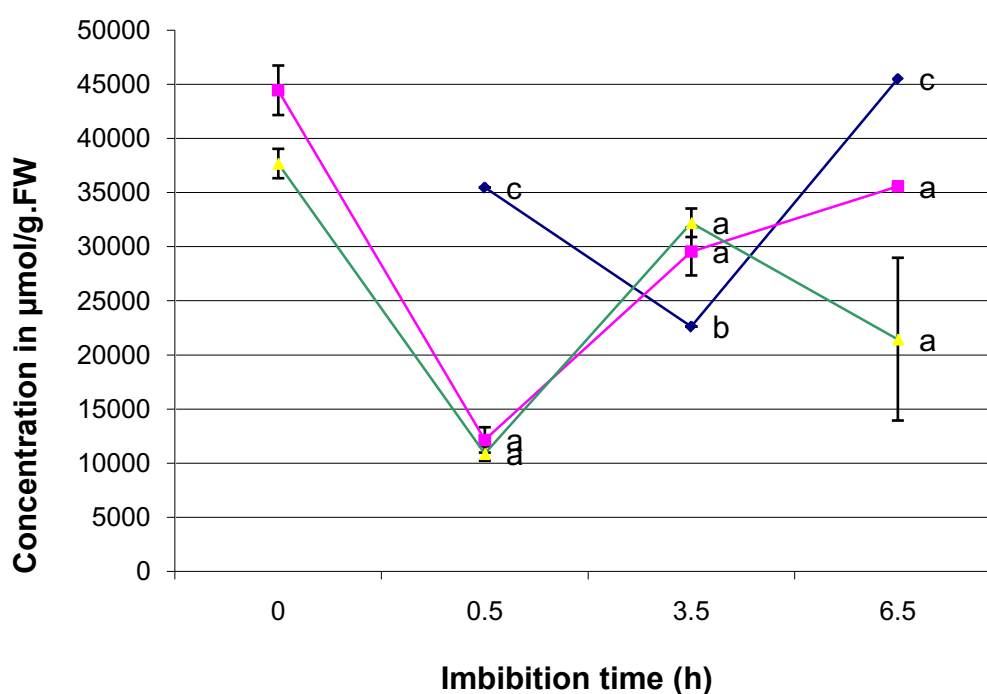
Alternatively, if previously accumulated proline (during maturation drying) within the cytoplasm of each cell in the radicle meristem (Verslues and Sharp 1999) is released at the onset of imbibition, this would provide a reasonable explanation for

the increase in proline content after 1.5 hours of imbibition (Fig. 4.12). It is postulated that as membrane restructuring/repair commences, the mobilised proline replaces water (Stanwood 1986), and later (between 1.5 and 3.5 hrs imbibition), becomes membrane bound, as seen in the decline in proline concentration between the 1.5 and 3.5 hrs imbibitions times (Fig. 4.12). Proline release, therefore, is a physical response to initial imbibition and can act as a possible defence mechanism against membrane damage.

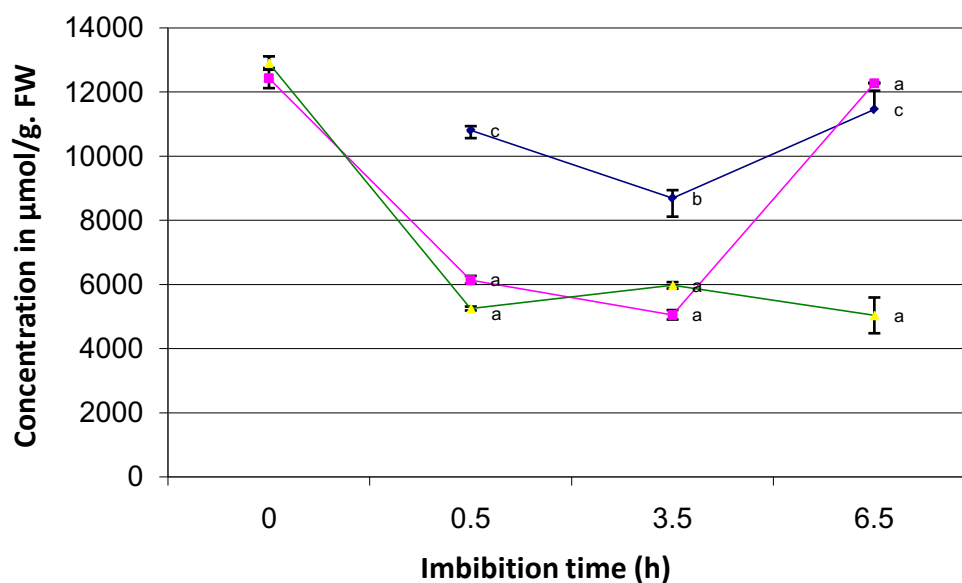
#### *Effects of cryostorage on biomolecules*

The total amino acid content was reduced considerably (Fig. 4.13) between original caryopses and stored caryopses. Tukey post hoc tests revealed a highly significant ( $F_{4,18}=11,7191$ ,  $P < 0.001$ ) difference, therefore, storage had a significant impact on the level of amino acids. However, at 3.5 hours of imbibition, detectable amino acids were slightly higher in concentration for both stored caryopses, before and after freezing, in comparison with the original caryopses. This trend was common in all examined solutes (see Appendix Two) excepting  $\gamma$ -amino butyric acid (Fig 4.14), and therefore, only these data will be examined. It is possible that the  $\gamma$ -amino butyric acid was not conserved during protracted periods of cold storage or became more tightly bound (see earlier) after 3.5 hours imbibition. This allowed for a greater success of seedling recovery post-cryostorage (Fig 4.14) and proved to be significantly different to the original tissue ( $F_{4,18}=11,989$ ,  $P < 0.0001$ ). At 3.5 hours imbibition there was a marked decline in solute concentration in the axis prior to cryostorage (Figs. 4.4, 4.6, 4.8, 4.10 and 4.12) and it was speculated that the solutes became biologically bound/incorporated into newly synthesised biomolecules and/or reconstituted with the membrane. These biomolecules were less vulnerable to the effects of freezing stress, and hence, were conserved (Figs. 4.13 - 4.15). Glucose, although present in smaller quantities, exhibited a similar trend to that of proline (Figs. 4.2 & Fig. 4.12), and as mentioned above, probably also contributed to cryostorage success. Tissue in cold storage for one year (stored tissue) demonstrated biochemical changes when compared with the original caryopses. Regardless of the

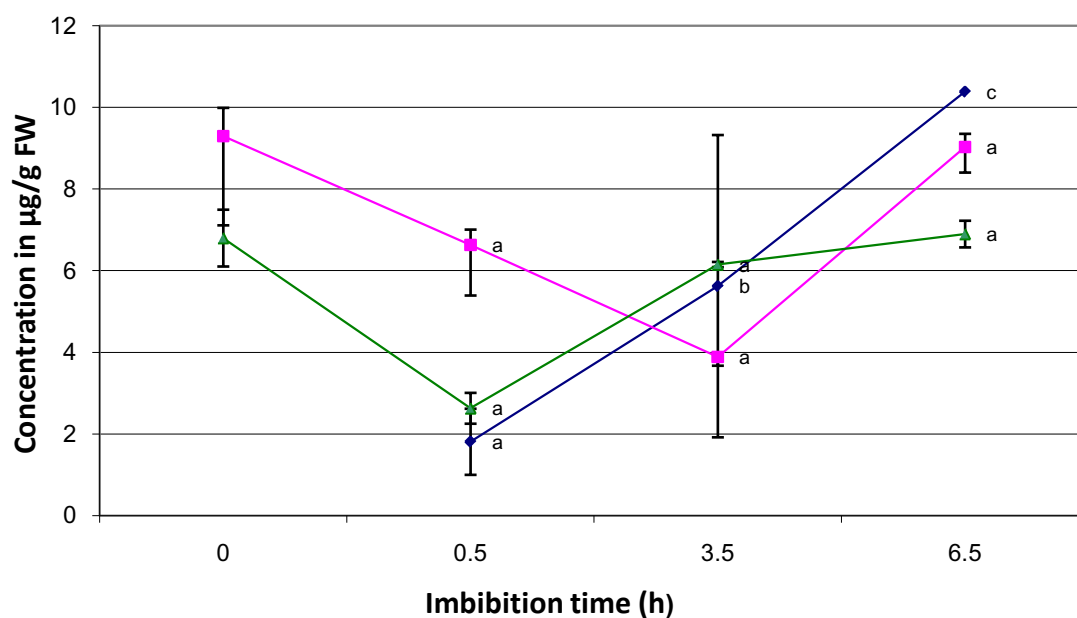
biomolecule in question (Figs. 4.13-4.15) the statistical comparisons revealed that the observed changes occurred during sample storage times and not on account of storage type. Despite the time spent in storage, the biochemistry of the caryopsis was maintained in cryostorage. It is possible that tissue had aged slightly in cold storage, and hence, differences in free solute concentration were observed between the original caryopses and the stored caryopses. Effects of cryostorage on these biomolecules became visible after 6.5 hours imbibition (Figs 4.13, 4.14 and 4.15), and upon further investigation and enquiry of the seed producer, it was established that the original seed lot had already aged prior to purchase.



**Figure 4.13.** Total amino acids found in embryos imbibed for 0 - 6 hrs in original caryopses (blue line), stored caryopses (in cold storage for 1 year – pink line) and then cryostored for 12 months (green line). Values with the same alphabet are not significantly different ( $P < 0.001$ , Tukey post hoc test).



**Figure 4. 14**  $\gamma$ -amino butyric acid found in embryos imbibed for 0-6hrs for original caryopses (blue line), stored caryopses in cold storage for one year - (pink line) and then cryostored for 12 months (green line). n = 40. Values with the same alphabet are not significantly different ( $P < 0.001$ , Tukey post hoc test).



**Figure 4. 15** Glucose content in embryos imbibed for 0-6 hrs in Original caryopses (blue line), Stored caryopses in cold storage for 1 year- (pink line) and then cryostored for 12 months (green line). n= 40. Values with the same alphabet are not significantly different ( $P < 0.001$ , Tukey post hoc test).

The levels of glucose and amino acid varied for this maize hybrid over two storage times, and subsequent to cryostorage, within the first 6 hours of imbibition. An additional freezing stress introduced another set of factors which influence the outcome of the developing plantlet post-cryostorage. These data therefore, provide information on the effects of cryostorage on individual biomolecules at particular hydration levels.

#### 4.4. Summary

Previous studies have revealed that the water content may affect the intracellular activities of seed tissues in a variety of ways. Firstly, by providing the media, for enabling the three dimensional conformation of active biomolecules (proteins, nucleic acids, substrates). Secondly, by mobilising substrates which are concentrated in certain particles and transporting them towards enzymes (or vice versa), and thirdly, by diluting substances which may exhibit inhibitional properties (Matthews 1985). Matthews, (1985) also observed that some biological activities occur only at water saturated stages and others occur mainly during water deficient stages. The present results have revealed that many biochemical changes commence at the earliest imbibition time, prior to the initiation of active metabolism. Based on these observations and the present data it is proposed that metabolism in *Zea mays* commences at or shortly after 3.5 hours of imbibition and this could well mark the beginning of desiccation sensitivity.

The observed success of plantlet recovery post-cryostorage at high moisture contents can be attributed to a number of factors. The solutes which accumulate during maturation drying (Helliot and Montain-Bernard 1999) are released, due to imbibitional forces, into the leachate and endogenously mobilised within the first 30 minutes of imbibition. The electrolytes released into the leachate are present in relatively low concentrations when compared with exogenously applied levels required for cryoprotection (Stanwood 1986; Withers and King 1979) and, therefore, probably do not individually provide cryoprotection for the embryo.



Combined concentrations of the investigated endogenous amino acids could, however, confer cryoprotection.

At 1.5 hours imbibition, proline concentrations were approximately 4000 $\mu$ mol/ml (Fig. 4.12). Optimum proline concentrations utilised as a cryoprotectant for cultured maize cells resulting in successful recovery post-cryostorage was in the range of 2.5 - 25% (Withers and King 1979). Proline at 10% applied to tissue in a supplemented medium resulted in minimal plasmolysis damage and adequate cryoprotection (Withers and King 1979). Conversion calculations show that 10% is equivalent to 100 000 $\mu$ mol/ml therefore; the measured 4000 $\mu$ mol/ml is only 4% of this total and may not be sufficient to behave as a cryoprotectant. Nevertheless, it has been shown that effective cryoprotectant concentrations vary with tissue type (Hitmi *et al.* 2000; Danso and Ford-Lloyd 2004).

Proline, when applied to recalcitrant seed tissues, resulted in great successes post-cryostorage, for example *Anthrrium* seeds (Stanwood 1986). When proline is used in high molar concentrations it removes water from the seed. For example, seeds placed into a 5.5 M solution of proline will become dehydrated within 72 hours, from moisture levels of approximately 50% to moisture levels of between 15 and 20% i.e. each 0.5 grams of proline replaces 1mg of water (Stanwood 1986). Moisture levels obtained by this method were low enough to limit freezing damage in this recalcitrant species. It is not known what concentration of proline is necessary to accomplish similar results in hydrated orthodox seeds, but, given that maize tissues imbibed for 30 minutes were hydrated to  $\pm$ 50%, it is estimated that comparable concentrations of proline would be required. For example, an alternative but equally adequate proline concentration used for cryoprotection of a recalcitrant species at water contents of 50% required as little as 633.05 $\mu$ g/ml or 5.5 M (Stanwood 1986). The quantities of proline detected in these hydrated orthodox tissues exceed the expected requirement for cryoprotection and provides a possible explanation for the survival of these hydrated embryos post-cryostorage.

From as early as 1971, Heber *et al.* indicated that proline may be only one of a range of amino acids which can afford protection, and Withers and King (1979)

have shown  $\gamma$ -amino butyric acid to confer cryoprotection upon maize cells. Both these amino acids could, therefore, be naturally cryoprotecting this tissue given the extensive quantities of free  $\gamma$ -amino butyric acid within the embryos (Fig. 4.6). The observed successes post-cryostorage, despite high water contents, could be attributed to the combined cryoprotective properties of proline (Figs. 4.11 and 4.12) and  $\gamma$ -amino butyric acid (Figs. 4.5 and 4.6). The other amino acids (Figs. 4.4, 4.8 and 4.10) and possibly other organic solutes such as the naturally occurring endogenous glucose within the embryo, could also contribute to protection during freezing. Effects of glycine on providing membrane stabilisation during chilling stress have also been documented (Peters *et al.* 1998) and, therefore, most certainly contribute to the cryoprotective role either in its unbound state or as part of LEA proteins known to confer membrane stabilisation and freezing tolerance. The degree of cryoprotection conferred on this tissue by each of these amino acids would need to be calculated and considered accumulatively. If, however, the optimal levels for total amino acid concentrations utilised as a cryoprotectant fell within the 2.5 - 25% levels, as for proline, adequate cryoprotection would be rendered by  $40244.7\mu\text{mol/ml}$ , which would be equivalent to  $\pm 40\%$ . Total amino acid values obtained in the embryos at 1.5 hours imbibition would therefore render effective cryoprotection. The total amino acid value in the dry embryonic axis (data not shown on graph so that values represented coincide with the data obtained for the leachate) was  $40443.3\mu\text{mol/ml}$ , which indicated that total amino acids present conferred the potential cryoprotection of up to 10 times greater than that of proline on its own.

As substrates are mobilised in response to increasing water content, the strategies of the tissue to counteract adverse effects are implemented and these are characterised by the multiple biochemical changes which are associated with imbibition (Bewley and Black 1994) and freezing tolerance (Gilmour *et al.* 2000). The release of glycine and subsequently proline, in response to imbibition therefore expedites membrane repair, and by 3.5 hours of imbibition this has already taken place, thereby explaining the ability of this tissue to withstand the effects of cryostorage. The implemented mechanisms allow the tissues to further improve their ability to

withstand cryostorage. This seems to be due to the reduction or reconstitution of the biomolecules with the membranes (Figs. 4.13 - 4.15). At the 0.5 hours imbibition time, however, membranes have not sufficiently reconstituted to totally withstand the effects of cryostorage, and hence, the reduction in vigour. At 3.5 hours, it appeared as if the biomolecules were reconstituted with the membranes and conferred protection against the subsequent effects of cryostorage.

The various sequences of biochemical events are postulated to occur in the absence of, or at very low concentrations of ATP i.e. between 0 and 3.5 hours imbibition (desiccation tolerant) and, proceed as the tissues become more hydrated (desiccation sensitive) and progressively more metabolically active. Therefore, at 3.5 hours imbibition (w/c  $\pm 58\%$ ) the embryonic axis had possibly reached the very early stages of desiccation sensitivity. For this reason cryostorage, post 3.5 hours imbibition, resulted in cryodamage.

In conclusion, the combined concentrations of proline,  $\gamma$ -amino butyric acid, glycine, glutamic acid and glucose {known cryoprotectants (Withers 1985)} are sufficiently high, and are therefore hypothesised to behave as natural cryoprotectants, but are only effective prior to the commencement of desiccation sensitivity. Whether axis meristematic tissue is considered metabolically active or not would appear to be dependent on the tissue developmental stages at specific hydration levels and by inference the sequence of biochemical reactions which have commenced.

## **Chapter Five                    The effect of age/storage on caryopsis vigour, viability and resilience to cryostorage**

### **5.1. Introduction**

The various means of storage and of treatment prior to storage can have very different effects on the desired prolongation of the viability of the seed (Cordova-Tellez and Burris 2002a). Specifically, stored seed is susceptible to ageing, genetic changes and microbial activity (Mycock and Berjak 1992; Cruz- Pérez *et al.* 2003). Whilst humans can control (to a certain extent) microbial activity, little can be done to retard natural ageing and genetic changes even when seed is stored under optimal conditions (Mycock 1990; Bajaj 1995).

One method of commercial seed storage, as discussed in Chapter One, includes the treatment of grain with a fungicide (e.g. Captan<sup>®</sup>) and the subsequent storage of grain in large 25kg bags in a cool shed. This method was employed by the seed producer from which the caryopses (*Hybrid 6363*) utilised in this study were purchased, and allowed for storage periods ranging from one month to five years i.e. this was carry-over seed. This particular method of storage is not regarded as optimal but is usually the method of storage employed by this seed producer.

It is also known that various changes occur under sub-optimal storage conditions which result in altered grain quality such as reduced germination capacity, increased micro-organism respiration, dry matter loss, visible moulding, and cooling aeration effects and, if efficacy of residual protectants is compromised, pest infestations result (Fleurat-Lessard 2002). The data presented in Chapter Four demonstrated that *Hybrid 6363* had aged in storage due to any one or a combination of the above mentioned factors in sub-optimal storage conditions. In addition to this, the caryopses were further stored in hermetically-sealed containers at 4°C for a subsequent year where further ageing damage had presumably occurred. The results for all previous studies were obtained on aged caryopses where damage incurred during imbibition was most likely exacerbated

by subsequent cryostorage. Freshly/newly harvested caryopses have been recommended as more suitable for long term germplasm preservation purposes (Walters *et al.* 2004). To ascertain the extent of the damage caused by ageing, new (*Hybrid 6321*) and aged (*Hybrid 6363*) caryopses are compared in this chapter.

The introduction of a new hybrid was required because the seed producer no longer produced *Hybrid 6363*. According to the seed producer, the best genetic match to *Hybrid 6363* was *Hybrid 6321*. Although the seed producer was prohibited from providing complete pedigree information they were able to provide the breeders codes showing how the crosses were obtained. They are as follows:

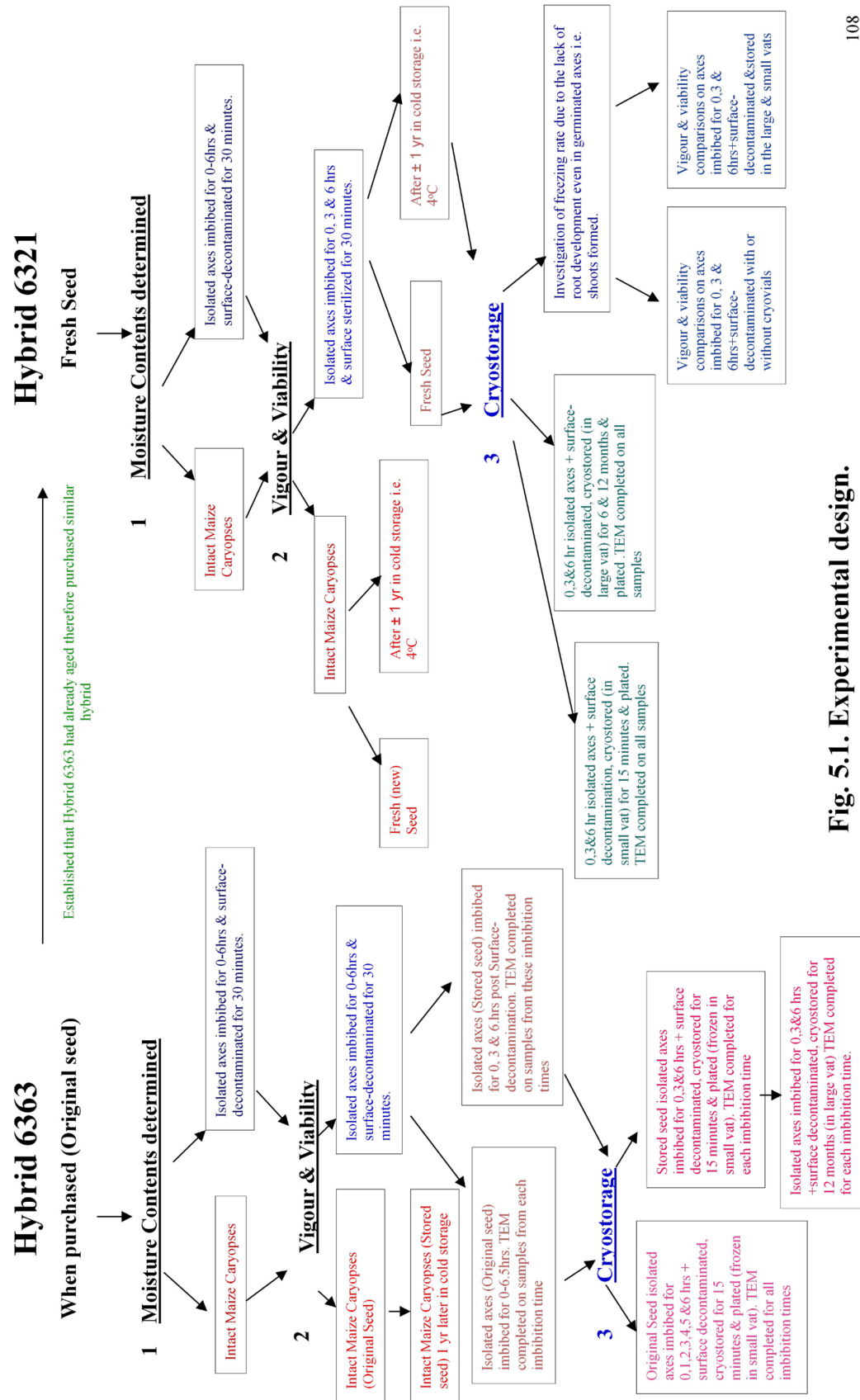
Pan 6321: 399 x 774 / 7196 x 507

Pan 6363: 774 x 507/ 399 x 22.

Usually a direct comparison of the performance of two genetically dissimilar hybrids would be avoided because any disparity arising from a specific treatment would necessitate consideration of genetic differences as well as the effects of the treatment. These crosses, however, demonstrate that at least 75% of the parent material was genetically the same, and because a comparison between stored and newly harvested caryopses would investigate whether ageing affected and exaggerated the effect of cryostorage, these hybrids were directly compared. The comparisons included water contents, biochemical constituents and ultra-structural analysis prior to and post-cryostorage.

## 5.2. Materials and methods

*Hybrid 6321* was selected as the comparison to *Hybrid 6363* due to similarities in size, shape, relative moisture contents and shared genetic material. They were both treated with the same fungicide (Captan<sup>®</sup>) at harvest. Both hybrids were selected by the seed producer because of their tolerance to drought and *Hybrid 6321* had replaced *Hybrid 6363*. The overall experimental design is represented in Fig. 5.1.



### 5.2.1. *Water content determinations*

Forty embryonic axes of *Hybrid 6363* were excised after their subjection to each of 0, 1, 2, 3, 4, 5 and 6 hrs of imbibition, and forty embryos of *Hybrid 6321* were excised after subjection to each of 0, 3 and 6 hrs of imbibition. The latter series of imbibition times was decreased as a result of the findings of the first. At 0 hrs, both hybrids had completed maturation drying. At 0.5 hrs the tissue had been surface-decontaminated. At 3.5 hrs it was presumed that desiccation tolerance was retained in the aged caryopses, and thereafter, at 6.5 hrs), became desiccation sensitive. Thus, these three times would provide data relevant to the full range of expected responses. The same procedures were then followed as described in 2.2.2 (see below).

The water contents, gravimetrically expressed on a fresh weight basis, of 40 individual embryos from each imbibition time, were determined post-surface-decontamination, and after drying for  $17 \pm 1$  hrs, in a ventilated oven at  $80^\circ\text{C}$  according to the modified ISTA Low Constant Oven Temperature Method (International Seed Testing Association 1985). The following equation was used to determine the fresh weight water content values:

$$\frac{\text{fresh weight} - \text{dry weight}}{\text{fresh weight}} \times 100$$

The water content values were expressed as a percentage. Each determination was performed three times.

### 5.2.2. *Vigour and viability assessments*

#### 5.2.2.a. *Intact caryopses*

Two hundred and fifty caryopses were placed into Petri dishes (10 caryopses per dish) for each hybrid and set to germinate on filter paper moistened with 10ml of

sterile distilled water. The caryopses were monitored for germination and growth every 24 hours. Emergence of the radicle served as an indicator of viability and the length of the radicle measured over time as an indication of vigour (rate of growth). The germination index (GI) (Czabator 1962) was determined as described in 2.2.3a.

#### 5.2.2.b. *Isolated embryonic axes*

Forty embryonic axes at imbibition intervals of 0, 3 and 6 hrs were excised and surface decontaminated, as previously described (2.2.2). This was undertaken using caryopses of *Hybrid 6363* at the time of purchase of the seed, after one year in cold storage (at 4°C) (hereafter referred to as original caryopses/tissues - all ultrastructural data presented in Chapter Three) and again after a further year in cold storage (hereafter referred to as stored tissues). This was repeated on caryopses of *Hybrid 6321* at the time of purchase and after  $\pm 1$  year in cold storage (4°C). The axes were then aseptically plated into tissue culture tubes with a standard Murashige and Skoog (1962) basal medium [see Appendix One], supplemented with 30 g.l<sup>-1</sup> sucrose, 2 g.l<sup>-1</sup> Gelrite R and equilibrated to a pH of 5.8. The material was incubated at 25°C with a 16:8 hrs light:dark photo period and at a light intensity of 200 E.m<sup>2</sup>.sec<sup>-1</sup>. Growth was monitored every 24 hours and observed for viability (number of axes germinated seen by the emergence of either the root, shoot or both) as well as vigour (rate of growth).

#### 5.2.3. *Cryostorage of isolated embryonic axes*

Forty embryos were excised after each imbibition time and surface decontaminated, as previously described (2.2.2). The axes were placed into cryovials (40 per vial) and frozen by plunging them directly into liquid nitrogen (temperature of -196°C). The liquid nitrogen ceased boiling after  $\pm 14$  seconds. After 15 minutes (short term cryostorage period) the axes in the cryovials were retrieved and thawed in a water bath of sterile water set at 30°C. The axes were plated into tubes with the MS basal medium (2.2.3. b) and tested for viability and vigour. Similarly-treated samples of *Hybrid 6363* were prepared and stored in liquid nitrogen for 12 months and *Hybrid 6321* for 6 and 12 months respectively. They were then thawed, plated and observed for vigour and viability.



#### 5.2.4. Biochemical analysis

Biochemical analyses were conducted as described in Chapter Four, section 4.2.5 on both hybrids. Conductivity data was not recorded for *Hybrid 6321*.

#### 5.2.5. Ultrastructural analysis

At the onset of this investigation, *Hybrid 6363* had been in cold storage for a year (original tissue - first TEM study conducted [Chapter Three, section 3.2.4]. After an additional year in storage, a second TEM study was completed on *Hybrid 6363*. For direct comparison, a control study was conducted on *Hybrid 6321*, where axes were prepared for TEM prior to and after each imbibition interval, and after cryostorage for 15 minutes and 12 months respectively (refer to Fig 5.1).

#### 5.2.6. Freezing protocol

The vigour and viability of isolated embryonic axes frozen in a large vat (Taylor Wharton 34 XT) and a small 1.5 litre flask shaped vat were compared. Samples frozen in the small vat were placed into four cryovials and mounted onto a cryocane. Each cryovial contained 40 embryos. The canes were plunged directly into liquid nitrogen and were frozen at a rate of greater than  $-200^{\circ}\text{C}.\text{min}^{-1}$  (boiling stopped within 14 seconds). The large vat of embryos (also in cryovials mounted in cryocanes) were frozen by placing the canes into a metal casing (maintained at  $-196^{\circ}\text{C}$ ). The casing was lowered directly into a larger vat filled with liquid nitrogen. The casing holding the cryocanes is presumed to have slowed the rate of freezing. These samples were maintained at  $-196^{\circ}\text{C}$  for 6 and 12 months respectively and the vat was filled regularly to ensure that samples were completely submerged in the cryogen.

#### 5.2.7. Data analysis

Water content data sets were Arcsine transformed to approximate normality and subjected to a one way ANOVA using Statistica 6.0 (Statsoft 2001). Tukey post

hoc comparisons were used to establish significant differences between each imbibition time. Viability data were analysed using contingency tables and Chi square tests using Instat version 3.0. (Graphpad software 2003). Germination indexes and percentage root formation data were analysed using Fisher's exact tests using Instat version 3.0. All biochemical data on amino acids and glucose were subjected to one way ANOVAs using Statistica 6.0 (Statsoft 2001). Tukey post hoc comparisons were used to establish significant differences between concentrations of biochemical molecules, at each imbibition time, in the leachate and the embryos for both *Hybrids 6363* and *6321*. Biochemical data obtained over different times, i.e. original tissue and stored tissue pre- and post-cryostorage, and for *Hybrid 6321* pre- and post-cryostorage, were subjected to a three way ANOVA using Statistica 6.0 (Statsoft 2001)' and, Tukey post hoc comparisons were used to establish significant differences between the two hybrids across treatments of tissue type, storage period and cryostorage.

### **5.3. Results and discussion**

#### *5.3.1. Water contents for aged and freshly harvested (new) caryopses.*

With each imbibition time for both aged and new caryopses, prior to and after cold storage, the water contents increased. When the responses to individual imbibition times were compared, it was found that the water contents between the 0 hrs sample for stored aged tissue and the freshly harvested tissue differed statistically. It was anticipated that the new tissue, on the whole, would be drier than the original tissue since an increase in overall seed water content is associated with ageing (Simon 1973; Walters 1998; Cruz- Pérez *et al.* 2003). However, in all hydrated samples, the new caryopses absorbed more water than the aged ones and even more so after a cold storage period. The water content of the stored aged tissue and the freshly harvested tissue after 3.5 hours of imbibition also differed statistically, again with the new caryopses more hydrated than the old (Table 5.1). This result may be indicative of new caryopses membrane status as a result of possible pre-treatments.

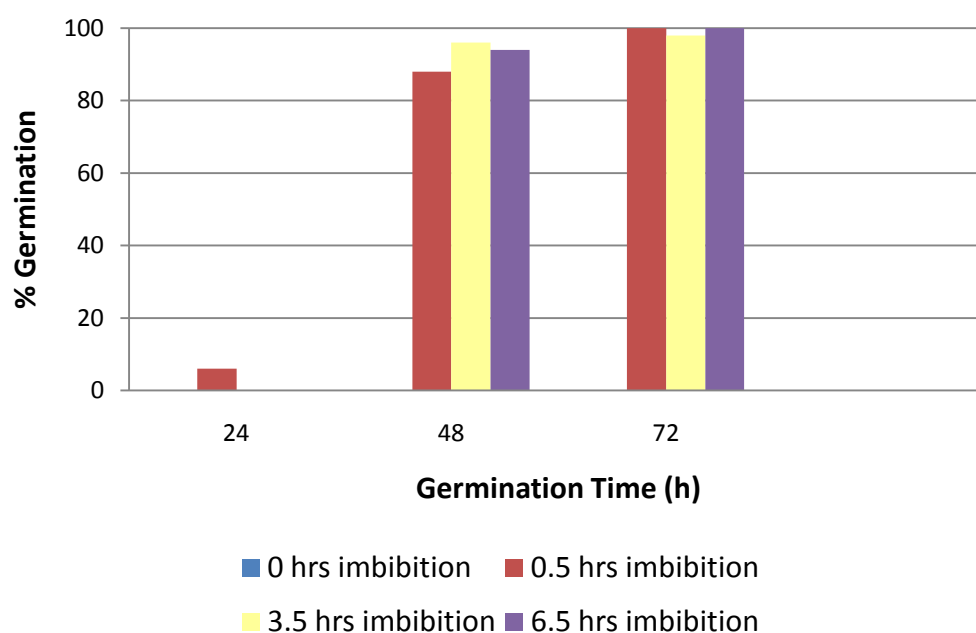
**Table 5.1. Percentage water content data for naturally-aged (*Hybrid 6363*) and newly-harvested (*Hybrid 6321*) maize caryopses.** Results for caryopsis type, water contents, imbibition time comparisons and their interactions with the same superscript alphabet are not significantly different ( $P < 0.001$ , Tukey post hoc test).

| Hybrid Type                          | Imbibition time (h) | % Water Content       |
|--------------------------------------|---------------------|-----------------------|
| <i>Hybrid 6363</i> (Original tissue) | 0                   | $12.4 \pm 0.8^{ab}$   |
|                                      | 0.5                 | $52 \pm 2.1^{ef}$     |
|                                      | 3.5                 | $58 \pm 3.5^{de}$     |
|                                      | 6.5                 | $62 \pm 10.2^f$       |
| <i>Hybrid 6363</i> (Stored tissue)   | 0                   | $15.4 \pm 1.7^b$      |
|                                      | 0.5                 | $55.8 \pm 4.3^{cd}$   |
|                                      | 3.5                 | $53.5 \pm 4.2^c$      |
|                                      | 6.5                 | $56.9 \pm 3.7^{cde}$  |
| <i>Hybrid 6321</i> (New tissue)      | 0                   | $8.66 \pm 0.96^a$     |
|                                      | 0.5                 | $56.74 \pm 3.9^{cde}$ |
|                                      | 3.5                 | $58.21 \pm 3.0^{def}$ |
|                                      | 6.5                 | $58.28 \pm 3.9^{def}$ |
| <i>Hybrid 6321</i> (Cold storage)    | 0                   | $13.7 \pm 1.2^{ab}$   |
|                                      | 0.5                 | $66.3 \pm 9.2^g$      |
|                                      | 3.5                 | $60.2 \pm 5.1^{ef}$   |
|                                      | 6.5                 | $62.2 \pm 7.6^{fg}$   |

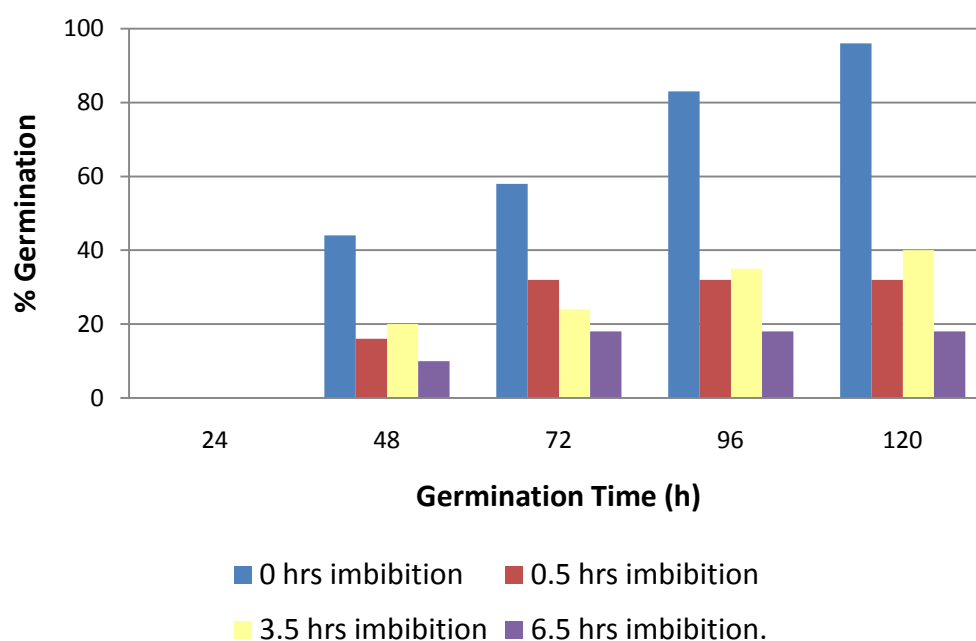
### 5.3.2. Vigour and viability

*Hybrid 6363* exhibited a reduction in vigour and viability and an increase in germination time indicating that these caryopses had aged (Begnami and Cortelazzo 1996; Cruz- Pérez *et al.* 2003). The vast majority of the isolated axes of new caryopses germinated within 72 hours (Fig. 5.2) and did not require the 120 hours needed by the majority of *Hybrid 6363* axes (Fig. 5.3). Additionally, after 72 hours, 99.6% of the developing seedlings germinated from intact caryopses and produced radicles longer than 5mm, indicating that the new caryopses were indeed more vigorous ( $\chi^2 = 4.045$ ,  $P = 0.399$ ). Although viability was maintained after each imbibition time (Fig. 5.4), the process of cryostorage appeared to impair vigour (Fig. 5.5) i.e. unimbibed cryostored axes all demonstrated high germination indices, but once imbibed, dropped in vigour by more than 60%. Hydration of the tissue was the primary cause of vigour loss. Subsequent freezing results in cryo-

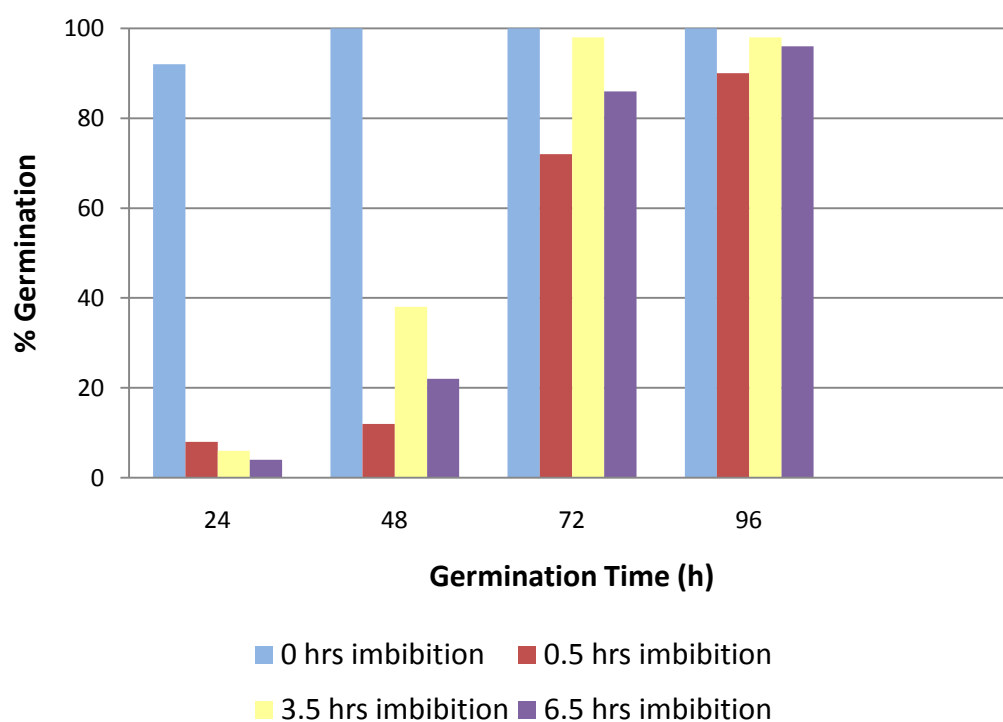
injury which is mostly incurred by ice crystal formation (Benson 2008). If this occurs extracellularly, i.e between cells, although it can be harmful, cells are still able to survive (Benson 2008).



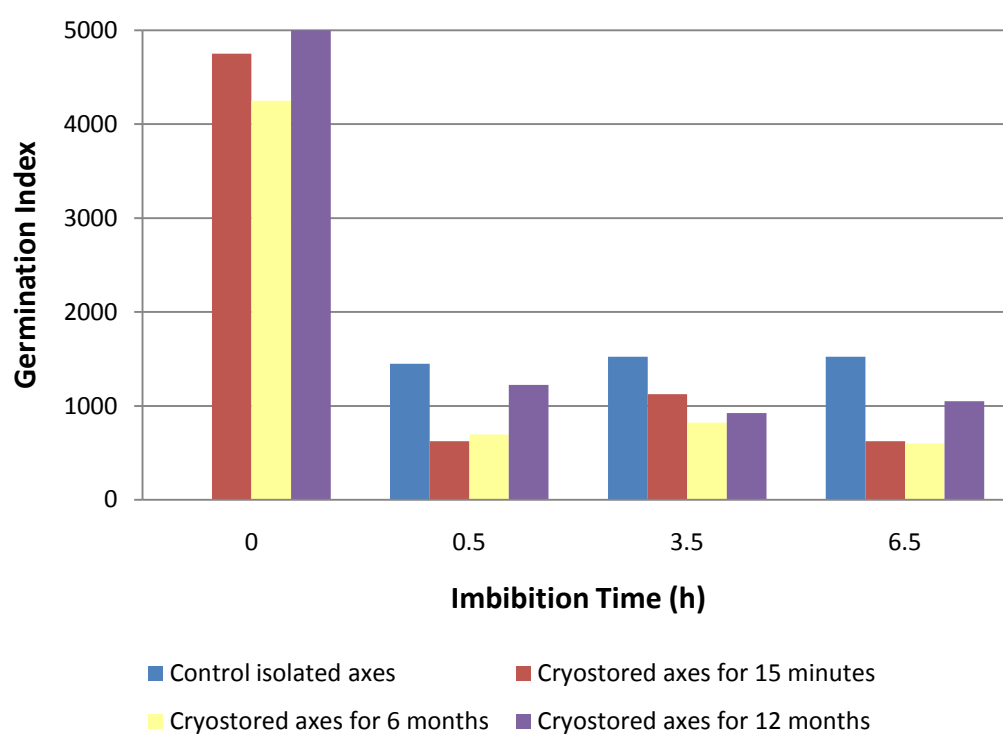
**Figure 5.2.** Viability of new caryopses (*Hybrid 6321*) isolated embryonic axes post-imbibition but prior to cryopreservation.



**Figure 5.3.** Viability of *Hybrid 6363* isolated embryonic axes post-imbibition and pre-cryostorage.



**Figure 5.4** Viability of *Hybrid 6321* isolated embryonic axes post-cryostorage.



**Figure 5.5** Germination indices for *Hybrid 6321* pre-and post-cryostored isolated axes. The cryostored samples include a 6 and 12 month long term cryostorage period.

Intracellular ice crystal formation however, is almost always lethal (Benson 2008). The removal of freezable water from the cytoplasm (Crowe *et al.* 1990) caused by submersion in liquid nitrogen (i.e. desiccation effect) also results in the concentration of solutes which results in colligative damage (Benson 1994, 2008). These combined factors could be contributing to the loss of viability after cryostorage. The differences observed within the various cryostorage durations are therefore dependent on the initial water contents when frozen.

### *5.3.3. Ultrastructure of Hybrid 6363 (original tissue and stored tissue) pre- and post- short-term cryostorage*

The two most distinguishing and prominent features, observed when comparing cells of the original tissue with those of the stored tissue (irrespective of imbibition time pre-or post-cryostorage), were: (1) the presence of lipid bodies aligned immediately beneath the plasmamembrane or scattered throughout the cytoplasm, and (2) the accumulation of vesicles/vacuoles in response to maturation drying, subsequent rehydration and cryostorage (see below for brief overview).

#### *5.3.3.1. Lipid bodies in seed embryo tissues - a general overview*

Lipid bodies, first observed scattered throughout the cytoplasm in radicle meristems of maize caryopses, migrate towards the plasmamembrane during artificial and field drying (Perdomo and Burris 1998). Although the mechanism(s) of alignment are not fully understood (Cordova- Tellez and Burris 2002a), it is thought that cell water loss might be associated with the movement of lipid bodies towards the cell wall i.e. during drying. As water leaves the cells, lipid bodies may move with it until they reach the plasma membrane (Cordova-Tellez and Burris 2002a). However, other possible processes cannot be disregarded. One such process could be that the cytoskeleton mediates the movement of the lipids to the plasmamembrane and, although actin-mediated movement of lipids has been described in many animal systems (Siddiqui *et al.* 1989; Rabello and Ludescher 1998) and in plasmodesmata plasmamembranes (Ding *et al.* 1992), it is possible that the cytoskeleton plays a role in mediating the movement of lipid bodies towards the plasmamembrane in the root meristems of maize.

Lipid bodies, therefore, migrate towards and align themselves by specific means along the plasmamembrane during drying, and play a very important role in the regulation of water loss during the process of maturation drying (Cordova-Tellez and Burris 2002a). Alignment of lipid bodies along the plasmamembrane is also associated with low cell solute leakage upon rehydration and enhanced germination (Peterson 1997; Perdomo and Burris 1998; Cordova-Tellez and Burris 2002a). Impairment of lipid body alignment caused by rapid mechanical drying rates, a commonly employed procedure by many seed producers (Cordova-Tellez and Burris 2002b), results in a reduction in overall seed quality i.e. reduced seed vigour (Cordova-Tellez and Burris 2002b; Samarah 2007), cell plasmolysis and coalescence of lipid bodies (Peterson 1997), and the ability of the seed to effectively acquire desiccation tolerance (Cordova-Tellez and Burris 2002b; Samarah 2007). If seeds, however, are dried slowly, lipid body alignment occurs progressively from the periphery of the radicle to the inner cortex cells in the embryo and result in a more organised dehydration during seed drying (Cordova-Telliz and Burris 2002a). This might be crucial in acquiring desiccation tolerance (Cordova-Tellez and Burris 2002b; Samarah 2007). Ultrastructural comparisons of orthodox embryos *Phaseolus vulgaris* and recalcitrant embryos of *Avicennia marina* revealed that lipid body alignment was evident in dry radicle meristems of the orthodox species but absent from the recalcitrant one (Farrant *et al.* 1997). This was also shown to be common in many other orthodox seed (Dasgupta *et al.* 1982; Leprince *et al.* 1990; Lyshed 1992; Misra *et al.* 1993). However, Cordova-Telliz and Burris (2002a) were the first to refer to lipid body alignment mechanisms and their participation in the acquisition of desiccation tolerance.

### 5.3.3.2. *Vacuoles/Vesicles in seed embryo tissues - a general overview*

Vacuoles and/or vesicles were observed throughout the studied tissue, pre- and post imbibition for 6 hours, as well as post-cryostorage, and, because vacuoles (protein storage vacuoles (PSV's) or vesicles (involved in the transport of biomolecules from the golgi apparatus or endoplasmic reticulum to the vacuoles (Hara-Nishimura and Matsushima 2003)) could not with certainty be distinguished, the term vesicles/vacuoles will be used. Storage protein bodies are deposited into protein storage vacuoles (PSV's) and occur as a natural consequence of the processes of plant seed development and maturation drying (Hiraiwa *et al.* 1993; Paris *et al.* 1996; Jiang *et al.* 2001; Neuhaus 2007) and more specifically in maize (Fransz and Schel 1991; Perdomo and Burris 1998). Vesicle formation also occurs from the endoplasmic reticulum in response to environmental stresses which are then transported to protein storage vacuoles (Hara-Nishimura and Matsushima 2003). These protein storage vacuoles found in orthodox desiccation tolerant seeds have been described as numerous small vacuoles rather than one central large vacuole (Pernollet 1978). Similar in appearance to the vacuoles found in desiccation tolerant seed are those formed in resurrection plants in response to dehydration, and their function has been reported as replacing volume occupied by water in the hydrated state (Van der Willigen *et al.* 2004). This water replacement strategy has been proposed to reduce the mechanical stress on the cellular substructure during dehydration (Farrant 2000), with numerous smaller vacuoles thought to be more mechanically stable than one large vacuole in desiccated tissues (Van der Willigen *et al.* 2004).

During dehydration, numerous metabolites in addition to proteins, such as proline and sucrose, accumulate in the small vacuoles within vegetative tissue (Van der Willigen *et al.* 2004) and stably accumulate to high levels within seeds (Jiang *et al.* 2001). Subsequently, during germination the proteins are rapidly degraded to provide nutrients for use by the embryo (Jiang *et al.* 2001) and these complex organelles demonstrate a very organised structure whereby their storage functions are separate from their lytic functions i.e. storage and lytic functions are partitioned within the same vacuole (Paris *et al.* 1996; Jiang *et al.* 2001 and Van der Willigen 2004). This information becomes pertinent to the occurrences in early hydration of maize embryos as well as their subsequent cryostorage, and,



although the formation of these maize vacuoles/vesicles are observed here, their contents have not been investigated, and their importance in the reduction of mechanical stress is, for this particular tissue, unclear. However, their presence and condition as observed from the ultrastructural results provide insight to the effects of cryostorage subsequent to rehydration.

The sample subjected to 0 hours of imbibition (Fig. 5.6a), i.e. unimbibed and not cryostored, was typical of the tissue in the desiccated state, as described by Berjak and Villiers (1972a). The plasmamembranes were withdrawn from the cell wall, which enclosed a dense, non-metabolically-active cytoplasm, as seen by electron translucent mitochondria in Fig 5.6a. Lipid molecules were situated immediately beneath the plasmamembrane, as would be expected in dry tissue. Cryostorage of this tissue resulted in similar cellular profiles (Fig. 5.6b). The plasmalemma of aged tissue appears more fragmented (Fig. 5.6c) than in fresh seed (Fig. 5.6a), and the cell walls more buckled. Such visible distortions in the plasmamembrane were also described for maize tissues subjected to accelerated ageing (Berjak and Villiers 1972a). *Hybrid 6363* had already aged prior to the purchase of the seed (and the evidence of having aged was more prominent in tissue after imbibition, i.e. exhibited lobed nuclei, more prominent vacuole formation and lipid coalescence). It is assumed that a further year of cold storage resulted in further ageing evidenced by fragmented plasma membranes, lobed nuclei and vesicle formation. Subsequent cryostorage would therefore have exacerbated the effects of ageing as indicated by the coalescence of the lipid molecules (Fig.5.6d) which were no longer restricted to the periphery of the cell but were scattered throughout the cytoplasm. The presence of vacuoles/vesicles with some electron dense particulate inclusions were noted (Fig. 5.6d) when compared with the cryostored tissue of the original tissue (Fig. 5.6b). Tissues cryostored for 15 minutes were compared ultrastructurally to aged tissue and subsequently cryostored for 15 minutes. Surface-decontamination and washing hydrated tissue had more water relative to imbibition time for both original tissue (effectively imbibed for 0.5 hrs) (Fig. 5.7a) and stored tissue (Fig. 5.7c). However, although the water contents were not significantly different, the tissue was markedly different in appearance.

**Figure 5.6a** Original seed (0 hrs unimbibed), pre-cryostored tissue exhibited plasmamembranes (**pl**) withdrawn from the cell wall (**cw**) which enclosed a dense cytoplasm (**c**). The plasmamembranes remained attached to the cell walls at the plasmodesmata (**pld**). Lipid (**l**) molecules were situated immediately below the plasmamembrane. Vacuoles (**v**) were present and appeared electron dense, possibly protein storage vacuoles (PSV) Scale bar: 1cm=2.2µm

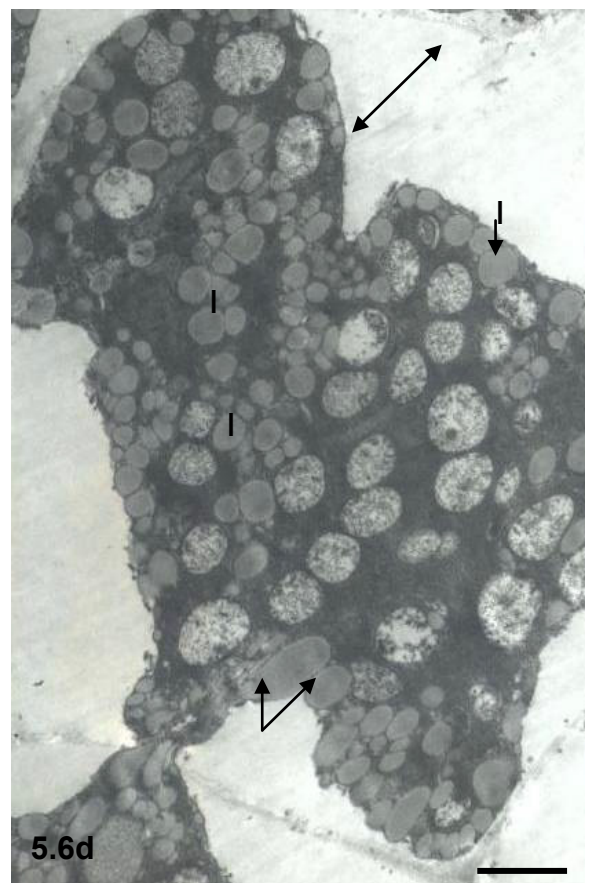
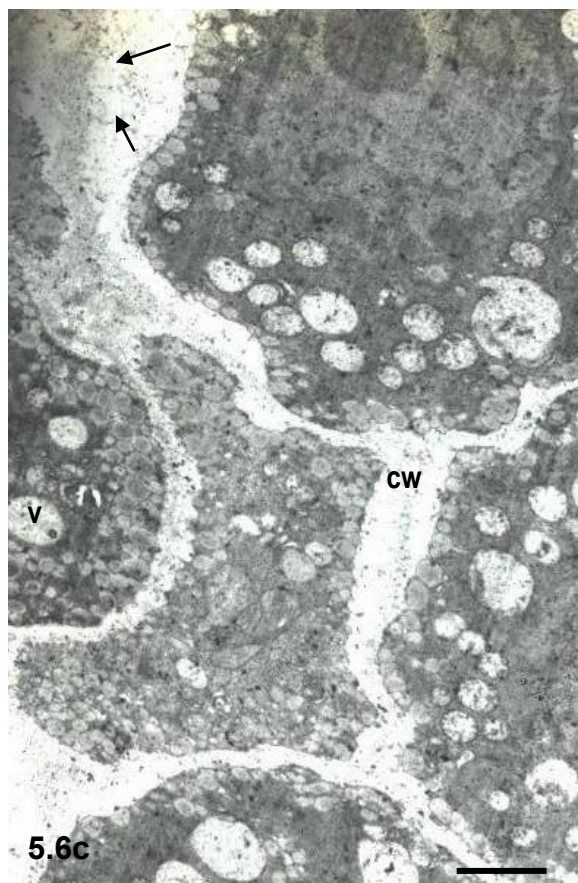
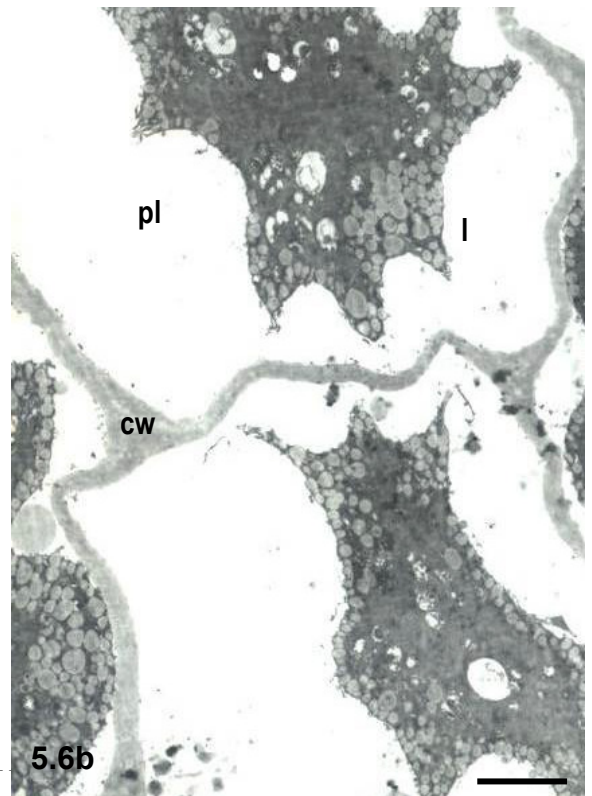
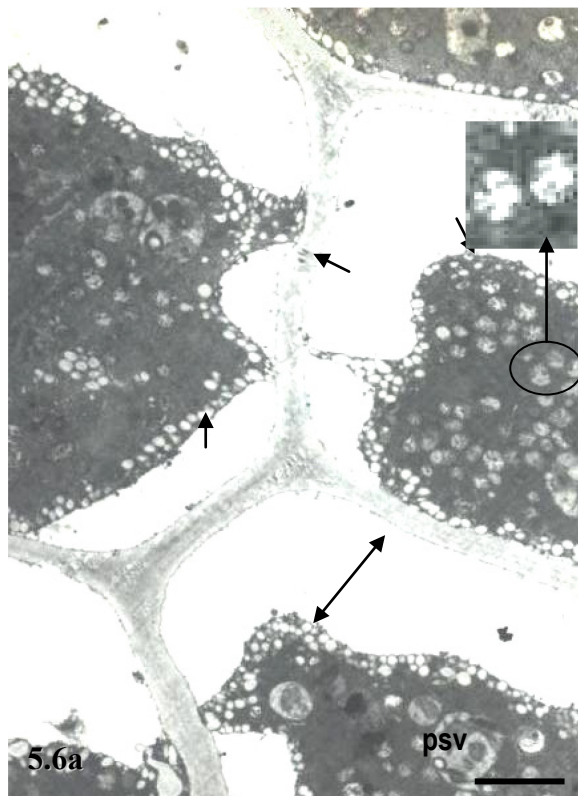
**Figure 5.6b** Original seed, (unimbibed), post-cryostored tissue was very similar in appearance to the pre-cryostored tissue. Scale bar: 1cm=2µm.

**Figure 5.6c** Stored seed (0hrs unimbibed) tissue demonstrated a more fragmentary plasmamembrane (**pl**) with buckled cell walls. Tissue appeared vacuolated (**v**) Scale bar: 1cm=0.2µm.

**Figure 5.6d** Stored seed (0hrs unimbibed), post-cryostorage. Lipid molecules (**l**) were no longer observed only at the periphery but appeared to have coalesced and scattered throughout the cytoplasm (**c**). Electron dense vacuoles (**v**) were observed throughout the cytoplasm. Scale bar: 1cm=0.15µm.<sup>2</sup>

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<sup>1</sup>For ease of access Fig. 5.6e follows after text describing long term cryostorage (12 months) results



Membrane bound vesicles/vacuoles observed in the aged tissue (Fig. 5.7c) contained more electron dense material than in the original tissue (Fig. 5.7a) and the nuclear envelopes lacked definition (Fig. 5.7c). In both original and aged tissue pre-cryostorage (Fig 5.7a and Fig 5.7c) resulted in the cytoplasm expanding to fill the entire cell and the lipid, although it remained peripheral in the original tissue (Fig. 5.7a), appeared diffuse in the aged stored tissue (Fig. 5.7c).

It has been shown by Cordovo-Tellez and Burris (2002a and 2002b), that lipid molecules aligned immediately below the plasma membrane are indicative of superior seed quality and possibly provides an indication that desiccation tolerance is maintained. In the absence of lipid alignment below the plasma membrane, the seed could have started to lose physiological integrity, including desiccation tolerance due to age. Chabot and Leopold (1982) suggested that large amounts of lipids and membrane vesicles in close proximity to the plasmamembrane may serve as a necessary source of additional membrane lipid during the initial process of imbibition. Numerous membrane-bound vesicles/vacuoles were observed in both samples for this imbibition time (0.5 hrs) and, as with the lipid deposits, their appearance may mark the beginning of membrane repair (Wu *et al.* 2001). In experiments where tissue age was accelerated (Berjak and Villiers 1972c) it was observed that even in aged caryopses, membrane repair occurred. It is hypothesised that the appearances of vesicles/vacuoles in older tissue serve as a source of stored reserves for membrane repair to occur, in the natural sequence of events upon imbibition.

Freezing tissue at water contents of  $\pm 52 - 60\%$  w/c resulted in a possible desiccation effect (Fig. 5.7b) (Crowe *et al.* 1990), i.e. the plasmamembrane, at various points of the cell wall plasmamembrane interface were separated, which occurs when water is removed from the cell. This “drying” effect was demonstrated in the original tissue cryostored for 15 minutes (Fig. 5.7b) but was not visible in the stored tissue cryostored for 15 minutes (Fig. 5.7d), possibly because an increase in water content occurred during storage, due to age, causing re-expansion of the plasmamembrane to the cell wall, and subsequent freezing did not result in as great a desiccation effect as observed in the original tissue.

**Figure 5.7a** Original seed tissue surface-decontaminated (0.5 hrs) demonstrated a cytoplasm expanded to the cell wall (**cw**) and lipids (**l**) remained peripheral. The nucleolus (**nu**) was spherical and clearly visible. Scale bar=2.4 $\mu$ m.

**Figure 5.7b** Original seed tissue surface-decontaminated tissue (0.5 hrs) post-cryostorage. Plasmamembrane (**pl**) had withdrawn from the cell wall (**cw**) possibly due to the dehydrating effect of the freezing process. Lipids (**l**) remained peripheral. The nucleolus (**nu**) was also spherical and clearly visible. Scale bar=2.1 $\mu$ m.

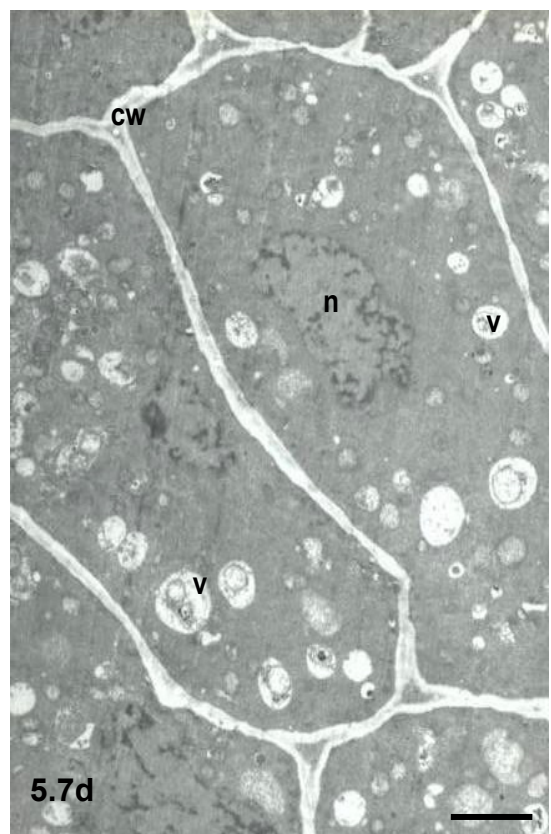
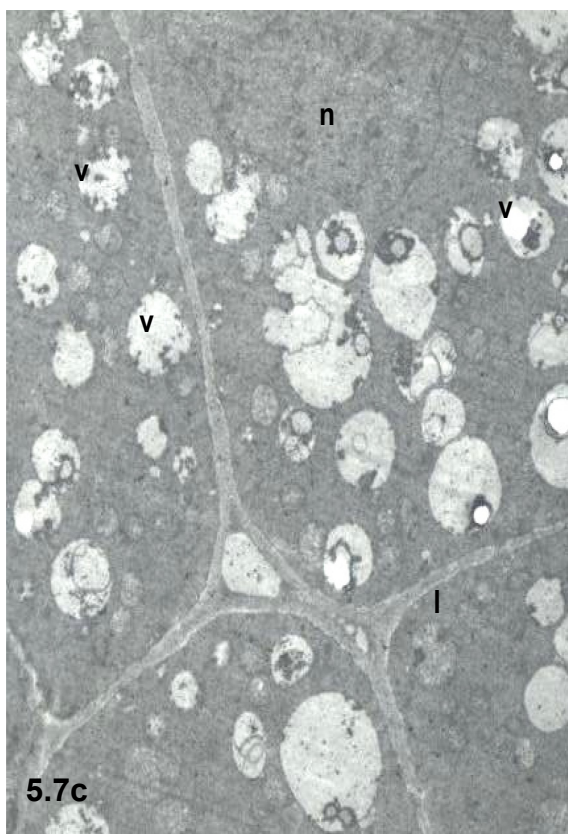
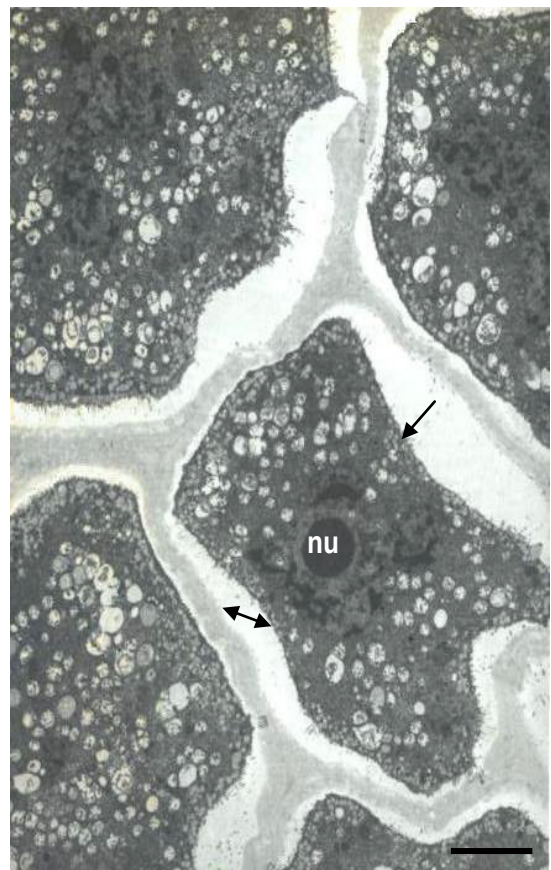
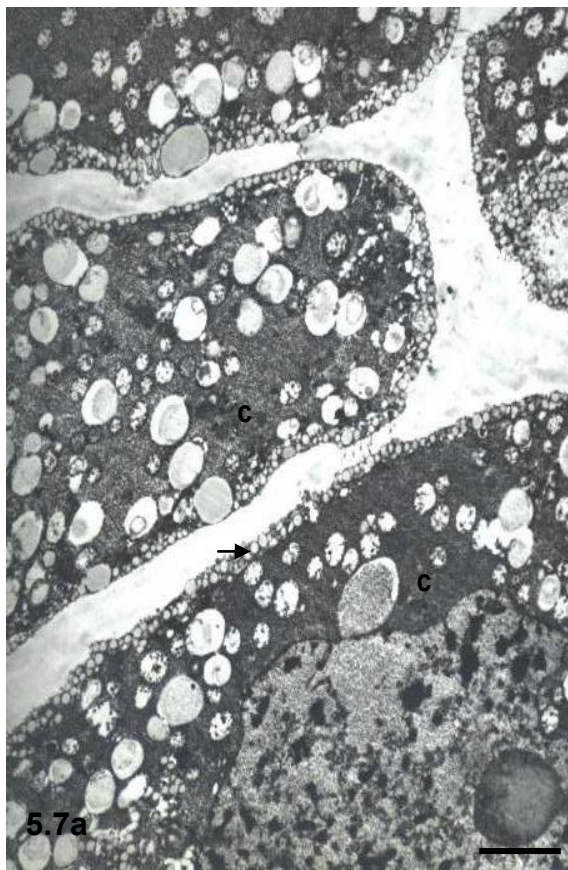
**Figure 5.7c** Stored seed tissues (0.5 hrs) surface-decontaminated. Cytoplasm had expanded to the cell wall (**cw**). The nuclear envelope, although visible, lacked definition and a greater amount of membrane bound vesicles (**v**) were observed. Scale bar=1.7 $\mu$ m.

**Figure 5.7d** Stored seed tissue (0.5 hrs) surface-decontaminated, post-cryostorage. The dehydrating effect observed in the original cryostored sample (Fig 5.7b) was not exhibited in this sample. The cytoplasm remained expanded to the cell wall (**cw**). Scale bar=2.1 $\mu$ m.<sup>3</sup>

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<sup>2</sup>For ease of access Fig. 5.7e follows after text describing long term cryostorage (12 months) results





A greater number of larger vesicles/vacuoles were observed in the stored tissues post-cryostorage, suggesting that age and subsequent cryostorage tissue possessed the resources and initiated the formation of vesicles/vacuoles. This was, therefore, a stress response to cryostorage as discussed by Hara-Nishimura and Matsushima (2003) and would ultimately participate in membrane repair rather than protection.

After 3.5 hours imbibition, the obvious differences between original (Fig. 5.8a) and stored tissue (Fig. 5.8c) were the amount of the vesicles/vacuoles present in the cytoplasm as well as the observation that in original tissue, the vesicles/vacuoles appeared to be electron translucent, whereas, in stored tissue the vesicles/vacuoles appeared to have electron dense particulate inclusions. These inclusions possibly contain protein bodies or the products resulting from damage due to age, and are localised in these vacuoles. These structures were more abundant in stored tissue and almost always associated with the lipid molecules immediately beneath the plasmamembrane (Fig. 5.8c), presumably readily available for membrane repair. Original cryostored tissue, once again, demonstrated freezing desiccation (Fig. 5.8b) as well as buckled cell walls, which were not seen in the stored cryostored tissue (Fig. 5.8d). This response was possibly due to the swelling of the cytoplasm which occurred as a natural consequence of imbibition, as described by Chabot and Leopold (1982). Swelling occurred to a greater extent in aged tissue, because these aged caryopses had a higher water content prior to imbibition (Table 5.1). It is further suggested that the dehydrating effects of the freezing process were not as extensive as that observed in original tissue. The greatest percentage of normal regenerated plantlets was recovered for both the original and stored caryopses at this imbibition time. A feasible explanation for the success in plantlet recovery post-cryostorage, notwithstanding the water content and age, was that the embryonic axis tissue had retained desiccation tolerance.

Tissue allowed to imbibe for 6.5 hours seemed typical of original tissue (Fig. 5.9a). However, the effects of ageing were evident in stored tissue (Fig. 5.9c).

**Figure 5.8a** Original seed tissue hydrated for 3.5 h demonstrated electron dense

vesicles (**v**) with the cytoplasm (**c**) completely expanded to the cell wall (**cw**). Scale bar = 0.9 $\mu$ m.

**Figure 5.8b** Original seed tissue hydrated for 3.5 h post-cryostorage exhibited freezing desiccation (see  $\leftrightarrow$ ) as well as buckled cell walls (**cw**). Scale bar = 2.1 $\mu$ m.

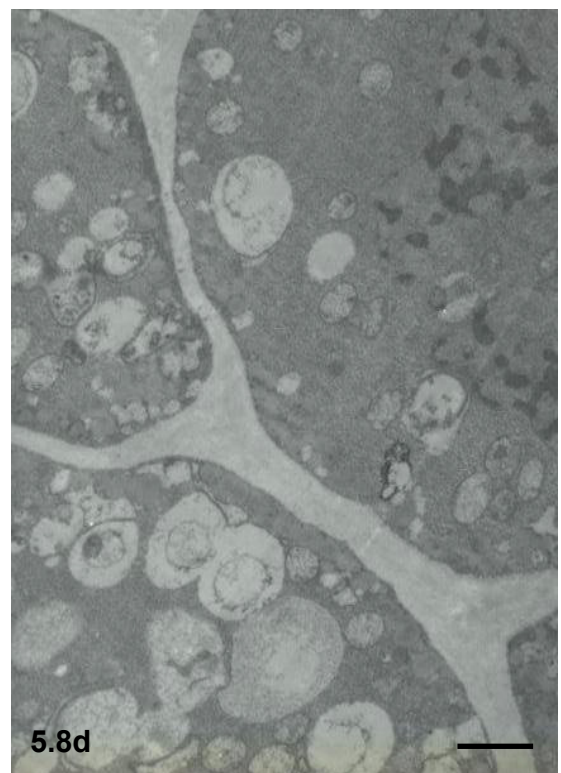
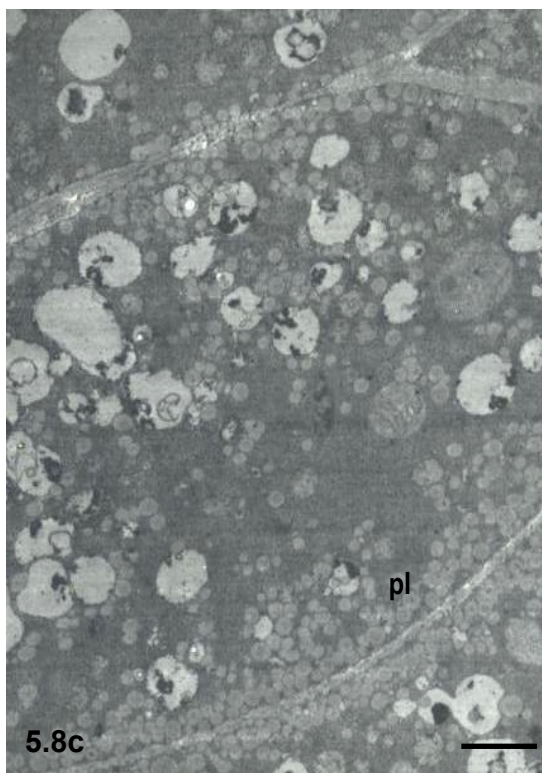
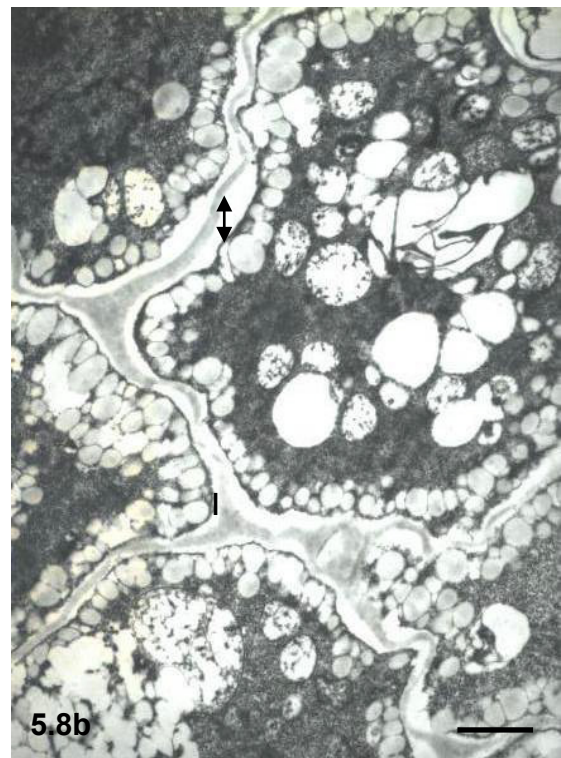
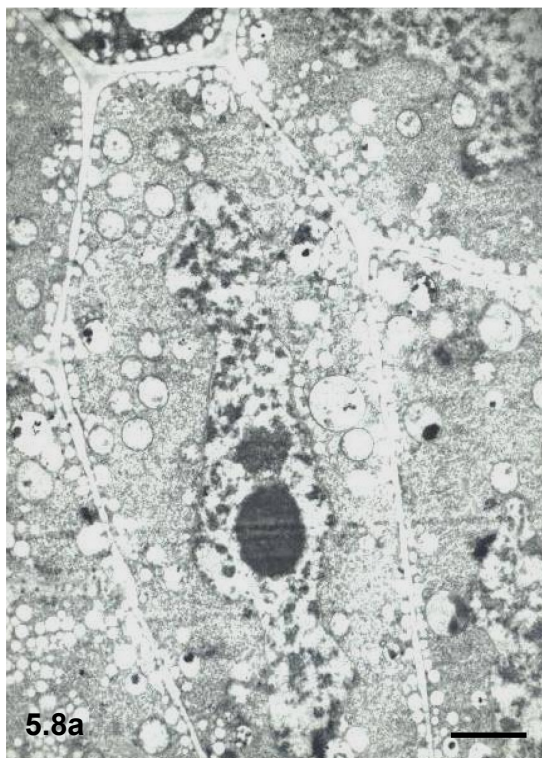
**Figure 5.8c** Stored seed tissue hydrated for 3.5 h- a larger amount of vesicles (**v**) were observed in this tissue when compared to the original seed tissue and they were almost always associated with the lipid (**l**) molecules immediately beneath the plasmamembrane (**pl**). Scale bar = 1.6 $\mu$ m.

**Figure 5.8d** Stored seed tissue hydrated for 3.5 hrs post-cryostorage did not exhibit buckled cell walls (**cw**), demonstrated a similar degree of vesiculation (**v**) but no dehydration effect was observed. Scale bar = 1.2 $\mu$ m.<sup>4</sup>

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<sup>3</sup>For ease of access Fig. 5.8e follows after text describing long term cryostorage (12 months) results





It appeared that the effects of extended imbibition times past 3.5 hours were exacerbated in *Hybrid 6363* that had been in cold storage for one year. A possible explanation for this observation was that membrane integrity had been compromised. Additional cryostorage on this tissue proved to be more deleterious in stored tissue. For example, pyknotic nuclei (irreversible condensation of chromatin in the nucleus of a cell undergoing programmed cell death or apoptosis), extensive cell wall buckling, and an observed increase in vesicle/vacuole number were evidenced (Fig. 5.9d). The original caryopses, however, (Fig. 5.9b) also demonstrated some damage i.e. distended endoplasmic reticulum, enlarged nuclei and some vesicles/vacuoles (Fig. 5.9b), but the observed damage was not as extensive as the stored cryostored tissue (Fig 5.9d). Some of the observed damage could have been due to a change in the rate of freezing, caused by using two different vats (see below) in the more hydrated tissue as proposed by Wesley-Smith *et al.* (2001). As mentioned in Chapter One, the rate of freezing could markedly impact hydrated tissue post- thawing and therefore, affect plantlet recovery (see below).

#### 5.3.4. Long term cryostorage of hybrid 6321

##### *Vigour and viability*

*Hybrid 6321* prepared for long term cryostorage was retrieved from the cryogen after 6 and 12 months. Viability studies conducted on the material revealed that although the tissue was viable, a large percentage of the originally hydrated axes developing into plantlets failed to produce roots (Fig. 5.10). For the 0.5 hrs and 6.5 hrs samples respectively only 10% of the axes produced roots and for the 3.5 hrs sample only 15% of the axes produced roots.

An explanation for the lack of root development post-cryostorage is incongruity in the rates of freezing. Discrepancies in rate of freezing could have occurred in one or both of two occasions, viz. ① the use of cryovials could have reduced freezing rates, and ② the use of two different vats could have resulted in different freezing rates. Firstly, the possibility that cryovials reduced freezing rate was investigated. Tissue frozen directly in liquid nitrogen, using a wire mesh basket, showed no significant difference in viability (Fisher's exact test at  $P=0.05$ ) to those frozen in

cryovials (Fig. 5.10). Hence, change in the rate of freezing due to cryovials is inconsequential when compared with the rate at which tissue is frozen when in direct contact with liquid nitrogen.

The second possibility for the change in the rate of freezing was due to the different vats utilised for the short term and long term cryopreservation studies. Rates of freezing and thawing are imperative to the survival of the tissue (Benson 2008) and this can be species specific (Stanwood and Bass 1981). This variable had to be accounted for and upon re-examination of the parameters a discrepancy was noted. The short term cryostorage studies i.e. 15 minutes, were administered in a small vat (1.5 litres) and the cryovials in the cryocanes were in direct contact with the cryogen, whereas, the long term studies were administered in a large vat (30 litres), where the canes were placed into metal baskets first and then submerged into the cryogen. A repeat experiment was conducted where axes in cryovials were frozen in the small and large vats, and their vigour, viability, germination indexes and presence or absence of root formation recorded (Fig. 5.10 & Fig. 5.12).

Viability appeared to be maintained for the isolated axes frozen in the small vat, with a percentage of the plantlets developing roots within the first 96 hours of growth. The greatest percentage of root formation occurred in the dry and the 3.5 hrs imbibed samples (Fig. 5.12.). For the samples frozen in the large vat, none of the imbibed tissue developed roots (Fig. 5.13) but, all of the unimbibed samples gave rise to roots. The failure of these samples to form roots was possibly due to the slowing in the rate of freezing caused by the introduction of the metal casing (refer to 5.2.6). Successes in cryostorage were also determined by the initial sample water content (Wesley-Smith *et al.* 2001), i.e. natural hydration levels obtained by tissue post maturation drying and prior to any exogenous processes. The changes in rate of freezing due to the increased water contents in the more imbibed tissue, combined with the introduction of the metal casing, contributed to the observed decline in root formation of these samples (Fig 5.13). Ultrastructural studies however, provided additional information for the lack of root development in freshly harvested cayopses. Tissues from both hybrids were directly compared, to determine if changes were due to age or cryostorage or both.

**Figure 5.9a** Original seed tissue imbibed for 6.5 hours possessed a fully expanded cytoplasm (**c**), peripheral lipid (**l**) and mitochondria (**m**) were distinct Mitochondria appeared to have more developed cristae possibly initiating ATP metabolism. Scale bar=0.2 $\mu$ m.

**Figure 5.9b** Original seed tissue imbibed for 6.5 hours post-cryostorage exhibited some damage, for example distended endoplasmic reticulum (**er**), an enlarged nucleus (n) and the appearance of some vesicles (**v**). Scale bar=1.4 $\mu$ m.

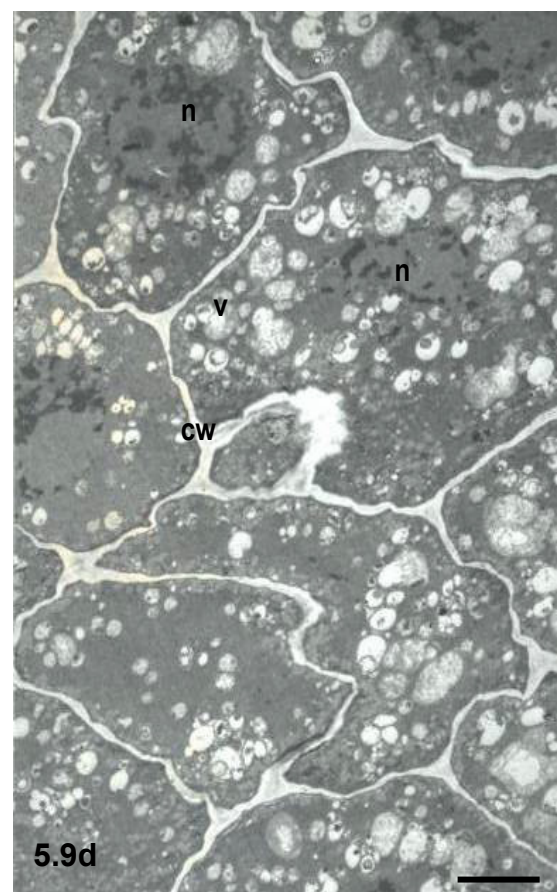
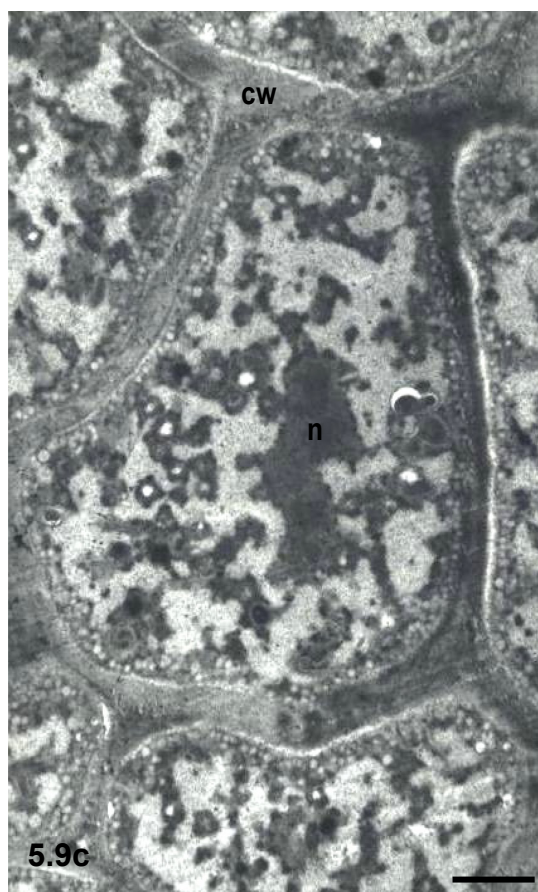
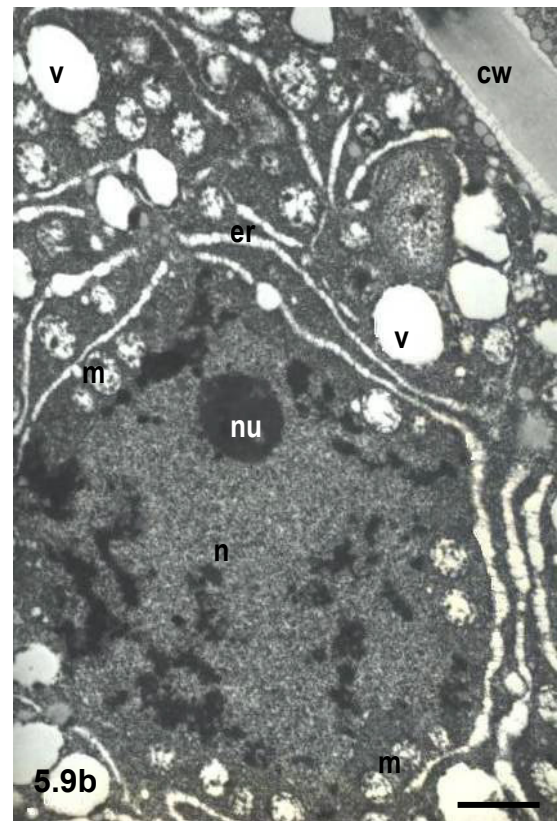
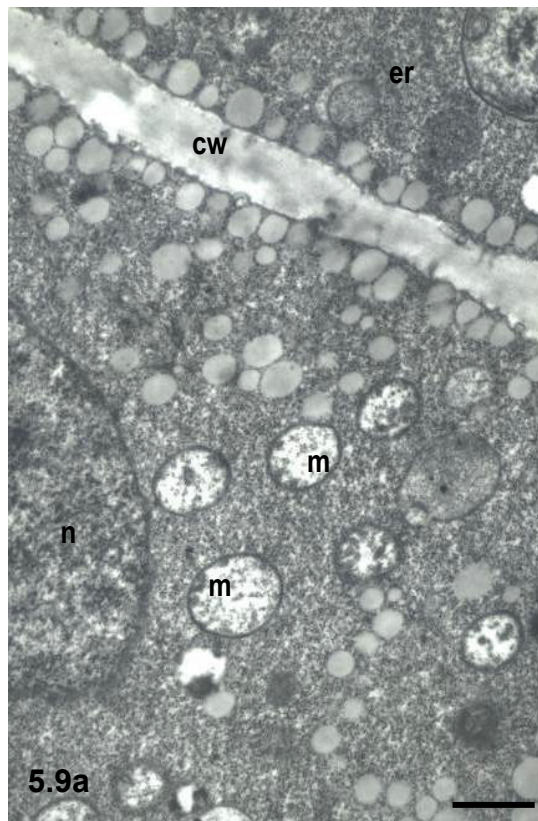
**Figure 5.9c** Stored seed tissue imbibed for 6.5 hours. Visible effects of ageing were seen. The nuclear envelope was completely degraded. Although extensive lipid molecules were present at the periphery of the cell, membrane integrity was compromised. The cytoplasm was void of defined organelles. Scale bar=2.0 $\mu$ m.

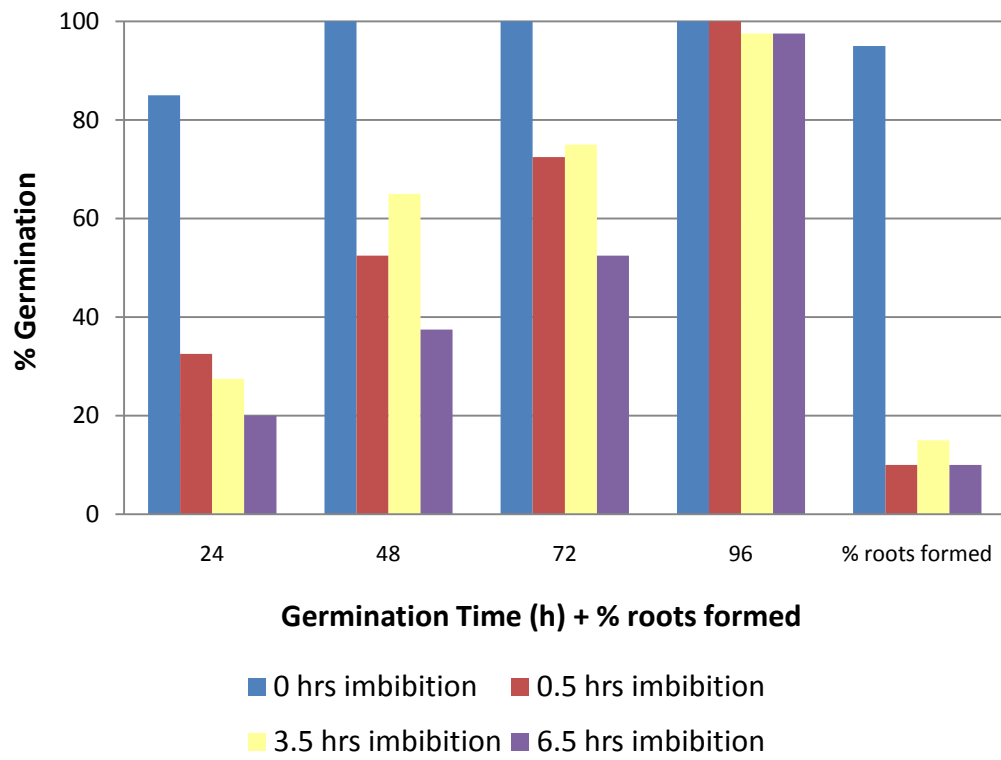
**Figure 5.9d** The effects of cryostorage on stored seed tissue imbibed for 6.5 hours proved to be more deleterious than the original seed tissue post-cryostorage (Fig. 5.9b). Nuclear membrane integrity was completely compromised, cell walls had buckled and large amounts of vesicles were observed. Scale bar=3.2 $\mu$ m.<sup>5</sup>

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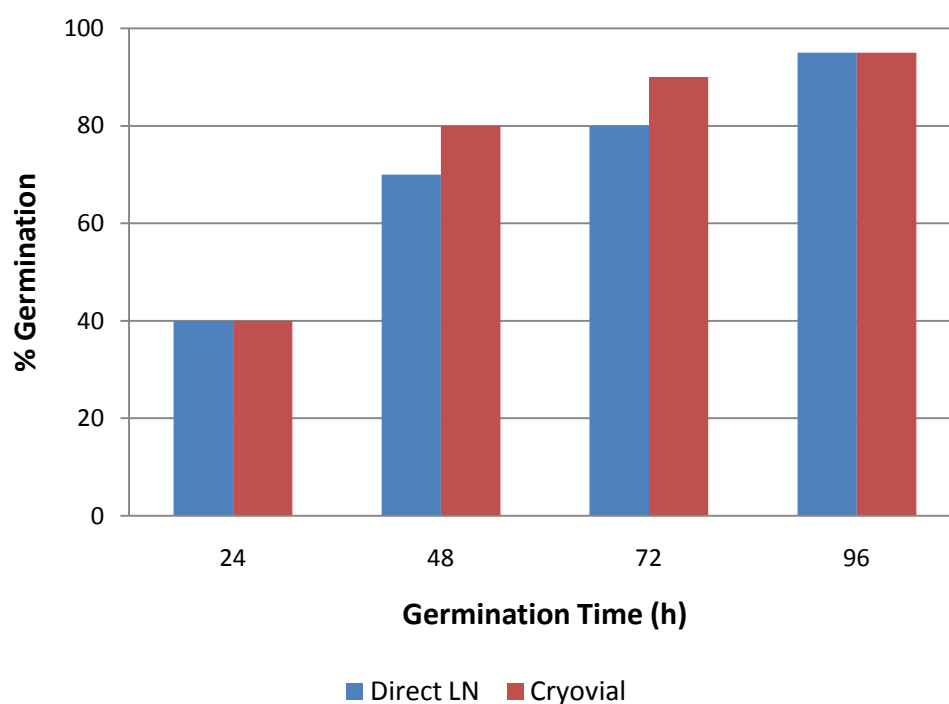
<sup>4</sup>For ease of access Fig 5.9e follows after text describing long term cryostorage (12 months) results



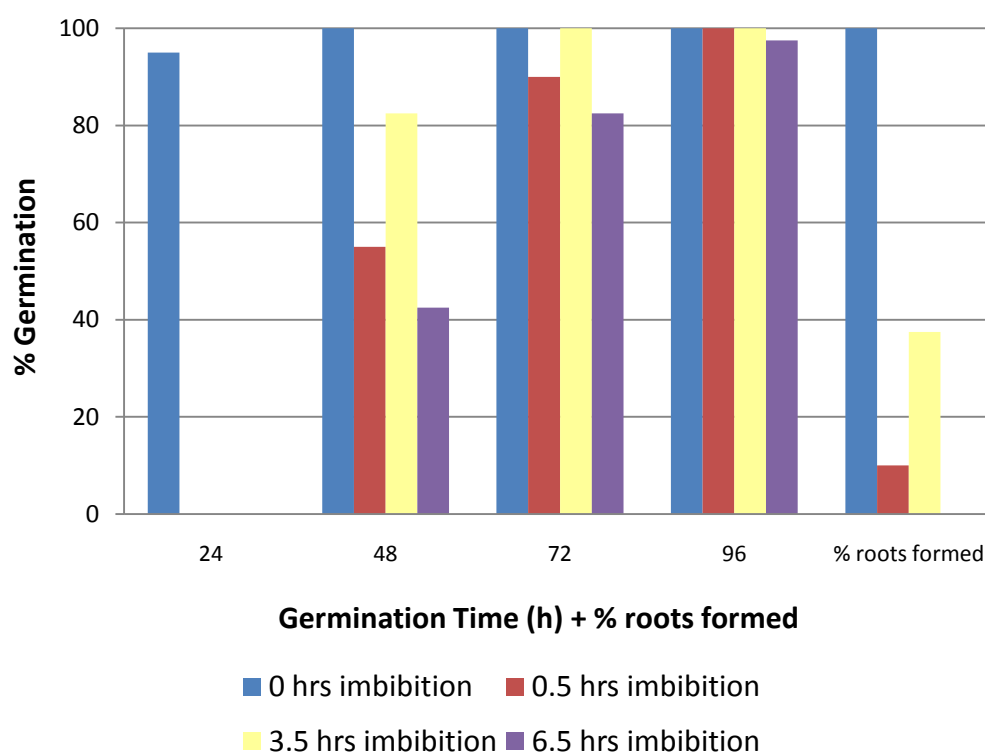




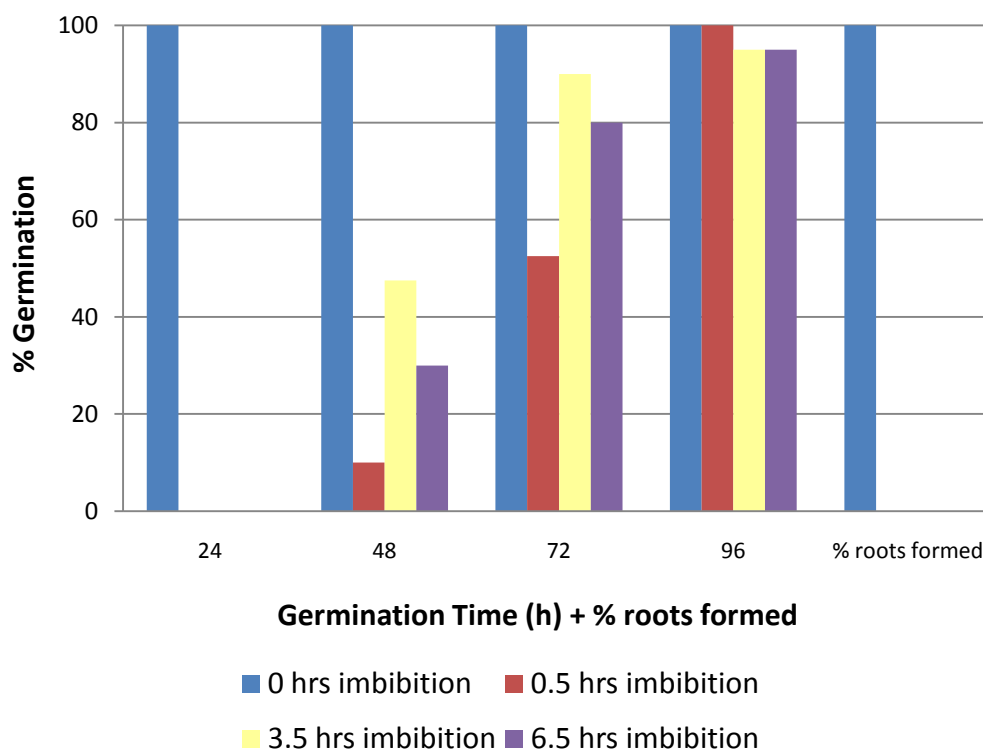
**Figure 5.10** *Hybrid 6321* -viability data after 6 months in cryostorage (large vat). Root formation was observed after two weeks in culture. n= 40



**Figure 5.11** Rate of freezing comparisons between *Hybrid 6321* samples placed into cryovials and those frozen directly in liquid nitrogen. n=40



**Figure 5.12** *Hybrid 6321* isolated axes, cryostored for 15 minutes in cryovials (small vat). Root formation was observed after two weeks in culture. n=40



**Figure. 5.13.** *Hybrid 6321* isolated axes, cryostored for 15 minutes in cryovials, placed into a metal casing, submerged into liquid nitrogen and stored in a large vat. Root formation was observed after two weeks in culture. n=40

#### 5.3.5. Differences in ultrastructural changes between *Hybrid 6363* and *Hybrid 6361* pre- and post- Cryostorage (15 minutes)

For the range of imbibition times tested (0 – 6 hrs) *Hybrid 6363* (Figs. 5.6 - 5.9) and the newly harvested *Hybrid 6321* (Figs. 5.14 - 5.17) caryopses were compared ultrastructurally pre- and post-cryostorage. For both seed batches, the pre-cryostorage samples were comparable in appearance in the dry tissues (Figs. 5.6a and 5.14a). Similarly, the cryostored samples appeared unaffected ultrastructurally by the cryoprocure (Figs. 5.6b and 5.14b). For example, plasmamembranes were withdrawn from the angular cell walls, the lipids were peripheral and the cytomatrix condensed. As the tissue became increasingly more hydrated with each imbibition time, the plasmamembranes and cytomatrix expanded to fill the entire cell volume. At each stage, the lipid molecules remained peripheral (Figs. 5.7a-5.9a and Figs. 5.15a-5.17a). Post-cryostorage, however, some variations were



noted as the tissue hydration levels increased. For *Hybrid 6363* tissue imbibed to  $\pm 52\%$  w/c, there was apparent freezing desiccation where plasmamembranes were, once again, withdrawn from the cell walls (Crowe *et al.* 1990). The cell walls were buckled (Fig. 5.7b) with this being more pronounced in the 3.5 hrs sample (Fig. 5.8b). The freshly harvested caryopses *Hybrid 6321*, however, did not exhibit any of these characteristics. The plasmamembranes remained expanded to the cell wall and cell walls maintained their structural integrity (Figs. 5.15b, 5.16b and 5.17b), but the nuclear profiles in this series appeared to have sustained some injury. The only visible similarity between the two hybrids post-cryostorage was for the 6.5 hrs samples (Figs. 5.9b and 5.17b). The peripheral lipid molecules were no longer as pronounced as for all the other imbibition times (Figs. 5.9b and 5.17b); they had become diffuse and the tissue appeared vacuolated. This observation resulted in the suggestion that the once peripheral lipids, (as seen in earlier imbibition times), were now required to participate in the membrane repair processes due to the possible loss of desiccation tolerance at 6.5 hours imbibition.

Platt *et al.* (1997) stated that desiccation-tolerant plants were thought to possess constitutive protective components to limit damage, such that a rehydration-induced repair mechanism would return the cell to a normal state. Lipid bodies are aligned on the inner surface of the plasmalemma during maturation drying (Cordova-Tellez and Burris 2002a) and these may act as a reserve for membrane expansion during imbibition (Vertucci and Farrant 1995). If this repair process is compromised at increased hydration levels (i.e. the onset of desiccation sensitivity due to the cryoprocure rather than the water content), then it is possible that the peripheral lipid molecules could be utilised in the repair process. This would explain the diminished appearance and/or coalescence of lipid molecules in the 6.5 hrs cryostored samples for both hybrids. However, at these hydration levels post-cryostorage, plantlets still germinated but they exhibited some morphological abnormalities when compared with the controls (Fig. 5.18). These observations suggest that the cryoprocure exacerbated the loss of repair potential and that it was further compromised in aged tissue.

Another change was noted in the caryopsis tissues post-imbibition and

cryostorage, viz. an increase in membrane vesiculation/vacuolation. The hydrated control tissues of *Hybrid 6363* all demonstrated a degree of vesiculation/vacuolation (Figs. 5.7a, 5.8a and 5.9a), whereas the caryopses tissue of the freshly harvested *Hybrid 6321*, only exhibited increased vesiculation/vacuolation after 3.5 hours of imbibition (Figs 5.15a, 5.16a and 5.17a). Increased vacuole formation is thought to provide a mechanical stabilisation during a dehydration process, whereby replacement of water in vacuoles with compatible solutes occurs (Sherwin and Farrant 1998). It has also been reported by Quartacci *et al.* (1997) that the break-up of a single large vacuole into numerous vesicular fractions occurs in dehydrating leaf cells. Responses to the effects of rehydration were also reported to include the formation of vacuoles/vesicles, which could be the result of the inability of the plasmamembrane to reconstitute properly, causing the lipid bilayer to vesiculate (Smith and Berjak 1995), as seen in *Hybrid 6363*. The suggestion is that, although water contents were similar for both samples, the resulting vesicles/vacuoles in *Hybrid 6363* after only 30 minutes of imbibition were due to responses initiated by the effects of imbibition of aged caryopses. *Hybrid 6321* only exhibited vesicle formation post 3.5 hrs imbibition, suggesting that the formation of vesicles/vacuoles in the freshly harvested tissue could be a mechanism of localising damage due to imbibition. This occurs after a longer period of imbibition as the rate of imbibitional damage accumulation is slower in freshly harvested tissue.

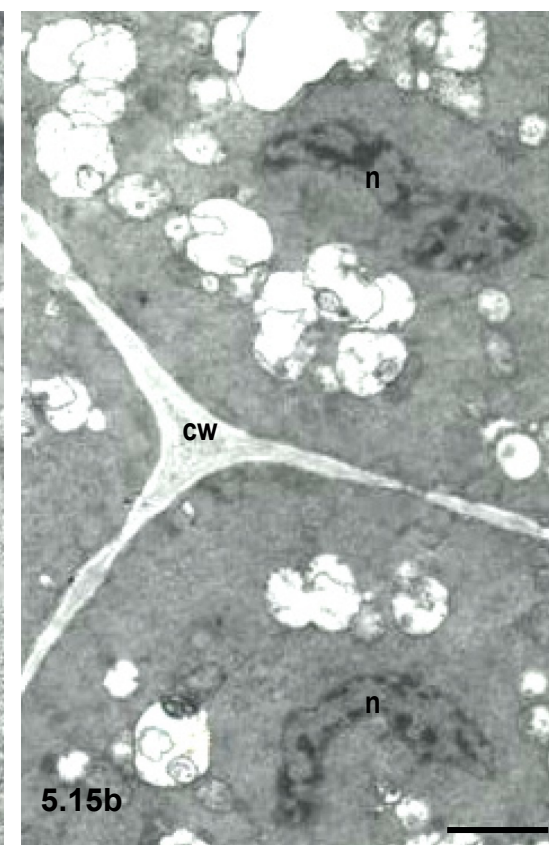
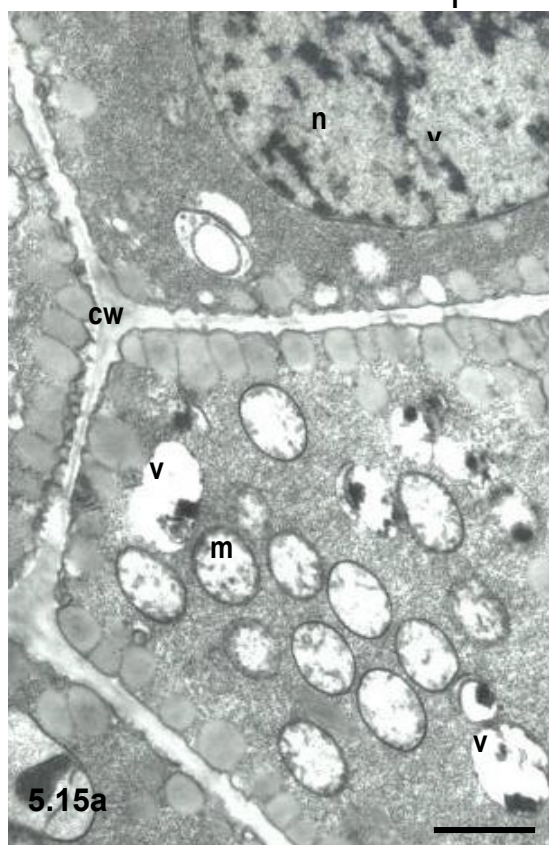
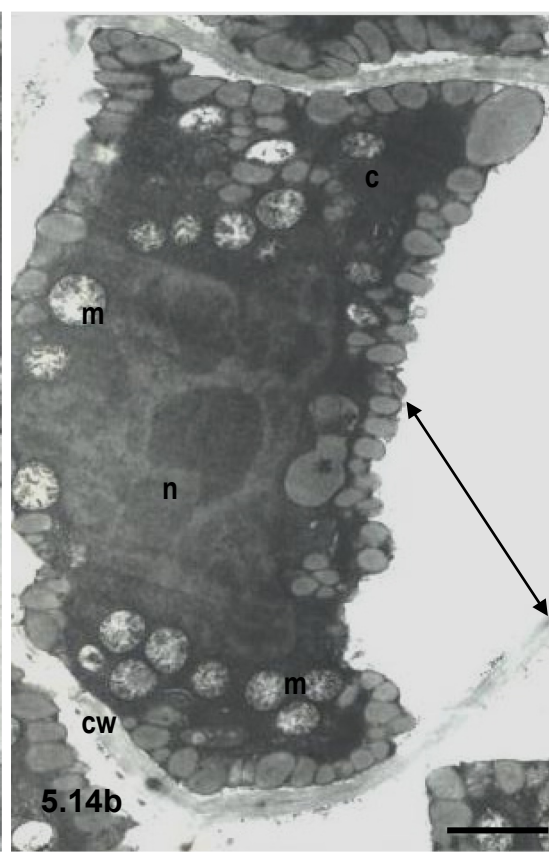
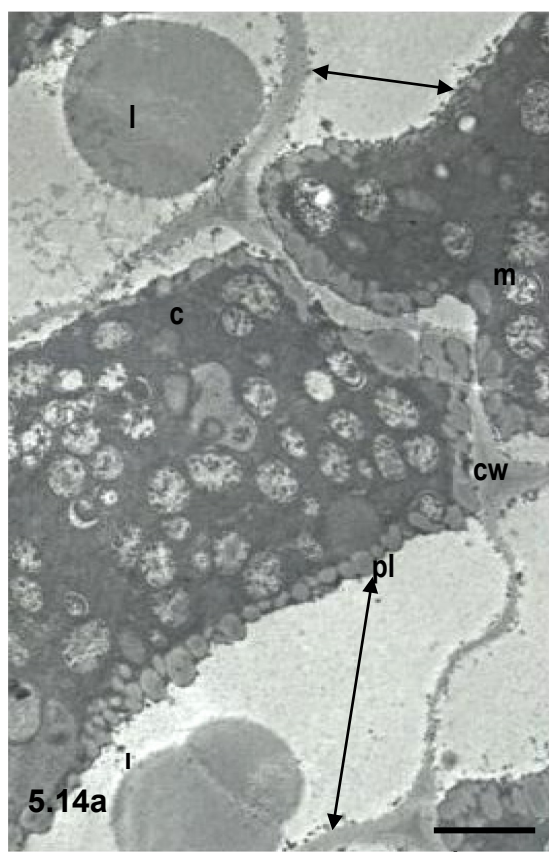
One additional factor which cannot be ignored is that although both lots of caryopses at this imbibition time were at similar hydration levels, the distribution of water within the tissue may have been different simply because they were not genetically identical and, hence, vesicle/vacuole formation in response to imbibition differed. The responses elicited by cryopreservation, however, at the 0.5 hrs imbibition time for the new tissue, resulted in vesicle/vacuole formation. The effects of cryopreservation elicited a similar response to that of initial imbibition in the aged tissue (Fig. 5.7a), suggesting that vesicle formation occurred as a protective response in containing or localising damage during imbibition and subsequent cryostorage.

**Figure 5.14a** *Hybrid 6321* referred to as new seed (NS) unimbibed tissue demonstrated cells typical of tissue post maturation drying. Plasmamembranes (**pl**) were withdrawn from the cell walls (**cw**), lipids (**l**) were peripheral and the cytomatrix (**c**) condensed. Scale bar=1.3 $\mu$ m.

**Figure 5.14b** (NS) Post-cryostorage (15 minutes) the 0 hrs tissue appeared very similar to the control exhibiting all the features seen in Fig. 5.6a. Scale bar=1.1 $\mu$ m.

**Figure 5.15a** (NS) 0.5 hrs imbibed tissue exhibiting very clear, electron translucent mitochondria (**m**) and very few vesicles (**v**). Lipids (**l**) remained peripheral and were situated immediately below the plasmamembrane. Scale bar=1.6 $\mu$ m.

**Figure 5.15b** Cryostorage (15 minutes) of the 0.5 hr imbibed tissue (NS) resulted in the extensive lobing of nuclei (**n**) and the formation of numerous vesicles (**v**). Scale bar = 2.0 $\mu$ m.



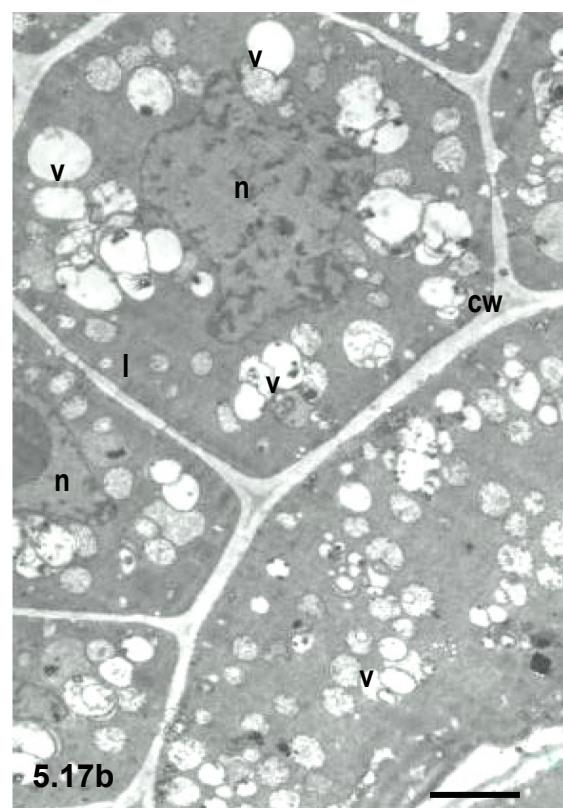
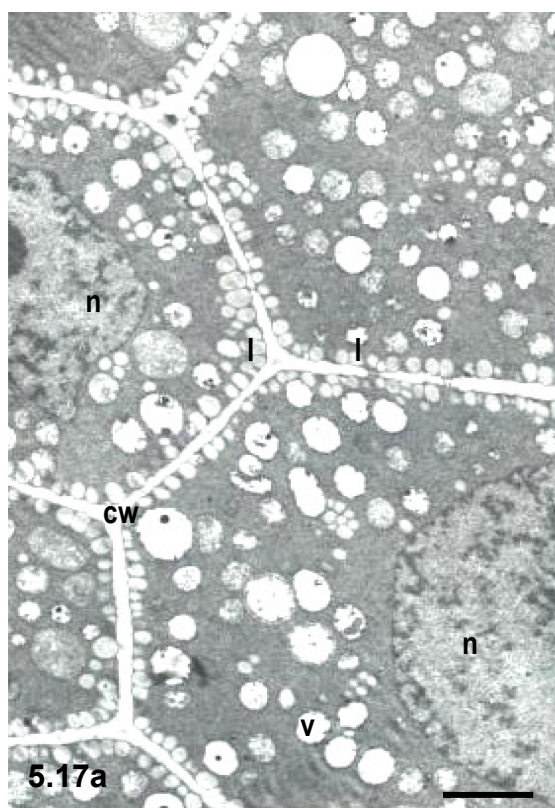
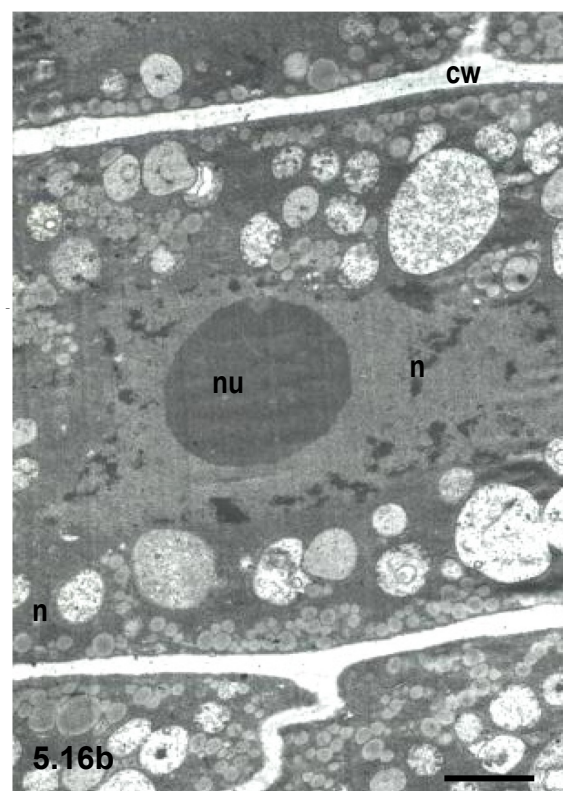
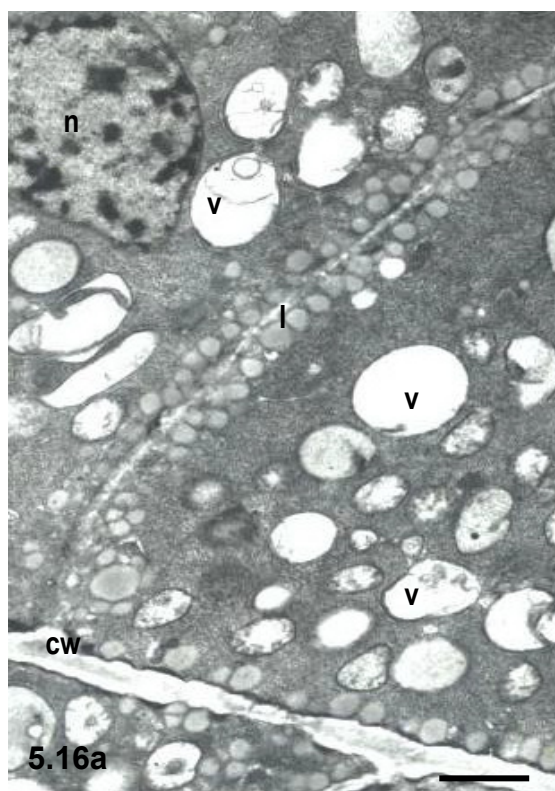
**Figure 5.16a** Tissue imbibed for 3.5 hours (NS) demonstrated spherical nuclei (**n**), an homogeneous matrix but with distinctively more vesicles (**v**) than the 0.5 hr imbibed tissue (Fig 5.15a). Scale bar=1.8 $\mu$ m.

**Figure 5.16b** Cryostorage of the 3.5 hrs imbibed tissue (NS) resulted in the buckling of cell walls (**cw**), lobing of nuclei (**n**) and the appearance of large vesicles (**v**). Scale bar=0.9 $\mu$ m.

**Figure 5.17a** (NS) imbibed for 6.5 hours demonstrated similar features to the 3.5 hrs sample. Nuclei (**n**) were spherical, the cytomatrix (**c**) homogeneous and the lipid (**l**) molecules were peripheral, situated immediately below the plasmamembrane. Scale bar=2.4 $\mu$ m.

**Figure 5.17b** Post-cryostorage, (NS) tissue imbibed for 6.5 hours demonstrated some lobed nuclei (**n**) and the appearance of numerous vesicles (**v**). The lipid (**l**) molecules, however, appeared to be scattered throughout the cytoplasm. Scale bar=2.5 $\mu$ m.





Cryostorage of this tissue at  $\pm 62\%$  water content resulted in the formation of vesicles/vacuoles for both old (Figs. 5.9b and 5.9d) and new caryopses (Fig. 5.17b). This showed that the formation of vesicles/vacuoles in the new tissue was possibly a direct response to the cryoprocurement, rather than the response of aged caryopses to the effects of rehydration and subsequent cryostorage, as seen in *Hybrid 6363*.

Extensive lobing of the nuclei in the older tissue pre- and post-cryostorage (Figs. 5.9a and 5.9b) was observed for all hydration levels. In the freshly harvested caryopses, nuclei were more spherical and the matrix homogeneous (Figs. 5.15a, 5.16a and 5.17a). This was similar to a report by Berjak *et al.* (1986) on newly harvested maize nuclear profiles. The process of cryostorage resulted in the extensive lobing of nuclei post-cryostorage for the new tissue (Figs. 5.15b, 5.16b and 5.17b). Nuclear lobing and chromatin clumping have been attributed to natural ageing associated with protracted storage periods (Berjak *et al.* 1986). The effects of cryostorage on the freshly harvested caryopses, of *Hybrid 6321*, were therefore very similar to the effects of ageing and desiccation as demonstrated in *Hybrid 6363*. The ability of the caryopses to withstand the deleterious effects of imbibition and cryostorage therefore, must be attributed to its freshness. Similarly, the condition of the tissue can be determined by its abilities to withstand the effects of the deleterious processes *viz.* initial imbibition and subsequent cryostorage. This said, the new caryopses, *Hybrid 6321* failed to produce roots post-cryostorage. Although the seed producer confirmed that these caryopses were freshly harvested, the ultrastructural profiles appeared very similar to that of the aged tissue (*Hybrid 6363*). The response of the new hybrid to imbibition and subsequent cryostorage could be similar to that of aged caryopses due to the possible pre-conditioning processes they were subjected to prior to purchase. This is speculation at this point and would need to be examined further, however, based on the observations made thus far it seems possible that these caryopses were possibly subjected to rapid preconditioning drying rates, and/or, the caryopses were primed and stored sub-optimally.

*5.3.6. A presentation of the results of the effects of both short and long-term cryostorage on the ultrastructure of the radicle meristem of isolated Zea mays. L. axes.*

*5.3.6.1. Hybrid 6363*

Material of this hybrid (in cold storage for one year stored tissue) was cryostored for both 15 minutes and 12 months. Control caryopses of stored tissue (0 hrs imbibition) demonstrated a range of cellular deterioration including lipid coalescence and lobed nuclei (Fig. 5.6c) which were not seen in tissues of original caryopses (Fig. 5.6a). The effects of protracted cryostorage on the 0 hrs tissue appeared to have resulted in a greater percentage of lipid coalescence, but the appearance of the plasmamembrane seemed to have maintained its integrity (Fig. 5.6d). The sample cryostored for 12 months (Fig. 5.6e) was similar to the 15 minutes cryostored sample, except for the apparent greater occurrence of larger membrane bound vesicles/vacuoles.

For the 30 minutes surface-decontaminated material, the stored tissue generally appeared to have sustained a degree of injury (Fig. 5.7c) when compared with the more vigorous original tissue (Fig. 5.7a). The nuclei of this tissue however, contained visible nucleoli, and the nuclear membrane was intact. Vesicles/vacuoles were present and appeared to contain electron dense material. Lipid molecules, although not easily detected, were present and still in close association with the plasmamembrane. The tissue cryostored for 15 minutes (Fig. 5.7d) had pyknotic nuclei, buckled cell walls and the presence of some vesicles/vacuoles. For the material stored in the small and the large vats for long periods, there were no apparent differences at the ultra-structural level for the 30 minutes sample (Fig. 5.7d and Fig. 5.7e).

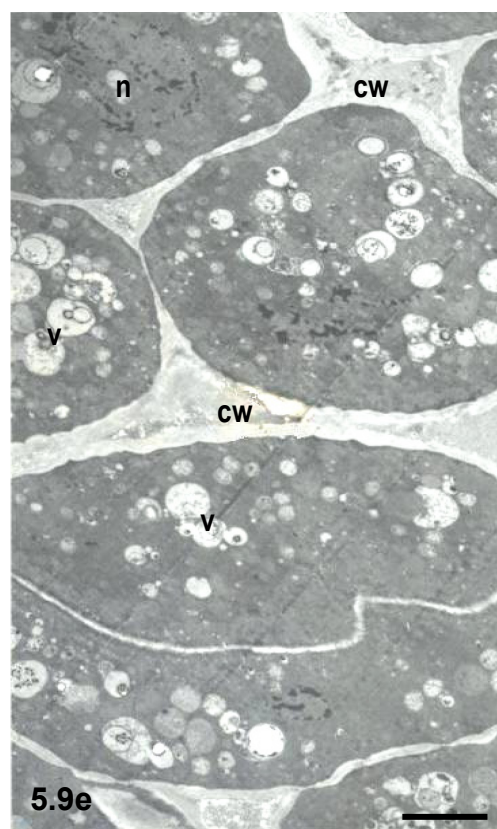
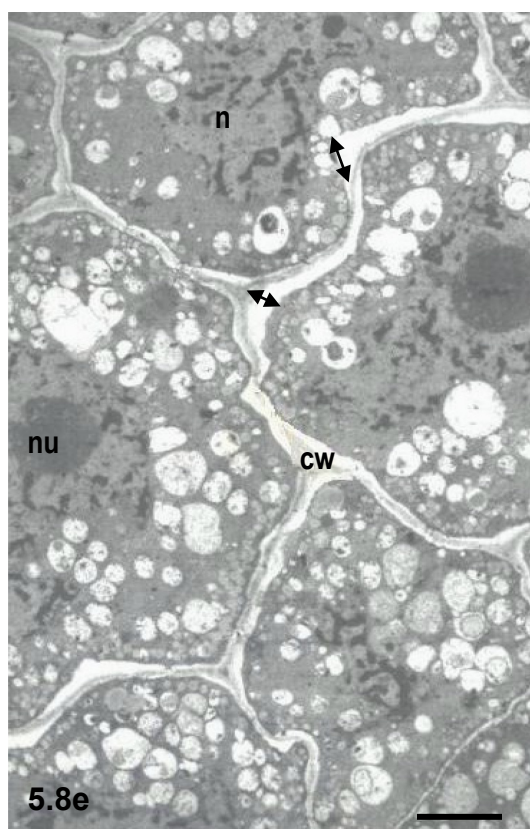
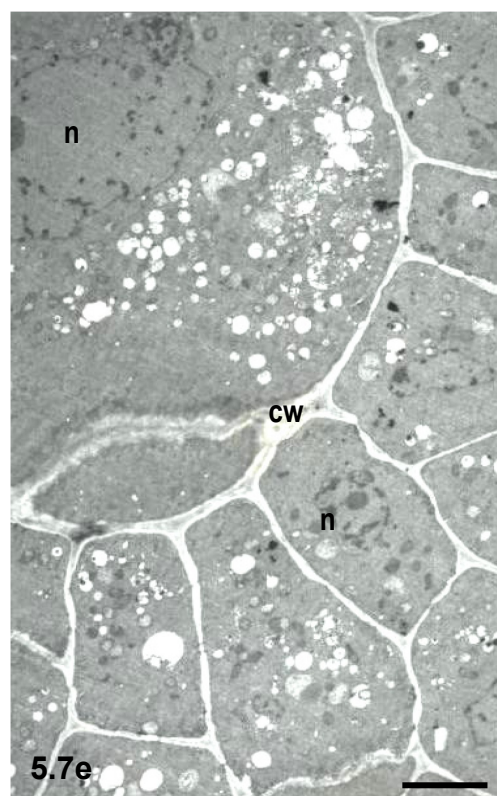
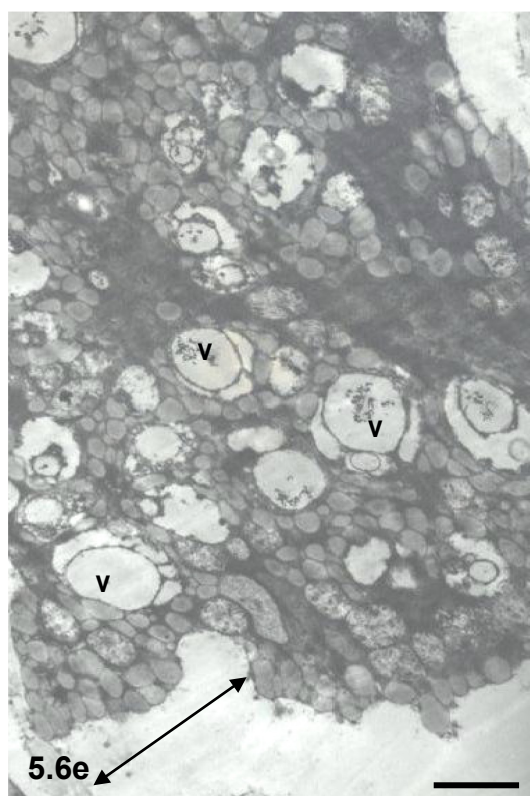


**Figure 5.6e** *Hybrid 6363* unimbibed tissue (0 hrs) cryostored for 12 months appeared to have an increase in lipid (**l**) coalescence and vesicle formation (**v**). However, the cytoplasm remained condensed and retracted from the cell wall (**cw**) while the plasmamembrane (**pl**) retained its integrity. Scale bar=0.2 $\mu$ m.

**Figure 5.7e** *Hybrid 6363* imbibed tissue (0.5 hrs) cryostored for 12 months was very similar to the tissue cryostored for 15 minutes (See Fig 5.7d). The prolonged cryostorage period therefore did not appear to result in any ultrastructural changes. Scale bar=1.1 $\mu$ m.

**Figure 5.8e** *Hybrid 6363* imbibed tissue (3.5 hrs) cryostored for 12 months. Plasmamembrane retracted from the cell wall and the cell walls (**cw**) appeared buckled which was not observed in the tissue cryostored for 15 minutes (Fig 5.8d). Scale bar=3.1 $\mu$ m.

**Figure 5.9e** *Hybrid 6363* imbibed tissue (6.5 hrs) cryostored for 12 months did not appear to be any different from Fig 5.9d. The cell walls (**cw**) were buckled, numerous vesicles (**v**) observed, plasmamembranes were diffuse, nuclei (**n**) when visible appeared pyknotic and these cells were presumed non-viable. Scale bar=2.1 $\mu$ m.



After 3.5 hours imbibition, however, notable changes were observed between the 15 minutes and the 12 months cryostored sample. For the latter, the process of cryostorage resulted in the retracting of the plasmamembrane from the cell wall (Fig. 5.8e) and more pronounced buckling of the cell wall which was not as obvious for tissue cryostored for 15 minutes (Fig. 5.8d). These observations could be attributed to a possible slowing in the rate of freezing caused by the increase in water content and the metal casing in the large vat. This 'slowing' in the freezing rate could exert a greater dehydration on the tissue than the relatively more rapid freezing protocol, and therefore, result in a greater freezing stress on this particular tissue. If the above occurred, then the lack of root development at all the imbibition times for both *Hybrid 6363* and *Hybrid 6321* can be explained by a change in freezing rate (see Chapter one) caused by the introduction of the metal casing in the large vat.

Tissue imbibed for 6.5 hours and cryostored for 15 minutes and 12 months, respectively showed no difference in cellular integrity. Cell walls were buckled, many vesicles/vacuoles were present, and although lipid molecules were present beneath the plasma membrane, they were diffuse in nature. Nuclei, when visible appeared pyknotic and such cells were presumably non-viable.

#### 5.3.6.1. *Hybrid 6321*

Material of this hybrid was also cryostored for 15 minutes, 6 and 12 month periods. The effects of long and short term cryostorage on the new tissue indicated some changes, but again, these changes were not thought to be due to the duration of exposure to the cryogen, but rather the possible changes in rate of freezing caused by the large and small vats respectively.

For the dry, unimbibed tissue frozen in the small vat for 15 minutes (Fig. 5.19b) there were no visible ultrastructural changes when compared with the control dry tissue (Fig. 5.19a). However, when compared with the samples cryostored for 6 and 12 months in the large vat (Figs. 5.19c and 5.19d) the plasmamembrane

appeared fragmented in areas, and the cytomatrix, although compact, revealed the appearance of many membrane bound vesicles/vacuoles. The nuclei in these cells were not easily discernable, but the lipid molecules were present and situated immediately beneath the plasmamembrane. The lipid molecules however, were less defined in appearance in the samples frozen in the larger vat and for the longer period. Mitochondria for all the unimbibed tissue, exhibited some internal development but were presumably metabolically inactive at this hydration level (8.66%).

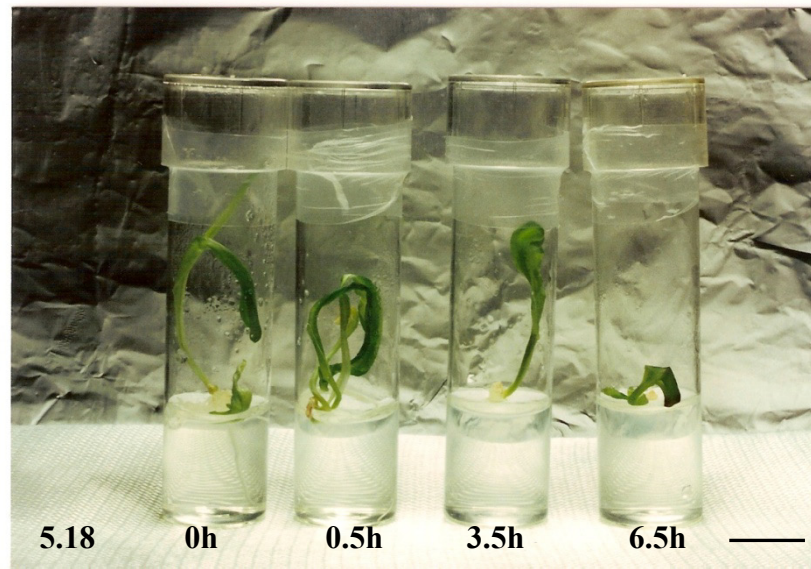
Samples hydrated to 56.74% w/c (i.e. surface decontaminated) and frozen for 15 minutes (Fig. 5.20b) demonstrated an increase in the number of vesicles/vacuoles. They also demonstrated lobed nuclei, which were not seen in the control tissue (Fig. 5.20a). The lipid molecules post-cryostorage displayed a less defined organisation when compared with the control, but remained closely associated with the plasmalemma which had expanded to fill the cells. After cryostorage for 6 months (Fig. 5.20c) in the large vat the plasmamembrane appeared to have retracted slightly, the number of vesicles/vacuoles had increased and nuclei remained lobed. The lipid molecules were diffuse but coalesced at points, and the mitochondria remained similar in appearance to the unimbibed tissue, and therefore, presumed metabolically inactive. After 12 months in cold storage (Fig. 5.20d) the cells appeared very similar to the control (Fig. 5.20a). The only difference between this tissue and the control was the presence of electron dense vesicles/vacuoles. The nuclei were rounded and the cells angular in shape with non-buckled cell walls. Lipids were defined and still aligned beneath the plasmamembrane.

The 3.5 hrs sample post 15 minutes cryostorage (small vat) appeared to be relatively unaffected by the process of cryostorage except for the slight buckling of the cell wall and lobing of the nucleus (Fig. 5.21b). Vesicles/vacuoles were present in both the control (Fig. 5.21a) and the cryostored tissue (Fig. 5.21b) and the mitochondria were relatively inactive in both sets of tissues. Lipids remained peripheral and in close association with the plasmalemma (Fig. 5.21b). After 6

months in cryostorage, however, many more vesicles/vacuoles were present (Fig. 5.21c). Once again, the plasmamembrane was slightly retracted from the cell wall possibly due to the freezing rate, but this was not visible in samples cryostored for 12 months (Fig. 5.21d). Nuclei were however, lobed, appeared abnormal and numerous vesicles/vacuoles (Fig. 5.21d) were present.

The 6.5 hrs samples at water contents of approximately 58% appeared (Fig. 5.22a) to be very similar to the other imbibition times i.e. 0.5 hrs (Fig. 5.20a) and 3.5 hrs (Fig. 5.21a) imbibition. The effects, therefore, of imbibition on *Hybrid 6321*, did not appear to be as deleterious as the effects of imbibition on the older seed lot (*Hybrid 6363*) as supported by the vigour and viability data (Fig 5.2). The subsequent effects of cryostorage for 15 minutes on the tissue at this hydration point were similar in appearance (Fig. 5.22b) to the effects of cryostorage observed earlier (Fig. 5.9b) e.g. nuclei where lobed and heterochromatic, lipid molecules were diffuse and many vesicles/vacuoles were observed. Once again, the sample frozen for 6 months (Fig. 5.22c) exhibited retracted plasmamembranes, lobed nuclei and the presence of numerous and relatively large vesicles/vacuoles, whereas, the 12 months sample (Fig. 5.22d) had fully expanded plasmamembranes, a rounded nucleus and very few vesicles/vacuoles. However, the appearance of organelles, collapsed around the nucleus in comparison to the more evenly distributed organelles visible in Fig. 5.22a suggests that the cytoskeleton may have been compromised as demonstrated by the lack of root formation in these samples.

Figure 5.18 *Hybrid 6321* at four hydration levels post-cryostorage, plantlets still germinated (0.5, 6.5 hrs samples) but exhibited some morphological abnormalities including no roots, when compared with the unimbibed (0 hrs) sample. Scale Bar 1cm=1.6cm.



**Figure 5.19a** *Hybrid 6321* referred to as new seed (NS), unimbibed tissue demonstrated cells typical of tissue post maturation drying. Plasmamembranes (**pl**) were withdrawn from the cell walls (**cw**), lipids (**l**) were peripheral and the cytomatrix (**c**) condensed. Scale bar=1.3 $\mu$ m.

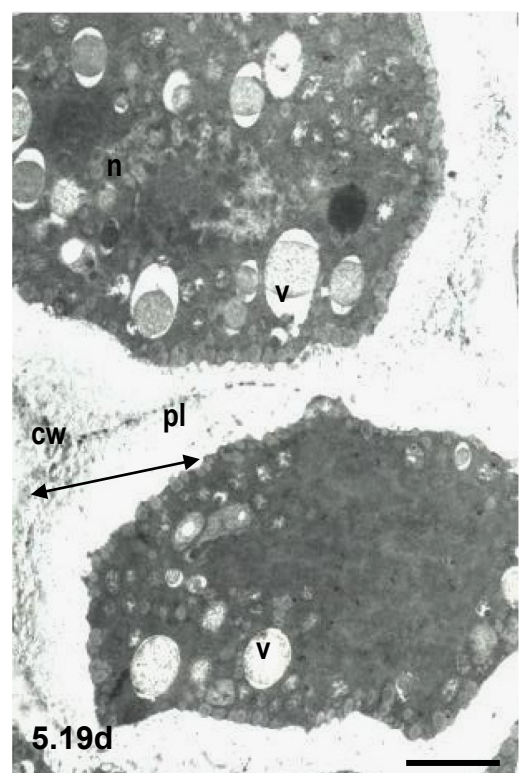
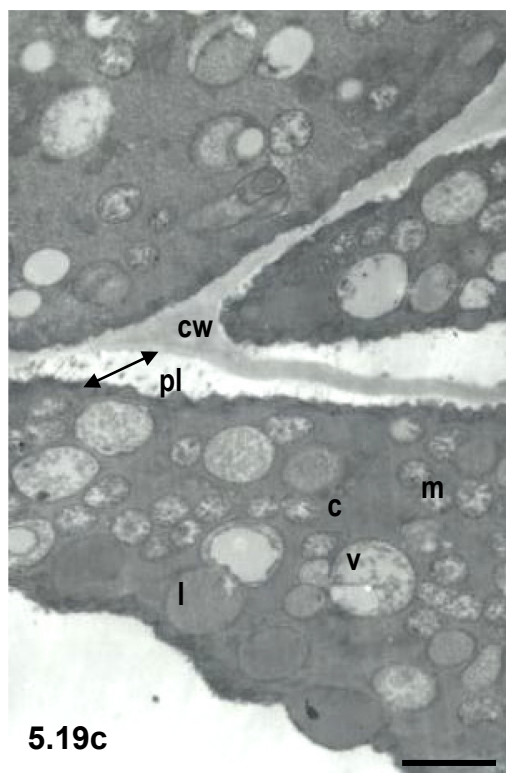
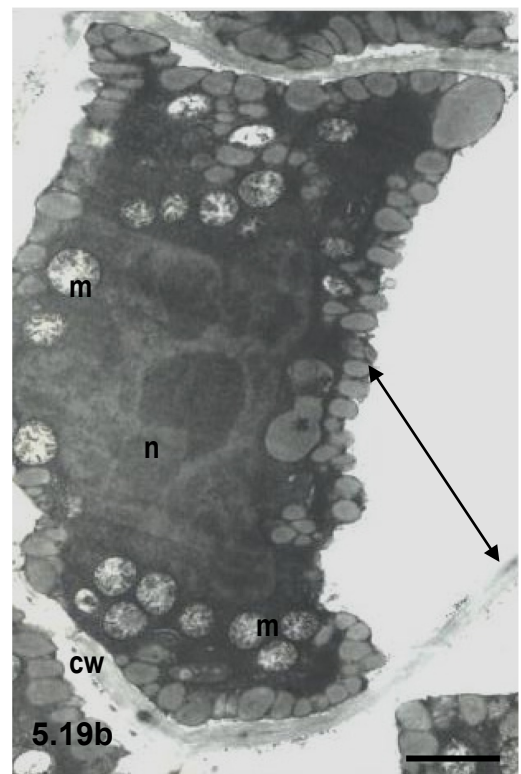
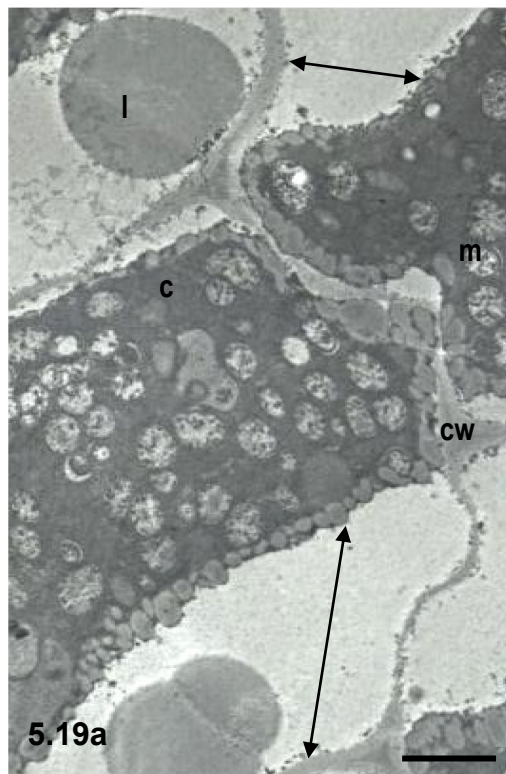
**Figure 5.19b** (NS) Post-cryostorage (15 minutes) the 0 hrs tissue appeared very similar to the control exhibiting all the features seen in **Fig. 5.6a**. Scale bar=1.1 $\mu$ m.

**Figure 5.19c** (NS) 0 hrs unimbibed sample after six months in cryostorage. The plasmamembranes (**pl**) appeared fragmented in areas and although the cytomatrix appeared compact, many membrane bound vesicles (**v**) were observed. Scale bar=0.7 $\mu$ m.

**Figure 5.19d:** (NS) 0 hrs unimbibed sample after 12 months in cryostorage appeared very similar to the samples cryostored for six months demonstrating fragmentary plasmamembranes (**pl**) and membrane bound vesicles (**v**). Scale bar=1.4 $\mu$ m.

Figs. 5.14 a&b are the same micrographs as Figs. 5.19 a&b. They are repeated here for direct comparison to the long term cryopreservation samples.





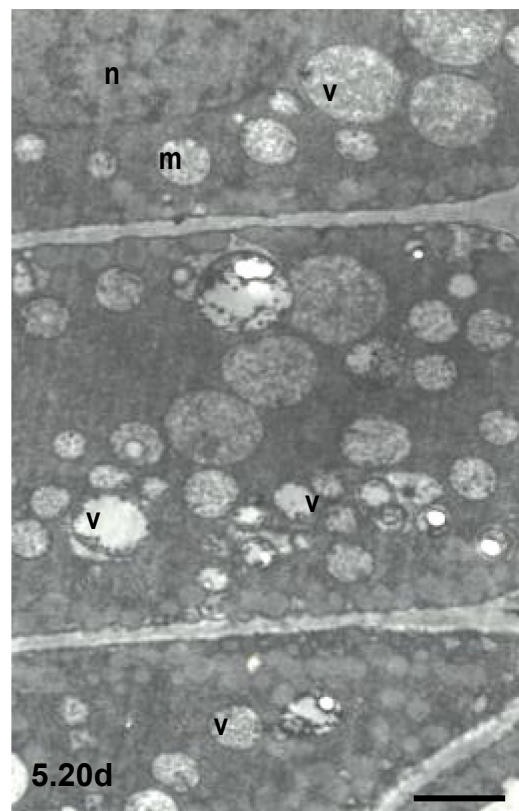
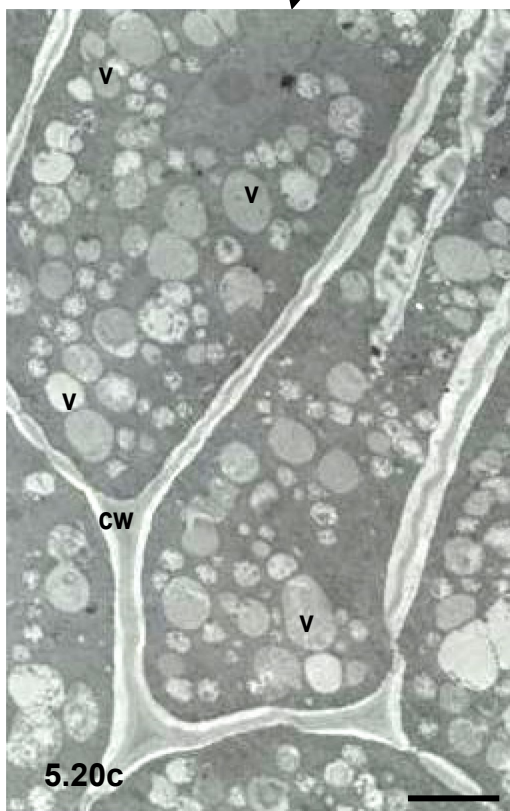
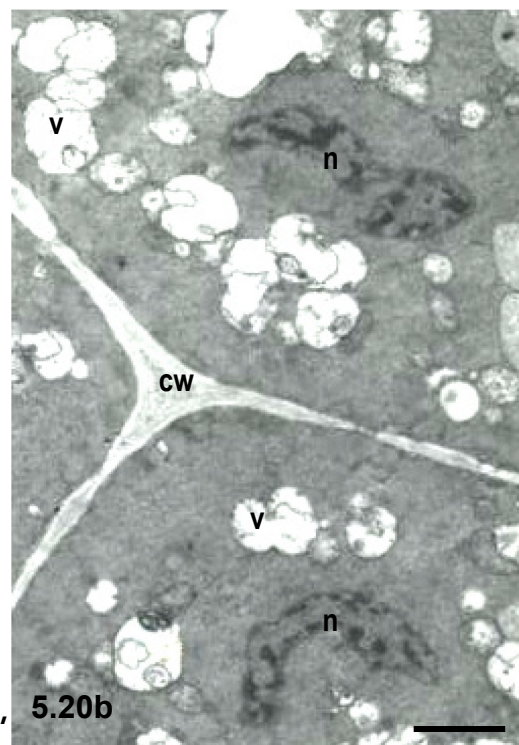
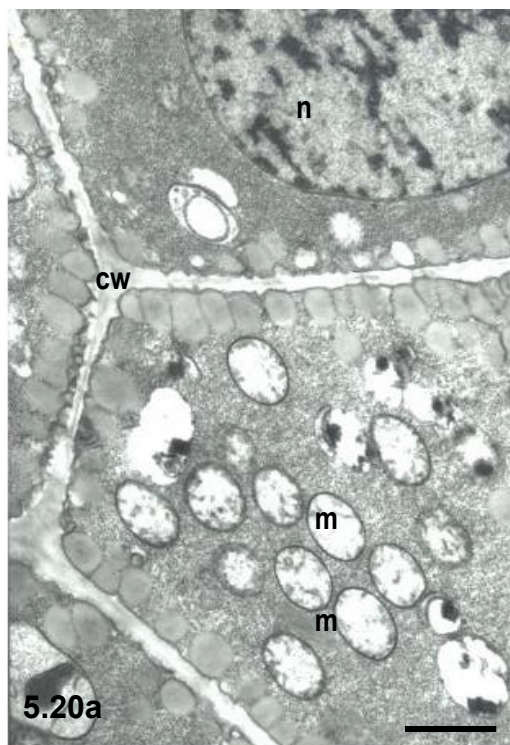
**Figure 5.20a** (NS) 0.5 hrs imbibed tissue exhibiting very clear, electron translucent mitochondria (**m**) and very few vesicles (**v**). Lipids (**l**) remained peripheral and were situated immediately below the plasmamembrane. Scale bar=1.6 $\mu$ m.

**Figure 5.20b** Cryostorage (15 minutes) of the 0.5 hr imbibed tissue (NS) resulted in the extensive lobing of nuclei (**n**) and the formation of numerous vesicles (**v**). Scale bar=2.0 $\mu$ m.

**Figure 5.20c** New seed tissue imbibed for 0.5 hours and cryostored for six months. Plasmamembranes were slightly retracted from the cell walls ( $\leftrightarrow$ ), nuclei (**n**) were lobed and the presence of vesicles (**v**) had increased. Scale bar=1.4 $\mu$ m.

**Figure 5.20d** New seed tissue imbibed for 0.5 hours and cryostored for 12 months was very similar to the control except for the increased number of vesicles (**v**). Scale bar=1.2 $\mu$ m.

Figs. 5.15 a&b are the same micrographs as Figs. 5.20 a&b. They are repeated here for direct comparison to the long term cryopreservation samples.



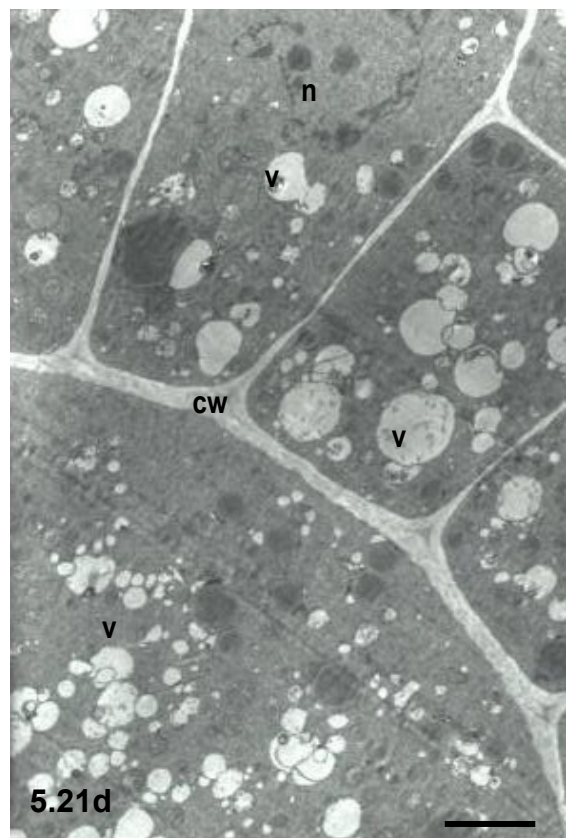
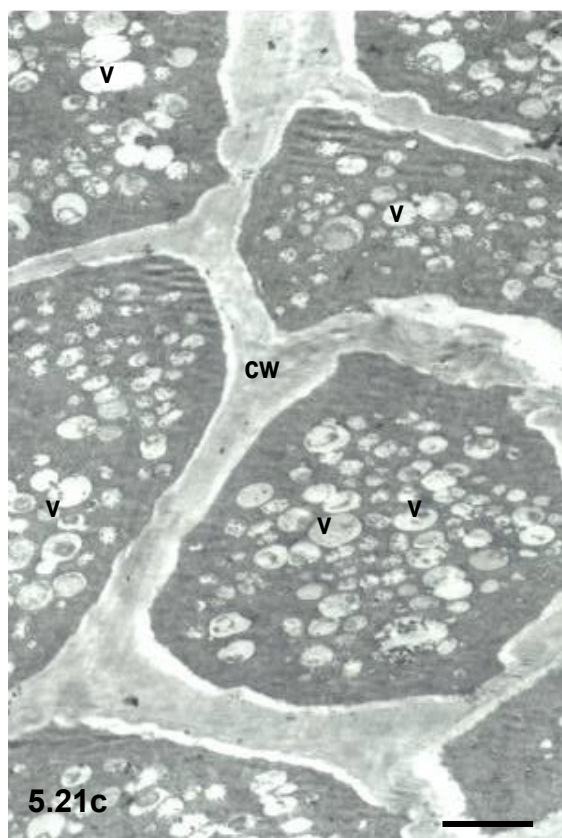
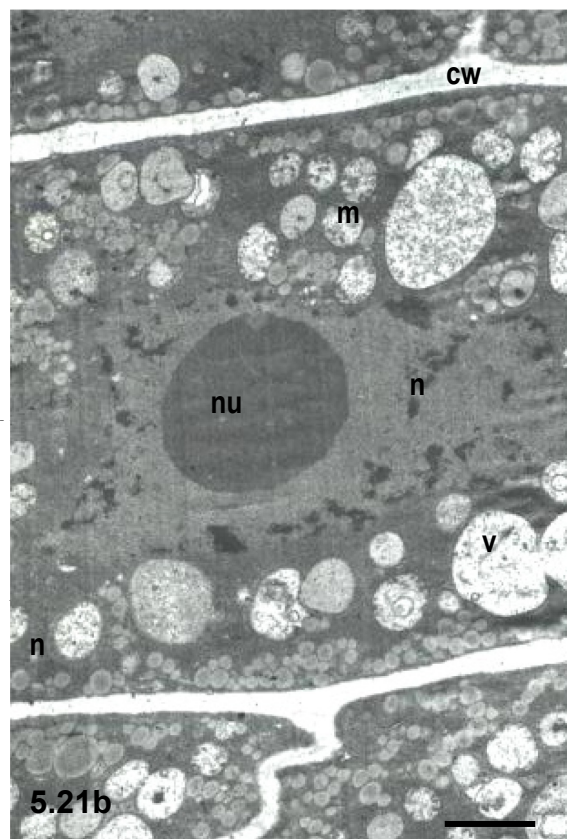
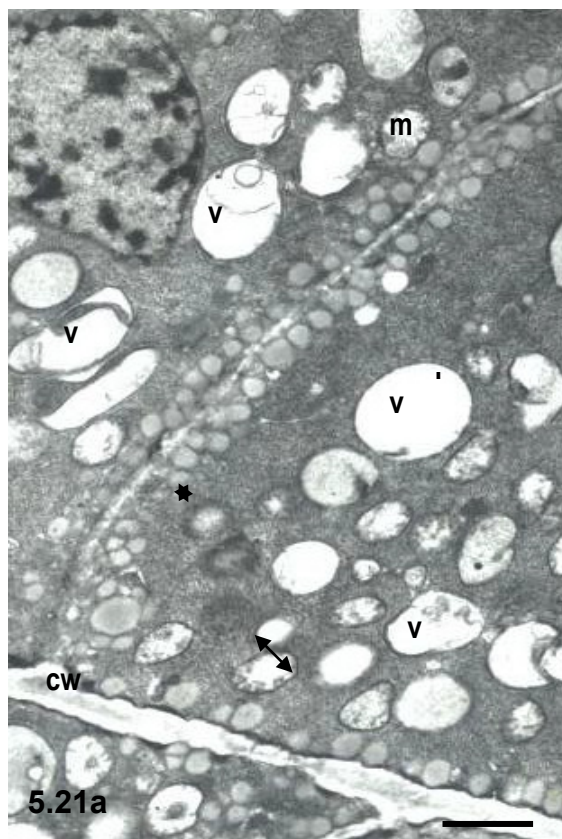
**Figure 5.21a** Tissue imbibed for 3.5 hours (NS) demonstrated spherical nuclei (**n**), an homogeneous matrix but with distinctively more vesicles (**v**) than the 0.5 hrs imbibed tissue (**Fig 5.15a**). Scale bar=1.8 $\mu$ m.

**Figure 5.21b** Cryostorage of the 3.5 hrs imbibed tissue (NS) resulted in the buckling of cell walls (**cw**), lobing of nuclei (**n**) and the appearance of large vesicles (**v**). Scale bar=0.9 $\mu$ m.

**Figure 5.21c** Tissue (NS) imbibed for 3.5 hours and cryostored for six months exhibited many more vesicles (**v**) and the plasmamembrane was once again slightly retracted form the cell wall ( $\leftrightarrow$ ). Scale bar=2.4 $\mu$ m.

**Figure 5.21d** Tissue (NS) imbibed for 3.5 hours and cryostored for 12 months exhibited lobed heterochromatic nuclei (**n**) and numerous vesicles (**v**). Cell walls (**cw**) were not buckled and the cytoplasms were fully expanded to the cell walls. Scale bar=2.1 $\mu$ m.



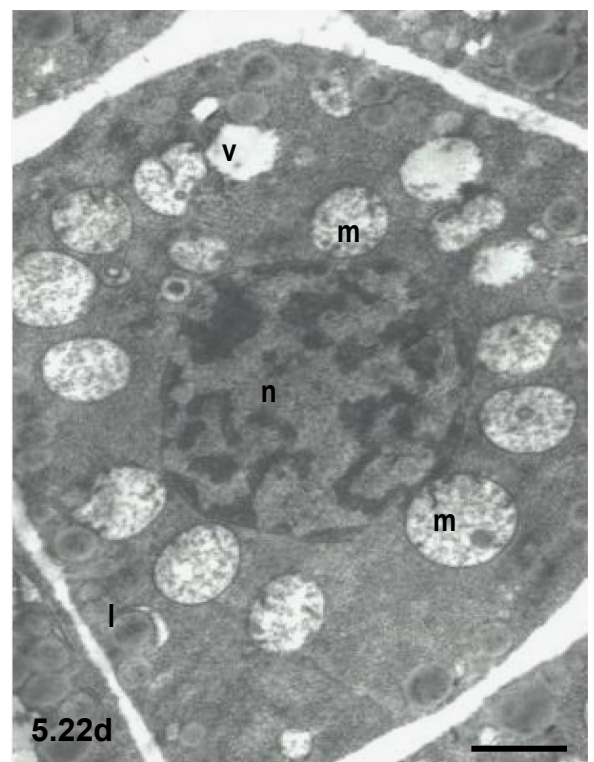
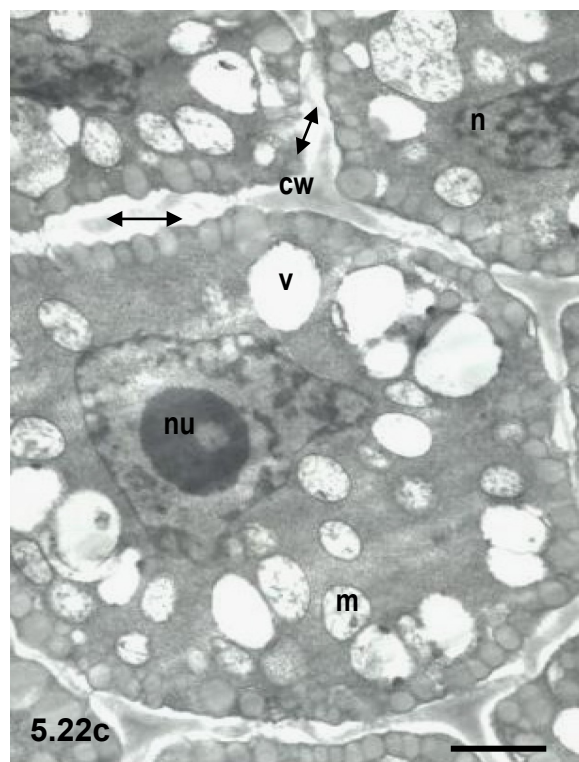
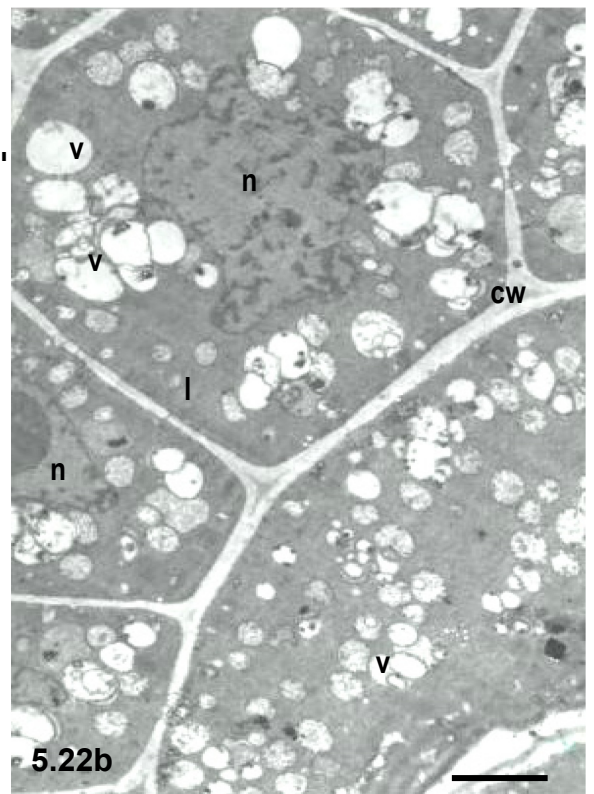
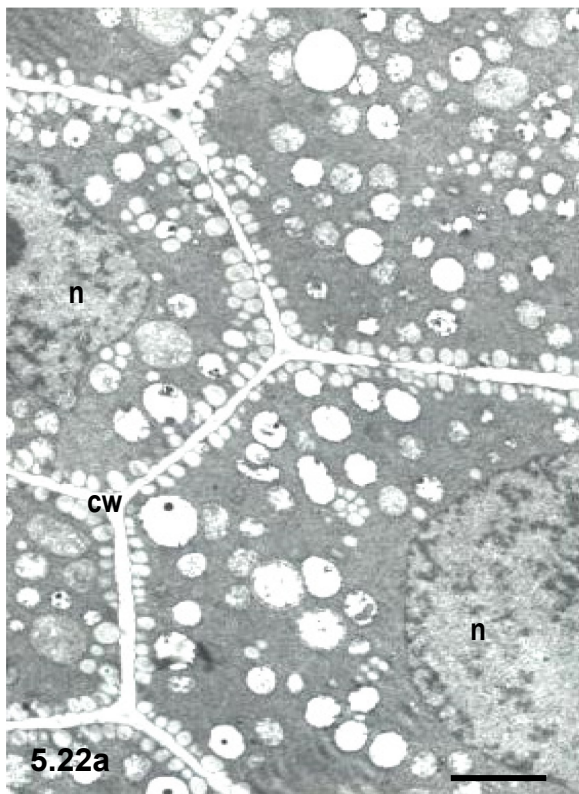


**Figure 5.22a** (NS) imbibed for 6.5 hours demonstrated similar features to the 3.5 hrs sample. Nuclei (**n**) were spherical, the cytomatrix homogeneous and the lipid (**l**) molecules were peripheral situated immediately below the plasmamembrane. Scale bar = 2.4 $\mu$ m.

**Figure 5.22b** Post-cryostorage, (NS) tissue imbibed for 6.5 hours demonstrated some lobed nuclei (**n**) and the appearance of numerous vesicles (**v**). The lipid (**l**) molecules however, appeared to be scattered throughout the cytoplasm. Scale bar=2.5 $\mu$ m.

**Figure 5.22c** Tissue (NS) imbibed for 6.5 hours and cryostored for six months exhibited retracted plasmamembranes ( $\leftrightarrow$ ), lobed nuclei (**n**) and the presence of numerous and relatively larger vesicles (**v**). Scale bar=1.1 $\mu$ m.

**Figure 5.22d** Tissue (NS) imbibed for 6.5 hours and cryostored for 12 months demonstrated fully expanded plasmamembranes, rounded nuclei (**n**) and fewer vesicles (**v**) but the organelles appear to have collapsed around the nucleus suggesting that the cytoskeleton has been compromised. Scale bar=0.6 $\mu$ m.



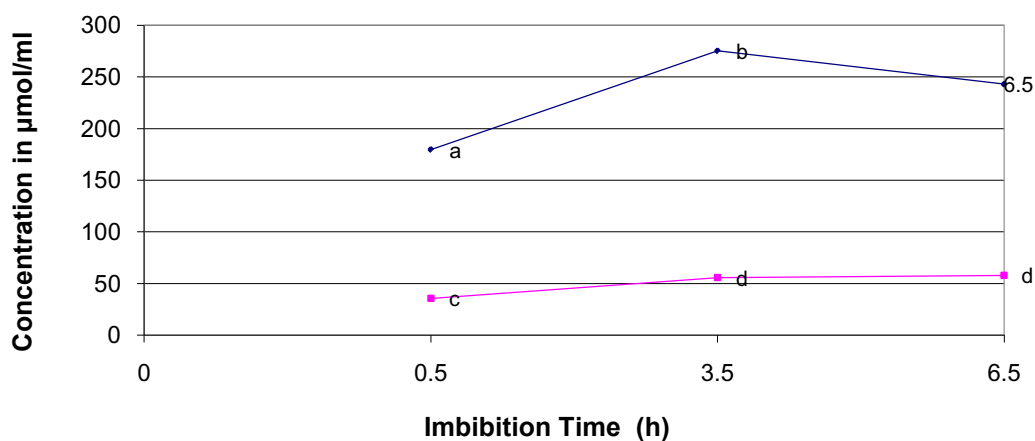


### 5.3.7. Biochemistry

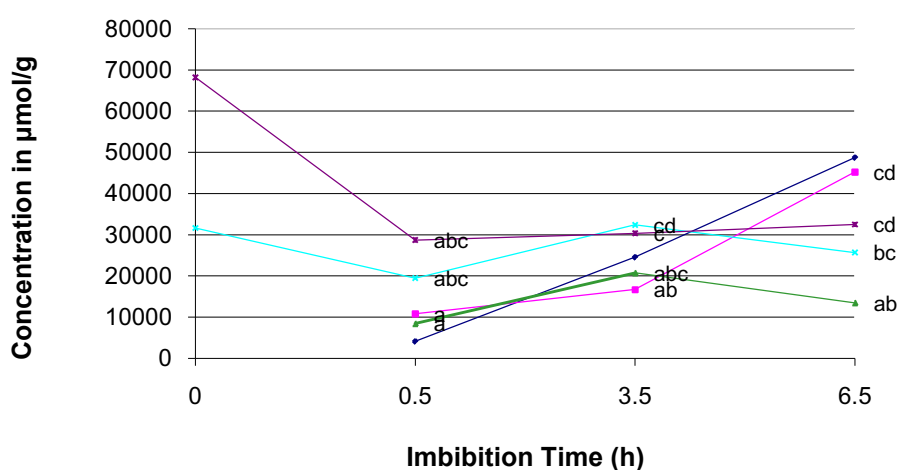
#### 5.3.7.1. Biochemical constituent comparisons between the leachates of original tissue (*Hybrid 6363 at purchase*) and freshly harvested caryopses (*Hybrid 6321*)

When biochemical constituents of the leachates of *Hybrid 6363* (aged caryopses) and *Hybrid 6321* (freshly harvested caryopses) were compared statistically, they were found to be significantly different ( $F_{1,12}=45,0784$ ,  $P < 0,001$ ). This result established that the older tissue had lost a significantly greater percentage of total amino acids ( $F_{2,12}=45,0784$ ,  $P < 0,001$ ) and glucose ( $F_{2,12}=8,12092$ ,  $P < 0,005$ ) than the fresh caryopses (Fig. 5.23 and Fig. 5.25). In fact, five times the amount of amino acids were leached from the older caryopses than from the new for each imbibition time, and three times the amount of glucose for the 3.5 hrs imbibition time (Fig. 5.25). Glucose quantities in the embryos of the new caryopses were statistically different to all the other treatments for each imbibition time (Fig. 5.26 and Table 5.2) indicating that the newer embryos retained a greater concentration of glucose within the tissues. This was correlated by the reduced levels in the leachate in comparison with the old caryopses. Notably, the amount of proline leached from the older embryos (Fig. 5.27) was minimal compared with the amount retained in the younger embryos (Fig. 5.28), but the amino acid illustrated a steady increase with each imbibition time. For the freshly harvested caryopses, proline was not detected in the leachate, demonstrating that all the proline was retained in the embryo (Fig. 5.28), possibly within the vacuoles (Leigh *et al.* 1981; Herman and Larkins, 1999; Heuer 1999). This has also been demonstrated in potato cell suspension cultures (Fricke and Pahlich 1990), yeasts (Matsuura and Takagi 2005) and vegetative plant tissue (Van der Willegen *et al.* 2004). Interestingly, most of the cryostored samples indicated a statistical difference in the controls of proline value for each imbibition time except for the 3.5 hrs imbibition time. Perhaps this was the reason why a greater degree of success was obtained for caryopses imbibed to this hydration level post-cryostorage.

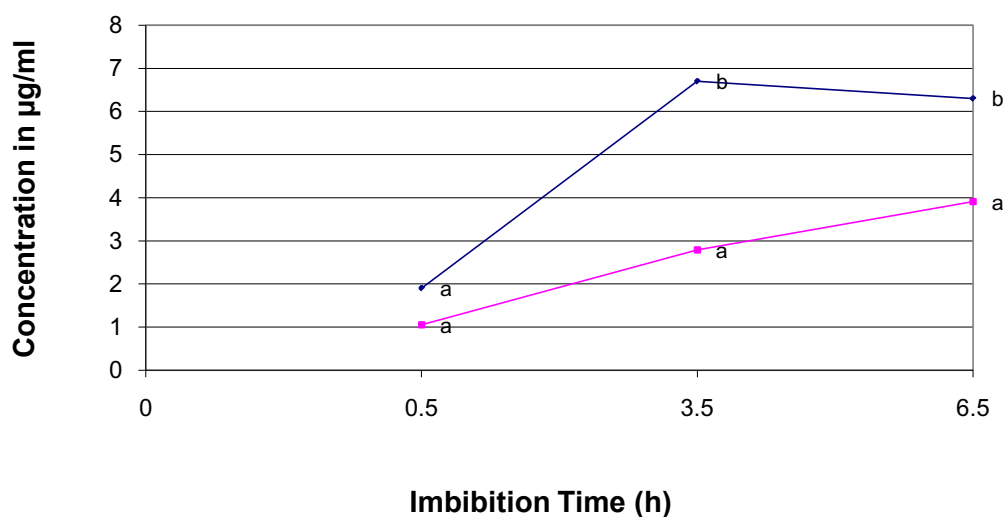




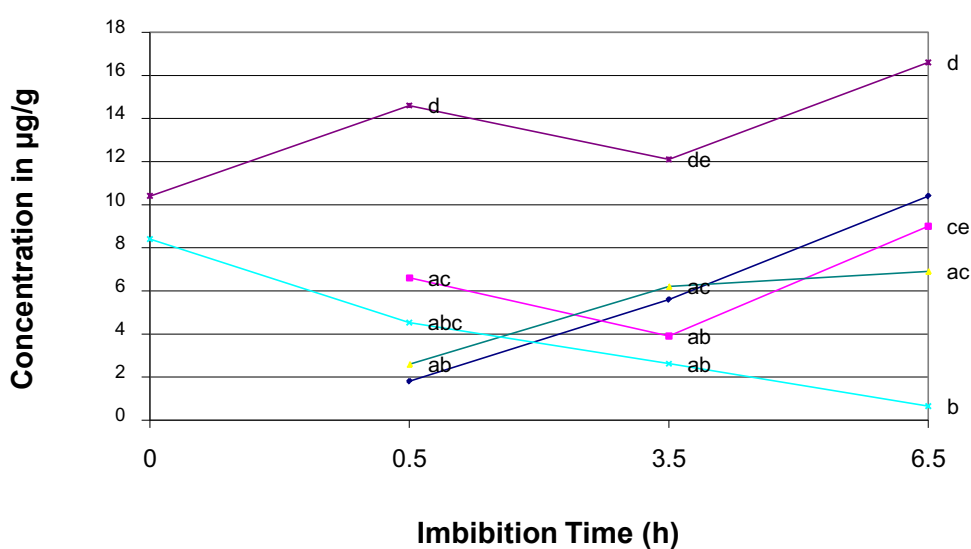
**Figure 5.23** Total amino acids in leachate for *Hybrid 6363* and *Hybrid 6321*.  $n=40$ . Values with the same alphabet are not significantly different ( $P < 0.001$ , Tukey post hoc test).



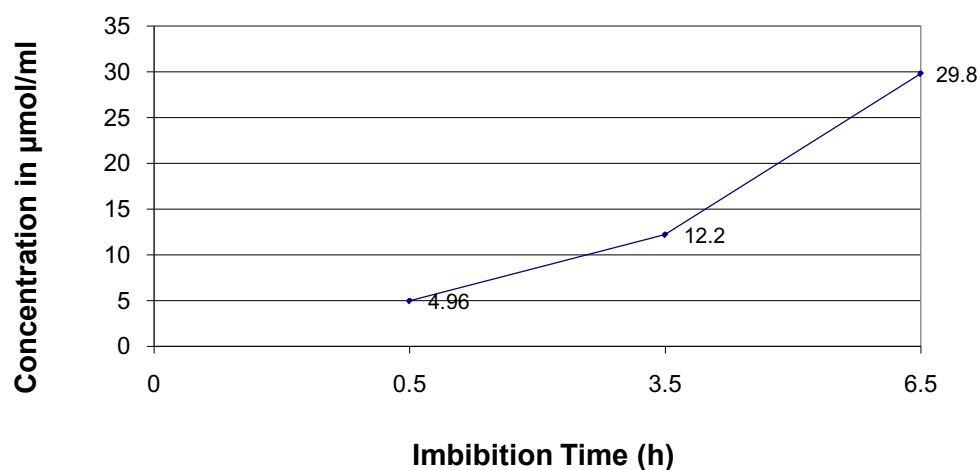
**Figure 5.24** Total amino acids found within the embryos/g tissue for *Hybrid 6363* and *Hybrid 6321* pre- and post-cryostorage. **Embryos from the original caryopses,** **embryos for stored caryopses,** **embryos for original caryopses after 12 months in cryostorage,** **embryos of new seed and embryos for new seeds after 12 months in cryostorage.**  $n=40$ . Values with the same alphabet are not significantly different ( $P < 0.001$ , Tukey post hoc test).



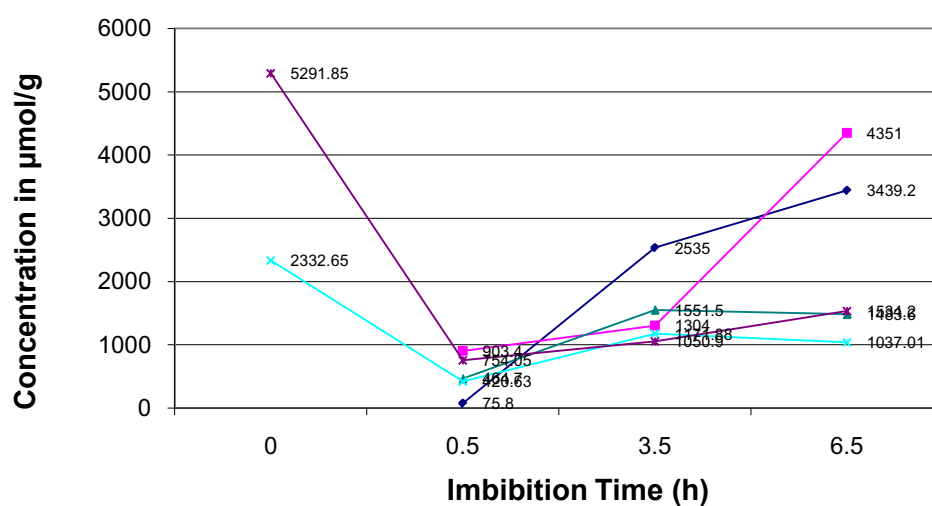
**Figure 5.25** Glucose quantities found within the leachate for *Hybrid 6363* and *Hybrid 6321*.  $n=40$ . Values with the same alphabet are not significantly different ( $P < 0.001$ , Tukey post hoc test).



**Figure. 5.26** Glucose quantities found within embryos/g tissue for *Hybrid 6363* and *Hybrid 6321* pre- and post- cryostorage. **Embryos from the original caryopses, embryos for stored caryopses, embryos for original caryopses after 12 months in cryostorage, embryos of new seed and embryos for new seeds after 12 months in cryostorage.**  $n=40$ . Values with the same alphabet are not significantly different ( $P < 0.001$ , Tukey post hoc test).



**Figure 5.27** Proline was only present in the leachate for *Hybrid 6363* (aged caryopses), it was not detectable or not present in *Hybrid 6321*. n= 40. Values with the same alphabet are not significantly different ( $P < 0.001$ , Tukey post hoc test).



**Figure 5.28.** Proline concentration present in embryos/g tissue for *Hybrid 6363* and *Hybrid 6321* pre-and post- cryostorage. **Embryos from the original caryopses, embryos for stored caryopses, embryos for original caryopses after 12 months in cryostorage, embryos of new seed and embryos for new seeds after 12 months in cryostorage.** n= 40. Values with the same alphabet are not significantly different ( $P < 0.001$ , Tukey post hoc test).

**Table 5.2** Statistical table indicating the individual treatment effects and their interaction effects. Caryopsis type (S/T), control tissue(C) vrs cryostored tissue (C/S), imbibition time (I/T) and the interaction of all three. Post hoc test results are listed in the table below. df = degree of freedom, Std E= standard error.

| <b>Biomolecule<br/>in Embryos</b> | <b>Comparisons</b> | <b>F</b> | <b>df</b> | <b>Std<br/>E</b> | <b>P</b> | <b>Interpretation</b> |
|-----------------------------------|--------------------|----------|-----------|------------------|----------|-----------------------|
| <b>Amino acids</b>                | S/T                | 39.6125  | 1         | 24               | 0.005    | Sig. diff.            |
|                                   | C vrs C/S          | 1.15     | 1         | 24               | 0.23     | Not sig.diff          |
|                                   | I/T                | 43,67    | 2         | 24               | 0.001    | Sig. diff             |
|                                   | Interaction        | 33.0812  | 2         | 24               | 0.001    | Sig.diff              |
| <b>Glucose</b>                    | S/T                | 22.7548  | 1         | 24               | 0.001    | Sig.diff              |
|                                   | C vrs C/S          | 91.0803  | 1         | 24               | 0.001    | Sig.diff              |
|                                   | I/T                | 4.919    | 2         | 24               | 0.016    | Sig.diff              |
|                                   | Interaction        | 8,3179   | 2         | 24               | 0.001    | Sig.diff              |
| <b>Proline</b>                    | S/T                | 88,072   | 1         | 24               | 0.001    | Sig.diff              |
|                                   | C vrs C/S          | 27,948   | 1         | 24               | 0.001    | Sig.diff              |
|                                   | I/T                | 136.065  | 2         | 24               | 0.001    | Sig.diff              |
|                                   | Interaction        | 56.062   | 2         | 24               | 0.001    | Sig.diff              |

All these observed differences could obviously be attributed to the difference in hybrid but it could also be due to the age of the caryopses. Amino acids like

glycine and glutamic were present, but only in very small quantities, once again suggesting that, much like proline, they too were retained to a large extent within the embryo. Due to the similarities in trends over the 6 hrs imbibition period, these amino acids will not be referred to. However, the data has been included in Appendix Two.

#### *5.3.7.2. Biochemical constituents in isolated embryos for stored tissue and new tissue pre- cryostorage and post 12 months cryostorage.*

Soluble or free amino acids and glucose were detected within the isolated embryos for both hybrids, and post hoc tests showed significant differences between the two hybrids (Table 5.2). With each imbibition time, water content increased (Table 5.1) and simultaneously triggered various biochemical processes. For *Hybrid 6363*, there was a steady increase in detectable total amino acids with each imbibition time (Fig. 5.24 and Table 5.2) i.e. the more hydrated the tissue became, the more amino acids were released from their bound state and possibly utilised in membrane repair (Oliver *et al.* 1997). Original tissue and stored tissue were not compared statistically for amino acids, glucose and proline, because stored tissue effectively became the control tissue for comparison with the 12 months cryostored sample. After one year in cold storage (stored tissue), the observed trend in amino acid found within the tissue remained statistically the same for 0.5 and 3.5 hours imbibition, but notably increased at 6.5 hours imbibition time (Fig. 5.24). This was possibly due to a greater percentage of the total amino acids becoming unbound with increasing imbibition time, and being made available for cellular repair of damage occurring during storage.

The subsequent effects of cryostorage on these biomolecules at 0.5 hours imbibition were not significantly different from the controls, but after 3.5 hours imbibition and at 6.5 hours, changes were noted (Fig. 5.24). At 0.5 hours, total amino acids were detected in greater concentrations post-cryostorage for the new tissue, and it was postulated that the freezing process itself was responsible for driving or pushing membrane bound molecules out of the membrane, resulting in their release. Alternatively, the actual production of these amino acids had increased to be used in the repair of membranes and other cellular components (Fig. 5.24) (Oliver *et al.* 1997) but, at 3.5 hours imbibition, these amino acids had

not as yet associated with the membranes. At 6.5 hours this value decreased considerably compared with the control (Table 5.2). The former explanation could no longer suffice unless, after 6.5 hours imbibition the reabsorption of previously free molecules were reincorporated into membranes and bound, possibly within vacuoles, rendering these molecules undetectable (Van der Willegen *et al.* 2001).

## 5.4. Summarising discussion and conclusions

### 5.4.1. Water content

The higher the moisture content of seeds and the higher the temperature at which they are stored the quicker viability is lost (Harrington 1959 and Simon 1973). By inference, seeds that have been in storage and which are found to have elevated water contents can be considered aged. As seeds age they germinate more slowly, respire slowly and become more susceptible to disease (Simon 1973) which, in itself, can cause increased water contents. The reduced vigour and viability observed in *Hybrid 6363* was again indicative of the ageing process. Several lines of evidence suggest that damage to cell membranes is a natural consequence of prolonged storage (Simon 1973; Bewley and Black 1994). The membrane aberrations which increase with age of the embryo (Berjak and Villiers 1972a; Begnami and Cortelazzo 1996) resulted in a greater rate of water uptake, the loss of biomolecules in the aged cells of *Hybrid 6363* and the attenuated viability and vigour, subsequent to the initial effects of imbibition was observed (i.e. aged tissue requires a longer period to repair). Cryostorage, therefore, proved more deleterious to this tissue since it had not yet repaired and was unable to withstand the effects of freezing and thawing, hence, the observation of increasing water contents, reduced viability and impaired rate of growth. It was expected that the freshly harvested caryopses, *Hybrid 6321*, would have demonstrated a greater ability to repair its membranes post-imbibition (at 3.5 hrs) given that they were freshly harvested and able to efficiently withstand the effects of cryostorage. However, these freshly harvested caryopses produced a larger percentage of abnormal plantlets i.e. lack of root formation, than the older caryopses, indicating that the tissue was more susceptible to loss of desiccation tolerance. The effects of imbibition and subsequent cryostorage were visible at the ultrastructural level for both these hybrids.

#### 5.4.2. Ultrastructural studies

Chabot and Leopold (1982) observed that as water is imbibed by seeds, rapid and extensive changes in physiology and structural changes occur. From the above data it can be concluded that the effects of imbibition on aged tissue were extensive, and in some cases deleterious, depending on the physiological state of the caryopsis as imbibition was initiated. Marked differences, at the ultrastructural level, were noted between *Hybrid 6363* and *Hybrid 6321*. These differences ranged from a compromise in plasmamembrane integrity, varying degrees of cell wall buckling in response to cryostorage, lack of defined lipid bodies, and lipid body alignment along the plasmamembrane, increased number of vesicles/vacuoles, and less defined nuclear membranes through the course of the imbibitional process and subsequent cryostorage. These characteristics were observed as a decrease in vigour and viability for *Hybrid 6363*, which is a feature associated with naturally aged seed (Bewley and Black 1994; Begnami and Cortelazzo 1996). At the ultrastructural level, it was clear that these caryopses had aged, and consequently, the axes were unable to withstand the effect of cryostorage. The combined effects of imbibition and cryostorage proved deleterious to the aged tissue, i.e. the subsequent effects of imbibition and cryostorage were exacerbated in the older caryopses. Whereas, the newer caryopses, *Hybrid 6321* appeared to sustain the effects of imbibition and demonstrated increased vigour and viability, but were unable to sustain the effects of subsequent cryostorage and gave rise to morphologically abnormal plantlets i.e. did not produce roots. One explanation for this observation, other than a change in freezing rate, could be that the caryopses were subjected to pre-conditioning treatments of rapid drying rates (Cordova-Tellez and Burris 2002b) which resulted in impairment of lipid migration to the plasmamembrane (Peterson 1997). This then compromised their ability to regulate water loss from the embryo tissues, resulting in reduced seed quality, and, although viability was maintained, ultimately, the acquisition of desiccation tolerance was compromised (Cordova-Tellez and Burris 2002a; Samarah 2007). For this reason, the freshly harvested caryopses were thought to be unable to “manage” the damage sustained during imbibition, and subsequent cryoprocurement proved to be deleterious.

#### 5.4.3. Biochemistry

The increase leakage of solutes by the material accompanies a decrease in viability (Nautiyal and Ravindra 1996; Thapliyal and Connor 1997; Shen and Oden 2000) and can, therefore, be associated with seed aging (Aiazzi *et al.* 1997). The present biochemical studies on the leachate showed that the older caryopses were more “leaky” than the recently harvested caryopses, as was described by Bewley and Black (1994), and therefore, the process of ageing had already occurred. In addition, those amino acids and glucose molecules retained within the embryo provide an indication of which solutes and their concentrations were necessary prerequisites for germination to reach completion and give rise to normal plantlets. With the overall quantities of these compounds reduced in aged caryopses, due to them being lost in the leachate, these axes were less likely to survive the effects of imbibition and subsequent cryostorage. Contrary to what was previously proposed, that the compounds released into the leachate acted as cryoprotectants, it would seem that newly harvested caryopses maintained the ability to retain amino acids and glucose, but had them mobilised within the tissue i.e. in an unbound state. Support by an uncompromised cytomatrix, which would be responsible for the motility of the organelles housing these molecules, ensured their availability for reconstitution into membranes and other metabolic activities, prior to the loss of desiccation tolerance. Hence, the observed increased viability percentages and rates of growth prior to cryostorage. The older tissue however, lost a greater percentage of electrolytes to the leachate, and probably, because total re-absorption did not occur within six hours of imbibition, they were unable to sustain the effects of cryostorage (See Chapter Four).

#### 5.4.4. Long Term Cryostorage

At every level, the effects of ageing could be seen, and as a direct consequence of these effects, *Hybrid 6363* was unable to survive cryostorage for any period. This was manifested as the lack of root formation in the developing plantlets. However, for *Hybrid 6321* at 0.5 hours imbibition and after 3.5 hours of imbibition, the lack of root development was noted in new caryopses for both short and long term cryostorage periods. Two possible explanations could provide adequate reasons for



the lack of root formation post-cryostorage. Firstly, the difference in rate of freezing and thawing, between the two vats, could have contributed to the observed differences. Secondly, possible damage incurred by the cytoskeleton during cryostorage could contribute to, or be the sole cause for, the lack of root formation. The extent of cytoskeletal damage post-cryostorage is investigated in the following chapter.

## Chapter Six                      Effects of early imbibition and cryostorage on cytoskeletal elements of cells in maize radicle tissues

### 6.1. Introduction

Cryopreservation of maize was investigated for the purposes of *in situ*, long term conservation. However, the successful cryopreservation of intact caryopses of a South African maize cultivar (*Hybrid 6363*) was not possible due to their large size, and thus, it was necessary to isolate the embryonic axes (which would give rise to a functional plant) and effectively reduce the size of the sample submerged in liquid nitrogen. Imbibition over a period of 30 minutes to 6.5 hours was employed to soften the testa and the pericarp, to successfully remove the intact axes from the caryopses. Extensive damage post-imbibition and subsequent cryostorage was noted in *Hybrid 6363*. Further investigation revealed that, due to unsatisfactory storage conditions, the ageing process had already commenced, and hence, *Hybrid 6363* was unable to withstand the effects of subsequent cryostorage (Walters *et al.* 2004). Young caryopses, for germplasm preservation (Smith and Berjak 1995; Bewley and Black 1994), was obtained (freshly harvested *Hybrid 6321*) from the same seed producer which was similar in size and shape, and treated with the same fungicide. The effects of direct immersion in liquid nitrogen on the entire caryopsis of *6321* were not determined, but due to the similarities in the two hybrids, it was assumed that they would respond in a similar manner (see Fig. 2.1). However, cryostorage of the more hydrated tissue proved deleterious to the development of functional roots within the freshly harvested material. The effects of early imbibition (within the first 6.5 hours), therefore, on the microtubule and microfilament elements of the radicle cells of maize zygotic embryos were investigated to determine if subsequent cryostorage of hydrated tissue altered cytoskeletal organisation. The data adds to our growing understanding of the ultrastructural changes which occur at the onset of imbibition and upon cryostorage (Isaacs and Mycock 1999). The response of maize embryonic tissue to cryostorage appears to be directly related to whether the tissue has retained or lost desiccation tolerance.

Many genes and integrated bio-processes are thought to be involved (Meagher *et al.*

1999) in the complex, multi-factorial trait of desiccation tolerance in seeds (Berjak *et al.* 1984; Bewley 1997; Alpert 2000; Faria *et al.* 2005). Their expression ultimately leads to mechanisms of both cellular protection and cellular repair. Cellular protection mechanisms are employed to sustain limited change/damage during drying itself. Cellular repair mechanisms would reverse any desiccation-induced change/damage, when the biologically appropriate hydration levels are re-established during imbibition (Kermode 1995). The specific imbibition time at which desiccation tolerance in seeds starts to be lost and desiccation sensitivity regained, is described by some authors, as being species specific (Seneratna and McKersie 1993; Bewley 1997), whereas, other authors view the concept of critical water content, on a species basis, as inappropriate, and should be viewed in terms of a continuum of behaviour rather than attempting precise categorisation (Pammenter and Berjak 1999; Cordova-Tellez and Burris 2002).

Water uptake during seed germination has been described as triphasic (Bewley and Black 1994; Bradford 1995; Bewley 1997) including an initial rapid period (phase I), followed by a plateau phase with little change in water content (phase II), and a subsequent increase in water content which coincides with radicle emergence and resumption of growth (phase III). Phases I and II are indicative of germination *sensu stricto*, during which imbibed seeds maintain their desiccation tolerance (Bewley 1997). According to Vertucci and Farrant (1995), orthodox tissue such as maize maintains desiccation tolerance between hydration levels of 8% (lowest hydration level prior to death by ultra-drying) and 45 to 70% water content, depending on the species. Phase III of water uptake is thought to occur above 70% water content.

Imbibition itself can be injurious (Bewley and Black 1994; Wojtyla *et al.* 2006; Garnczarska *et al.* 2007) and such damage is thought to be exacerbated during cryostorage, particularly if the material were to be frozen/thawed at a point after desiccation tolerance is lost. Possible changes in the plane of cell division as a result of the perturbation of the cytoskeleton, due to the effects of imbibition and cryostorage, are thought to result in the observed lack of radicle development into a functional root. This observation is speculated to be associated with the loss of desiccation tolerance prior to cryostorage. It is also surmised that the tissue are unable

to effectively protect itself from, or repair itself after the negative impacts of cryostorage.

Gallardo *et al.* (2001) have investigated a number of proteins, including the cytoskeletal proteins actin and tubulin, associated with the different phases of seed germination and priming. Both actin and tubulin ( $\alpha$  and  $\beta$ ) were found to be at increased levels during germination *sensu stricto* as well as increased levels during osmopriming and hydropriming (Gallardo *et al.* 2001). Considerable evidence has been obtained on the role of cytoskeletal elements within the cells of metabolically active tissue. Most of the studies reported on cytoskeletal function during cell division (e.g. formation of the pre-prophase band), cell growth (Overall *et al.* 2001), interactions between the cytoskeleton and the cell wall (Shibaoka and Nagai 1994), and how cytoskeletal elements in response to external stimuli are activated (McDowell *et al.* 1996). Seeds undergo metabolic adjustments, from the dehydrated resting embryo to the hydrated state, where desiccation tolerance (and the ability to be cryostored) that characterises the resting embryo, is lost (Vertucci and Farrant 1995). The cytoskeleton (key components in the metabolic regulation of the cell (Masters 1996)), therefore, plays a significant role in the biochemistry occurring during maturation drying and subsequent imbibition, as part of the seed's complex developmental programme and acquisition/loss of desiccation tolerance.

Cytoskeletal polymers are highly dynamic, capable of extending, shortening and moving within the cytomatrix in a time scale of seconds to minutes (Desai and Mitchison 1997). Plant cells are fundamentally different from most animal cells in that they are surrounded by walls that define their shape and fix their position within tissues (Derksen *et al.* 1990; Smith 2001). To achieve functional growth and development, cell division and expansion within plant tissues must occur in a highly coordinated fashion (Derksen *et al.* 1990; Díaz-Camino *et al.* 2004). Growing evidence suggests that the various elements of the cytoskeleton are responsible for the directional control of plant cell expansion, and that they respond in a coordinated way to environmental signals (Blancaflor 2000). The cytoskeleton is involved in cell division (Smith *et al.* 2001; Tylicki *et al.* 2003) and therefore central in the co-ordination of plant growth (Overall *et al.* 2001).

Kirschner and Mitchison (1986) stated that assembly of cytoskeletal elements in response to wounding in animal cell occurs in three phases. Initially there is a lag phase, followed by an exponential assembly phase, and finally, a stable plateau is reached in both abundance and polymerisation of these elements (Kirschner and Mitchison 1986). In response to wounding in animal tissue, cytoplasm viscosity is altered and assembly of cytoskeletal elements occurs commensurate with the polymer levels within the cytoplasm (Kirschner and Mitchison 1986). Water uptake by the embryo, which is injurious to the tissue, is speculated to induce a similar cytoskeletal response seen in the example above, and that the level of organisation seen in the cytoskeletal elements must be associated with tissue hydration levels. The following study focuses on the first 6.5 hours of water uptake, and attempts to provide further understanding on cytoskeletal activities in response to imbibitions and subsequent cryostorage.

## **6.2. Materials and methods**

### ***6.2.1. Imbibition of caryopses***

Intact caryopses of the *Zea mays Hybrid 6321* (PANNAR) were imbibed in sterile distilled water for 0, 3 and 6 hours. Embryonic axes in each treatment were then isolated, surface-decontaminated in a 2% v/v sodium hypochlorite solution for 15 minutes and washed in sterile ultra pure water for a further 15 minutes, thereby potentially increasing each imbibition time by a further 30 minutes.

### ***6.2.2. Water content determinations***

Individual embryo (40 from each imbibition time) water contents, gravimetrically expressed on a fresh weight basis, were determined post-surface-decontamination and after drying for  $17 \pm 1$  hours in a ventilated oven at 80°C according to the modified ISTA Low Constant Oven Temperature Method (International Seed Testing Association, 1985).

### **6.2.3. Vigour and viability assessment**

A further 40 isolated axes per treatment were placed into tissue culture tubes in an aseptic manner with a standard Murashige and Skoog (MS) (1962) basal medium (Appendix 1). The MS medium was supplemented with 30 g.l<sup>-1</sup> sucrose, 2 g.l<sup>-1</sup> Gelrite and adjusted to pH 5.8 prior to autoclaving at 121°C for 20 minutes. The material was incubated at 25°C with a 16:8h ; light:dark cycle with a light intensity of 200  $\mu\text{E.m}^2.\text{sec}^{-1}$ . Developing seedlings were monitored every 24 hours and viability recorded as the number of axes germinated. Germination was determined by the emergence of either the root or shoot, or both. Vigour was assessed by the rate of growth. The germination index devised by Czabator (1962) (see 2.2), which provides an indication of the percentage germination and rate of growth, was calculated for all treatments.

### **6.2.4. Cryostorage**

Isolated embryonic axes from each treatment (10 per treatment) were placed into cryovials on cryocanes and plunged directly into liquid nitrogen, cooling the tissue at a rate of approximately -200°C/min. The samples were held in liquid nitrogen for 15 minutes, after which, the cryocanes were immersed into a 30°C water bath, thereby thawing the embryonic axes. Each treatment was repeated a minimum of three times.

### **6.2.5. Fixation and wax-embedding**

After each imbibition time, the terminal 5mm of the isolated embryonic radicles (10 replicates), including the meristems, was excised and placed into microtubule-stabilising buffer (MTSB) (Baluška *et al.* 1993; Vitha *et al.* 1997) which contained 0.5ml of dimethyl sulfoxide (DMSO, Sigma). All specimens were incubated for 15 minutes at room temperature and then fixed with 4% (v/v) paraformaldehyde in the MTSB and DMSO mixture for one hour. Following a brief rinse in MTSB, the specimens were dehydrated through a graded ethanol series diluted with phosphate buffered saline (PBS). Tissue segments were infiltrated at 37°C with mixtures of absolute ethanol plus Steedman's wax (Vitha *et al.* 1997) made up in the proportions

of 2:1 (v/v) overnight; 1:1 and 1:2 (v/v) at two hours for each step, followed by two changes of pure wax (of 15 minutes each) under vacuum. The specimens were then transferred to metal moulds with fresh wax at 37°C overnight, again under vacuum. The following day, all specimens were optimally re-orientated for sectioning and allowed to polymerise at room temperature.

#### **6.2.6. Microtoming and de-waxing**

Transverse 3 - 5  $\mu\text{m}$  sections were cut from these wax blocks with a Leitz Wetzlar 1512 microtome and the sections were expanded on a drop of warm water. The sections were then mounted onto albumen adhesive microscope slides (Brown *et al.* 1989) and allowed to dry. De-waxing with ethanol as described by Vitha *et al.* (1997) was initially attempted, but the wax was not completely removed. Xylene was used to de-wax the sections with greater success and they were subsequently rehydrated through an ethanol series as described by Vitha *et al.* (1997) for 3 - 4 minutes, with the final step in distilled water.

#### **6.2.7. Application of fluorescent tags**

Monoclonal Anti Beta Tubulin Cy<sub>3</sub> (Sigma) was utilised to localise microtubules (Siddiqui *et al.* 1989) and TRITC - Phalloidin (Sigma) utilised for the localisation of filamentous actin (Wehland *et al.* 1977). Each was mixed in its appropriate buffer (Brown *et al.* 1989) together with combinations of 2% (v/v) methanol, 1% (v/v) Triton X and 1% (v/v) DMSO, to facilitate the movement of the tagged reactants into the tissue (Brown *et al.* 1989). The tissue was exposed to the antibodies for 75 minutes in the dark and washed three times with the appropriate buffer (See Appendix 3). The sections were then mounted in an antifade agent (CitiFluor) and cover slips were sealed with clear nail varnish. Unimbibed tissue, not treated with either of the tagged reactants, was also observed for any possible autofluorescence.

### **6.2.8. Viewing and photography**

The meristematic region of each radicle was sectioned transversely to reveal a number of tissue layers, viz. the epidermis, outer and inner cortex, meristem, pro-endodermis and parenchyma cells. Dry, unimbibed (control) tissue was examined using a Zeiss fluorescence microscope to ascertain the extent of autofluorescence. Observation of the microtubules and microfilaments, in tissues of all treatments including the control, was carried out using a Zeiss LSM 3.90 set to a pinhole of 22 equipped with confocal software. TRITC is optimally excited at 554 nm with emission between 570 and 580 nm, while Cy<sub>3</sub> was excited at 554 nm with emission at 568 nm. Given the limitations of this microscope, both fluorochromes were detected in this window. Optical sections were acquired at 0.3  $\mu$ m intervals and processed using Adobe Photoshop 7.0. The images presented are the stacked projections of a series of optical sections.

### **6.2.9. Statistical analysis**

Water content data sets were arcsine transformed to approximate normality and subjected to a one-way ANOVA using Statistica 6.0 (Statsoft 2001). Tukey post-hoc tests were used to establish significant differences between imbibition times. Percentage viability prior to and post-cryostorage, were statistically compared using Fisher's exact tests, and the percentage of radicle and shoot formation were analysed using Contingency tables and Chi square testing, all using Instat version 3.0 (GraphPad software 2003).

## **6.3. Results and discussion**

The extent of cryodamage on imbibed tissue, upon initial examination of developing seedlings, was visualised by the presence or absence of shoot and root formation. Almost 100% viability was attained for each treatment with no significant difference between them (Fisher's exact test:  $P=1,000$ ). However, only a very small percentage of the embryos developed roots, and only these developed into normal seedlings (Table 6.1). The remainder of the embryos produced shoots only, and developed into



deformed seedlings which remained viable within culture tubes for approximately two weeks, after which they died.

**Table 6.1.** Water content, viability and root/shoot development of isolated maize axes after various imbibition times, followed by surface-decontamination and cryostorage. Standard deviations (SD) with the same letter were not significantly different at the  $P=0.05$  level.

| Parameters                                            | Imbibition time (hours)     |                             |                                          |                                          |
|-------------------------------------------------------|-----------------------------|-----------------------------|------------------------------------------|------------------------------------------|
|                                                       | 0(SD)                       | 0.5(SD)                     | 3.5(SD)                                  | 6.5(SD)                                  |
| Water content                                         | 8.66 <sup>x</sup><br>(0.96) | 56.74 <sup>y</sup><br>(3.9) | 58.21 <sup>■</sup><br>(3.0) <sup>y</sup> | 58.28 <sup>■</sup><br>(3.9) <sup>y</sup> |
| Viability and root formation prior to cryostorage (%) | 100 <sup>°</sup>            | 100                         | 100                                      | 100                                      |
| Root development after cryostorage (%)                | 100 <sup>°</sup>            | 10                          | 37.5 <sup>■</sup>                        | 0 <sup>■</sup>                           |
| Shoot development after cryostorage (%)               | 100                         | 90                          | 100                                      | 97.5                                     |

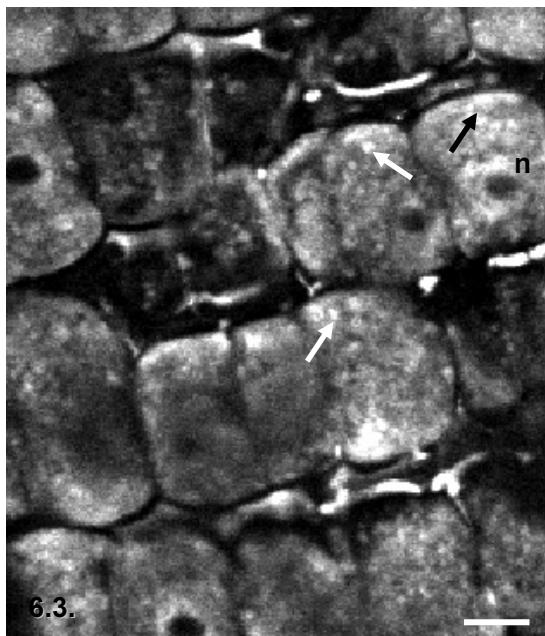
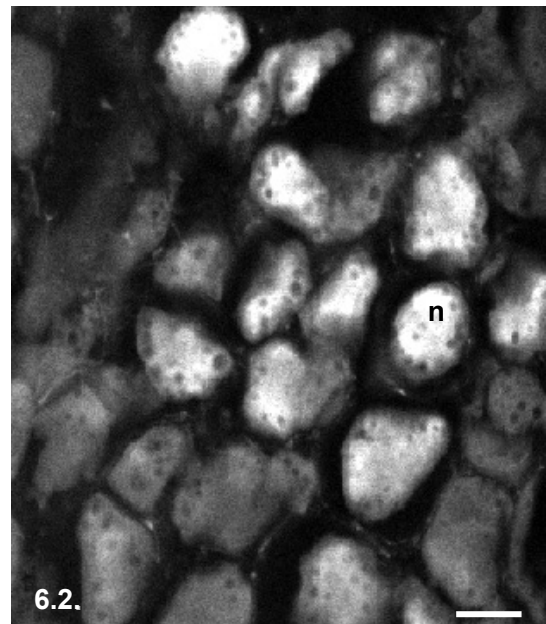
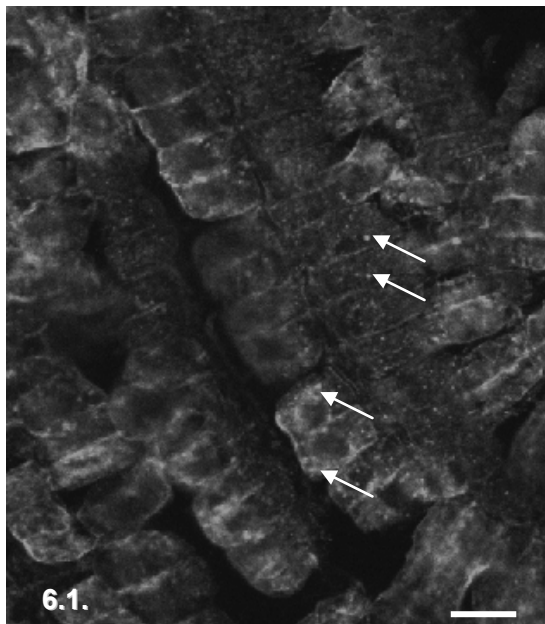
<sup>°</sup> In dry tissue there is no significant difference in viability pre and post-cryostorage ( $\chi^2=3.02$ ,  $P=0.389$ ).

<sup>■</sup> A significantly higher percentage of the 3.5 hours embryos than the 6.5 hours imbibed embryos (Fischer's exact test:  $P<0.0075$ ) developed into normal seedlings.

<sup>■</sup> w/c's at 3.5 hours and 6.5 hours after imbibition were not significantly different (Fischer's exact test:  $P<0.0075$ ).

It was postulated that the cytoskeletal elements within the cells of maize zygotic embryonic axes disassociate into their constituent subunits during maturation drying, as has been demonstrated to occur in the seeds of *Medicago truncatula* (Faria *et al.* 2005). Therefore, it would be expected that cytoskeletal elements are in a disassembled state in dehydrated, quiescent tissue and within the early imbibition phases of this maize hybrid. Auto-fluorescence of cell walls and cellular content was not detected in the unimbibed (control) tissue (*Hybrid 6321*). After fluorescent tagging, a small positive signal, for assembled tubulin (Fig. 6.1) in the outer cortical cells in the meristematic region of the dry root apices, was observed.

**Figures 6.1 - 6.4** Confocal images of unimbibed radicle/root in the vicinity of the meristem tissue pre (Figs 6.1 & 6.2) and post-cryostorage (Figs. 6.3 & 6.4). **Fig. 6.1.** Dry tissue tagged for tubulin indicating aggregations of tubulin. The brighter spots as indicated by the arrows are possibly polar views of these aggregations. Scale bar =10.16  $\mu\text{m}$ . **Fig. 6.2.** Dry tissue tagged for F actin note fluorescent patches in certain outer cortical cells and, where visible, in close association with the nucleus. Scale bar = 3.5  $\mu\text{m}$ . **Fig. 6.3.** Diffuse microtubule subunit arrangements were present (see arrows). Scale bar=5  $\mu\text{m}$ . **Fig. 6.4.** Diffuse actin in the meristematic zones appeared to be in a disassembled form. Scale bar=6.94  $\mu\text{m}$ .



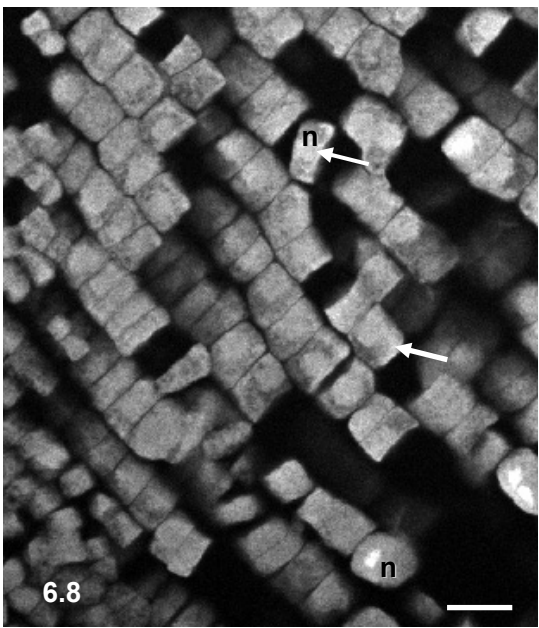
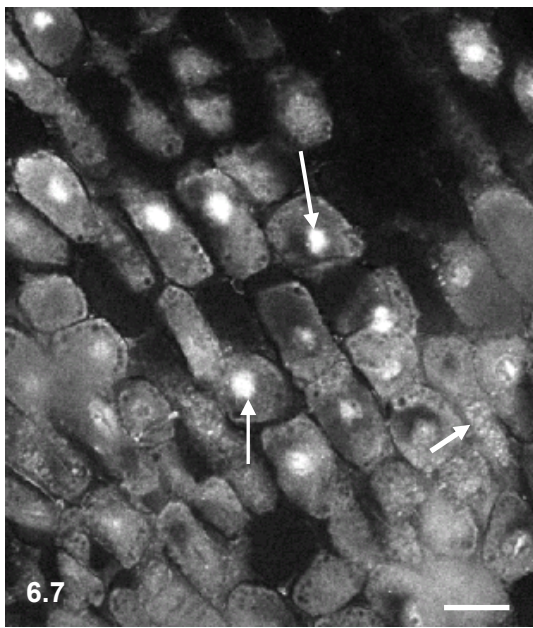
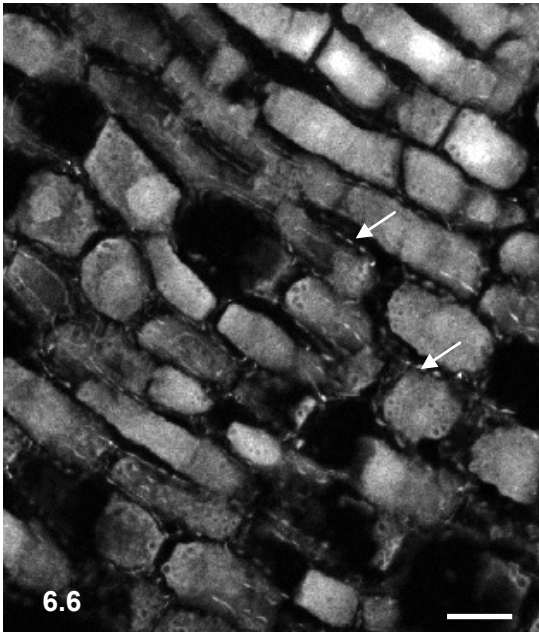
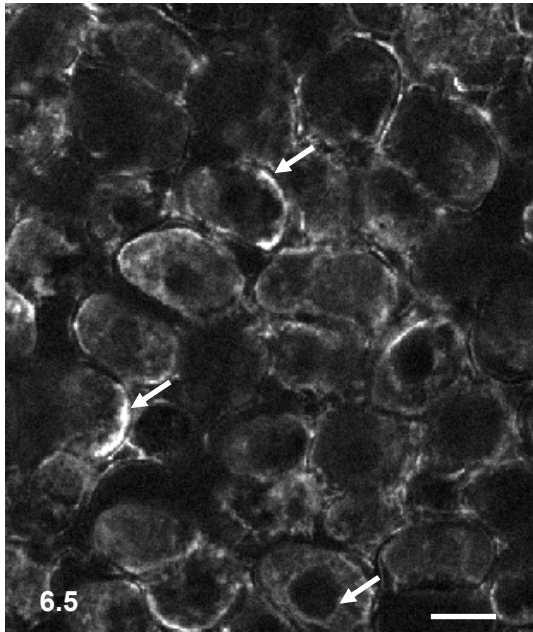
Where present, the signal was restricted to regions directly underlying the cell wall. Both actin and tubulin (in assembled form) have been detected in orthodox seeds before and after the priming processes (Gallardo *et al.* 2001). Overall *et al.* (2001) have shown similar patterns in cells of maize roots and described these as the microtubules which form the transverse arrays. There is strong evidence that microtubules control the precise alignment of deposition of the cellulose microfibrils in the cell wall (Cyr 1994) and subsequently impact on the direction of cell expansion (Overall *et al.* 2001). Although this tissue was in a quiescent state, the cortex of these cells did have aggregations of tubulin, the polar views of which should appear as brighter spots, and where observed in Fig. 6.1, indicated that a degree of microtubule polymerisation had commenced. Polymerisation could have occurred during the surface-decontamination step, as the formation of microtubules can occur within seconds of hydration (Desai and Mitchison 1997) or the presence of polymerised tubulin indicates *Hybrid 6321* caryopses were primed (Gallardo *et al.* 2001).

The dry tissue stained with the F actin-specific-phalloidin only displayed fluorescent patches in some outer cortical cells and, where visible, these were closely associated with the nucleus (Fig. 6. 2), which would be expected from previous ultrastructural data (Isaacs and Mycock 1999). Actin filaments and bundles have been described as a complex network that reaches into nearly every part of the cytoplasm, also forming a basket around the nucleus and connecting to the cortical cytoskeleton (Meagher *et al.* 1999). Combinations of various actin-mediated processes are thought to be necessary for the rapid response of plant cells to internal or external signals (Meagher *et al.* 1999). Although this general arrangement of F-actin was recorded for fully-hydrated *Arabidopsis* cells grown in suspension, it is interesting that, in some of the cells in this dry maize tissue, actin bundles/filaments were also seen in association with the nuclear region (Fig. 6.2). In cells where fluorescence was not visible it was assumed that the actin was in a disassembled form, or was not obviously connected to the main network, and possibly, in the form of short filaments. It has been reported that cells in this state (i.e. where MFs and microtubules are semi-polymerised) are able to survive preservation in liquid nitrogen better (Morisset *et al.* 1993) than cells with polymerised microtubules.

Lower freezing temperatures induce a higher degree of actin depolymerisation and result in a stable state (Egierszдорff and Kacperska 2001). Microtubules with differing cold sensitivities can exist within the same cell (Kerr and Carter 1990), and perhaps, the more cold-stable cytoskeletal elements in depolymerised form, provide one of the reasons why cryo-success is obtained at this hydration level. It is expected that the freezing induced changes in the structure of actin filaments may be an important element in development of cell resistance to freezing (Egierszдорff and Kacperska 2001). Depolymerised cytoskeletal elements are also less likely to provide nucleating sites for ice crystal formation than in the polymerised form, and could reduce cellular damage from cryostorage. The tissue in the current study may be sufficiently dry, with too little water available for ice crystal formation to occur. Depolymerised actin and tubulin also contribute to changes in membrane fluidity (Browse and Xin 2001) which is crucial for survival post-cold stress (Xiong *et al.* 2002), although this would be more applicable to tissue in a hydrated state.

After cryostorage (Fig. 6.3), microtubule disposition was similar to that in the non-cryostored tissue (Fig. 6.1) i.e. present but diffuse. Actin filament arrangements of the cryostored unimbibed material (Fig. 6.4) also appeared diffuse and in a disassembled form. Therefore, dry tissue (0 hrs imbibition) is typified by depolymerised cytoskeletal elements. Cryostorage of dry axes were not significantly different to the uncryostored dry material and produced viable seedlings (Table 6.1). Survival is therefore presumably a factor of the reduced water content, i.e. <10% w/c, at which there is little likelihood of ice crystal formation or increased cytoplasm viscosity, and a paucity of organised cytoskeletal elements. Collectively, the data showed that cryostorage had no apparent effect on the physical make-up of the dry tissue or its ability to recover from its effects. This result is in accordance with the observations of Kerr and Carter (1990), who postulated that microtubule depolymerisation is important for achieving maximal freezing tolerance. In the context of the present study, the depolymerised state is probably an inevitable consequence of seed maturation drying to water contents of less than 10%. The added effect of low-temperature-induced microtubule depolymerisation (Kerr and Carter 1990) (the extent of which would need to be the focus of another study) by exposing the tissue to liquid nitrogen, must contribute to the stability of the cytoskeletal elements and confer maximal freezing tolerance.

**Figures 6.5 -6.8** Cross sections of the terminal 5mm radicle ends at the first hydration level ( $\pm 56.71\%$ , imbibed 0.5 hrs) pre (Figs. 6.5 & 6.6) and post-cryostorage (Figs. 6.7 & 6.8). **Fig. 6.5.** Tubulin fluorescence was prominent in stained cortical tissue, associated with the cell periphery, and in some cells, around the nuclei ( $\leftarrow$ ). Scale bar=4.23 $\mu$ m. **Fig. 6.6.** Epidermal cells stained for actin, fluorescence indicates short filaments associated with the cell wall( $\leftrightarrow$ ). Scale bar=5.15  $\mu$ m. **Fig. 6.7.** Cryostored meristematic tissue showed microtubular subunits around the nuclear region as well as many polar aggregations of tubulin. Scale bar=7.95  $\mu$ m. **Fig. 6.8.** Microfilaments within the near meristematic tissue appeared to be in close association with the cell wall. However, fluorescence within the cytoplasm appeared diffuse. Scale bar=15.9  $\mu$ m



It is, therefore, not surprising that the dry unimbibed embryos survived the cryostorage process and developed into normal seedlings.

Cortical cells at the first hydration interval (0.5 hrs surface-decontamination; water content (w/c)  $\pm 57\%$ ), displayed little tubulin fluorescence, and when present, such fluorescence was not restricted (Fig. 6.5). A reduction in microtubule density in most cases limits the resolution of fluorescence microscopy (Williamson 1991), and therefore, the microtubules were presumed to be in a depolymerised state. The same amount of polymerised tubulin is speculated to be present, as in the dry tissue, but was dispersed throughout a more hydrated cytomatrix. Nevertheless, fluorescence, when visible, was associated with the cellular cortex immediately below the cell wall and could represent the early development of a cortical array of developing microtubules. Similarly, actin within epidermal cells, at the same hydration interval, appeared as short filaments associated with the cell wall regions or surrounding the nucleus (Fig. 6.6), but in many cells fluorescence was diffuse.

The samples hydrated to the same level, but subsequently cryostored, demonstrated the presence of microtubular subunits in the centre of meristematic cells (Fig. 6.7). This aggregation of tubulin/microtubular elements around the nucleus was probably due to the dehydrating effects of the cryoprotocols (Crowe *et al.* 1990) i.e. upon submersion in liquid nitrogen; water freezing intracellularly resulted in damage to the cytoskeleton and possibly led to its collapse around the nuclear region. Similar “collapse” of the cellular matrix and organelles to the peri-nuclear region has been observed by Berjak *et al.* (1999) as a response of recalcitrant desiccation-sensitive zygotic axes to physical drying, and this was ascribed to the derangement of the cytoskeleton. Instead of the microtubules being transversely-aligned about the nucleus as seen in *Lolium* leaf tissue (Clearly and Hardham 1993) and pea roots (Hush and Overall 1992) prior to wounding, they were found to be aligned longitudinally (Fig 6.7). This change in spatial orientation occurs as a direct response to wounding (Hush and Overall 1992), and the maize cellular response to cryostorage could be similar to that of wound-induced re-orientation observed in pea roots. Tissue tagged with phalloidin displayed an arrangement of actin filament fluorescence (Fig. 6.8) similar to that of the non-cryostored material (Fig. 6.6). In meristematic tissue, faint fluorescence appeared in close association with the cell



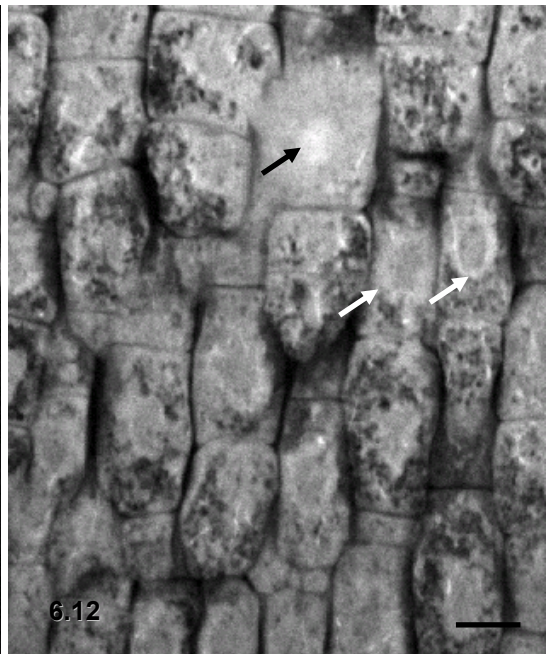
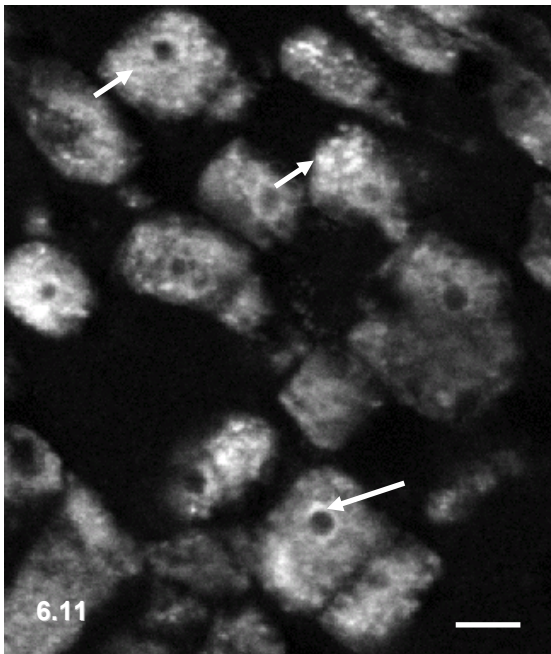
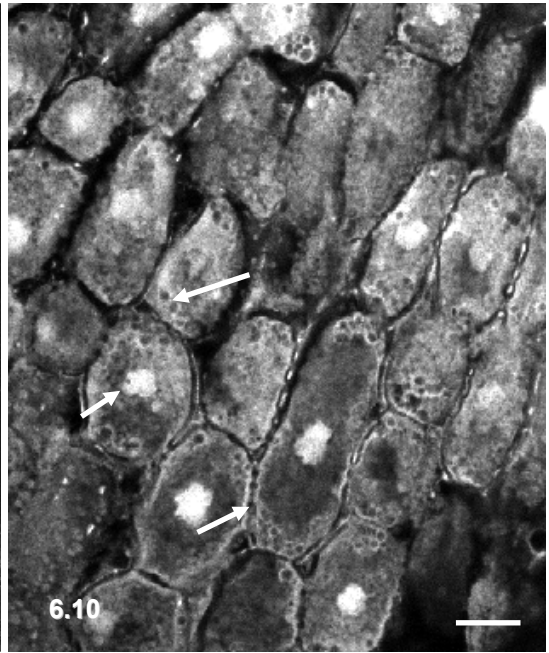
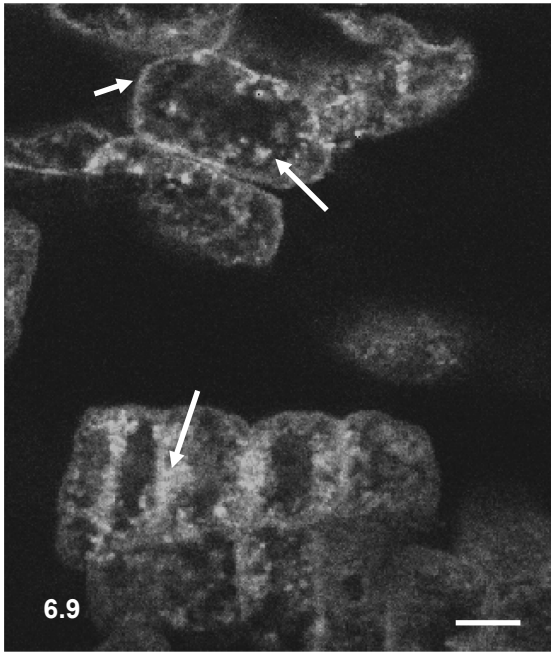
wall and in some cells around the nucleus (Fig. 6.8). However, fluorescence within the cytomatrix appeared generally diffuse (Fig. 6.8) indicating that the microfilaments were labile and possibly disassembled as a result of the cryo-procedure at this hydration level.

At the second hydration level (3.5 hrs imbibition, w/c  $\pm 58\%$ ) both polymerised tubulin (Fig. 6.9) and actin microfilaments (Fig. 6.10) within cells of the meristematic zones were more prominent, despite the fact that the water content was not statistically different from the tissue at the first hydration interval. Throughout the cytomatrix, tubulin reorganisation, possible nucleating microtubular aggregations, of varying sizes, were observed (Fig. 6.9). Although present throughout the cytomatrix, microtubules were concentrated at the plasma membrane, and consequently, underlying the cell wall. Microfilaments predominated around the nucleus and clearly delineated a variety of cytomatrical structures, presumably organelles, and continued to be associated with the cell walls (Fig. 6.10).

Fluorescence in tissue subjected to cryostorage at this hydration level (3.5hrs), for both tubulin and actin filaments, were very different from those seen at the first hydration interval. In post-cryostored material, tubulin was found to be diffuse in the cytoplasm of some cells, and concentrated around the nuclei (Fig. 6.11). This accumulation around the nucleus was absent in tissue that was not cryostored (Fig. 6.9). An interpretation of this is that many of the microtubules depolymerised in response to cryostorage and retracted to their nucleation sites at the microtubule organising centres (MTOC's). MTOC's are considered to predominate at the periphery of the nucleus during interphase (Becker *et al.* 2009). Interphase cells are characterised by the cortical microtubules aligned in roughly parallel arrays under the plasmamembrane (Sonesson *et al.* 1997), the orientation of which, is under strict developmental control (Blancaflor and Hasenstein 1993). Fig. 6.11 also shows cells exhibiting aggregations of tubulin, in polar view, as many brighter spots. Microtubules have been reported to depolymerise at least partially in response to

**Figures 6.9-6.12**

Tissue at the second hydration interval ( $\pm 58\%$ , imbibed for 3.5 hours). **Fig. 6.9** Tubulin localisation, meristem, pre-cryostorage shows reactivity of tubulin (Scale bar= $5.65\mu\text{m}$ ). **Fig. 6.10.** Actin localisation, of meristem pre-cryostorage, is well resolved. (Scale bar= $7.14\mu\text{m}$ ). Microfilaments appeared to surround the nuclear regions and individual organelles, and fluorescence was maintained in association with the cell walls **Fig. 6.10.** Tubulin however, appeared to occur as a possible combination of both its polymerising form i.e. the appearance of microtubule organising centres (MTOCs larger dots) and subunit form (smaller fluorescent speckles) **Fig. 6.11.** Tubulin localisation, root cortex, post-cryostorage demonstrated fluorescence around the nuclear region (not seen in the pre-cryostored tissue [Fig. 6.9]) as well as what are interpreted as randomly distributed cortical microtubular arrays. (Scale bar= $4.35\mu\text{m}$ ). **Fig. 6.12.** Actin localisation, meristematic zone, post-cryostorage. Some cells fluoresced slightly; however, fluorescence was diffuse, except where visualised in the perinuclear area in some cells. (Scale bar= $4.69\mu\text{m}$ ).



freezing, leading to the predominance of short microtubule fragments (Kerr and Carter 1990) and/or cryostorage-disrupted microtubule orientation which explains the observed specks and clumps of tubulin arranged randomly within the cytoplasm (Fig. 6.11). Assembled tubulin polymers were associated with the plasma membrane (Fig. 6.11), and their interactions are important for the regulation of cellular activities, including cellulose microfibril deposition essential for determining the direction for cellular expansion (Sonesson *et al.* 1997). This would indicate that cryostorage at this hydration level did not completely deter cellular functionality. The root cellular response, particularly to osmotic stress, necessitates rapid and reversible retractions of their protoplasts from the cell walls (Oparka and Crawford 1994; Komis *et al.* 2003), and, to maintain mechanical integrity, must retract their protoplasts from their cell walls immediately (Baluška *et al.* 2003) presumed to be facilitated by the tubulin polymers associated with the plasmamembrane.

In all tissues subjected to cryostorage after imbibition, there was a loss of organisation of actin (compare Fig. 6.12 with Fig. 6.10). Diffuse fluorescence was visible in some cells, which is indicative of depolymerisation, in response to cold temperatures, as stated previously (Egierszdorff and Kacperska 2001). Although the temperature in the current investigation ( $-196^{\circ}\text{C}$ ) was much colder than those in that study (from  $-3^{\circ}\text{C}$  to  $-25^{\circ}\text{C}$ , Egierszdorff and Kacperska 2001), freezing maize radicle tissue resulted in a similarly substantial loss in the organisation of actin seen during cryostorage. Therefore, cryostorage infers massive depolymerisation of actin at a water content of  $\pm 58\%$ . Prior to cryostorage, microfilaments appeared highly organised (Fig. 6.10). Post-cryostorage, cellular integrity was maintained; the isolated axes survived the effects of cryostorage and developed into seedlings with roots. Statistically, a significantly higher percentage of the 3.5 hours embryos than the 6.5 hours imbibed embryos (Table 6.1) developed into normal seedlings, despite the water contents not being significantly different between these times (Table 6.1). Therefore, at the second hydration interval, it appears that this tissue maintained its tolerance to desiccation and hence was able to withstand the effects of cryostorage (Vertucci and Farrant 1995).

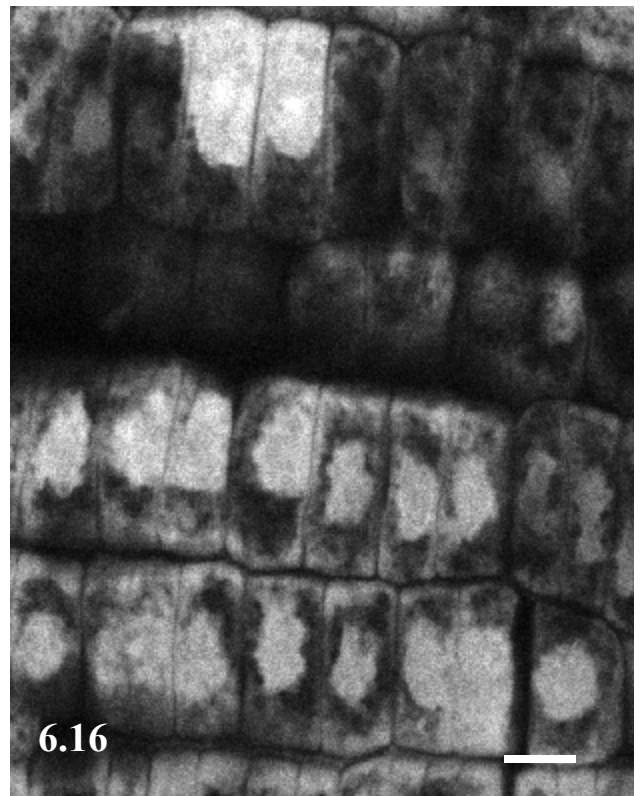
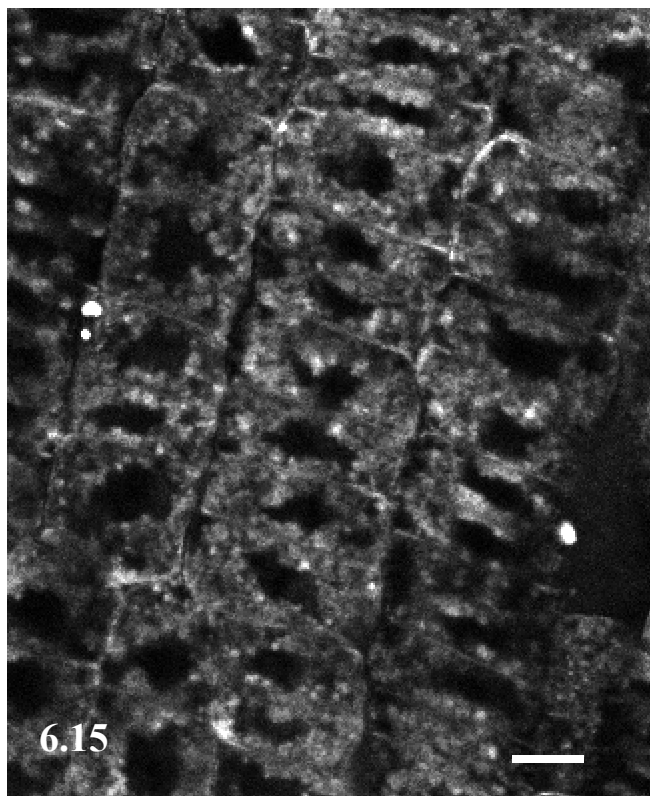
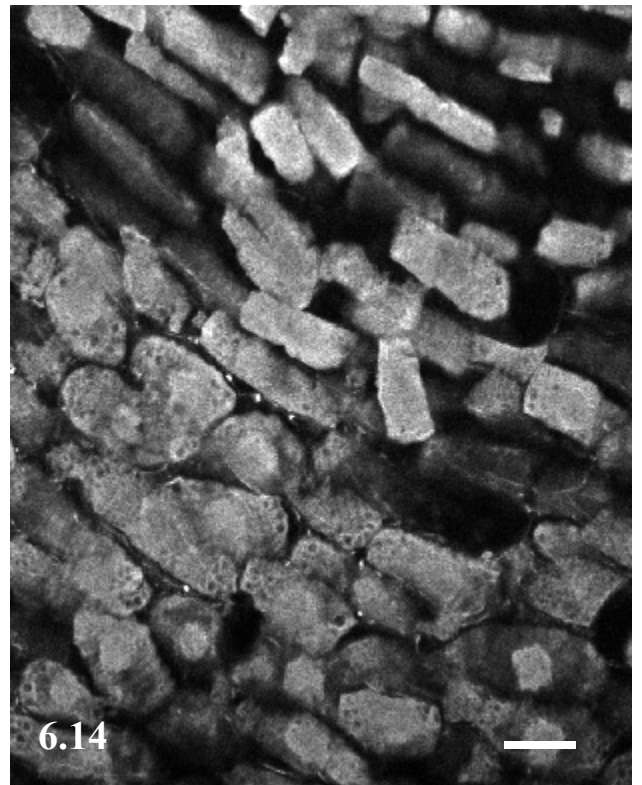
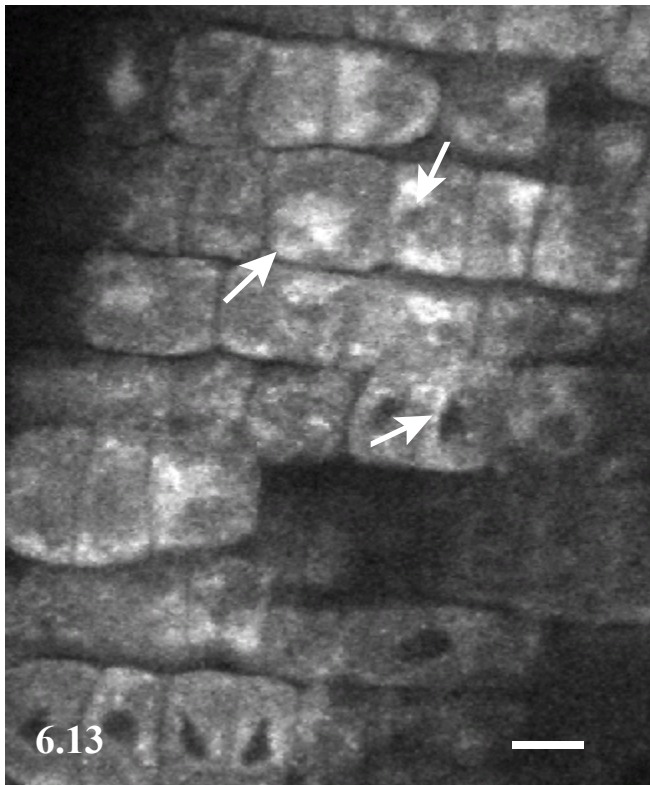
At the third hydration interval (6.5 hrs imbibition; w/c  $\pm 58.28\%$ ), microtubule assembly was evident around the nucleus and in the cortex of the cells (Fig. 6.13). Actin filaments (Fig. 6.14) were visible in the assembled form as randomly orientated strands throughout the cell, at the periphery of the cell and around the nuclei. Clearly, the cytoskeleton was at a higher level of organisation (Fig. 6.13) than observed after 3.5 hours imbibition (Fig. 6.10). Subsequent cryostorage at this hydration level resulted in the collapse of the actin network.

In summary, at the third hydration level, cytoskeletal elements were further along their developmental pathway (Figs. 6.13 and 6.14) and the tissue itself had become hydrated beyond its threshold to withstand the negative impact of cryostorage (Table 6.1). Cryostorage at this hydration level, led to the depolymerisation of tubulin (Fig. 6.15) and actin (Fig. 6.16) resulting in cryo-lability. The tissue at the third hydration level, although not significantly different in water content from the second hydration level (Table 6.1), appeared to be at a more progressed stage of microtubule and microfilament assembly. Exposure to the cryogen proved to be totally disruptive to the radicle cells, therefore, the axes failed to produce roots.

Within the dry mature maize caryopsis, water content is between 5 - 15% (Bewley and Black 1994) and metabolic activity is suspended, viz. the caryopsis is quiescent (Bewley and Black 1994; Gallardo *et al.* 2001). For germination to occur, the seed needs to be hydrated to levels that promote metabolism, at a suitable temperature and in the presence of oxygen (Bewley 1997; Gallardo *et al.* 2001). In the present experiment the caryopses hydrated rapidly within the first 30 minutes and had possibly completed Phase I of water uptake. Phase II of water uptake in seeds is associated with a number of metabolic initiations/stages other than cytoskeletal assembly e.g. replication of mitochondria (Morohashi 1986; Bewley 1997), protein synthesis, and repair of DNA damage during the rehydration of tissue (Osborne and Boubriak 1994). It is the initiation of these metabolic processes that causes the lag phase seen in germination, and it is their activity that ultimately results in radicle protrusion (Phase III). Faria *et al.* (2005) show that radicle emergence occurs at water contents above 70%.

**Figure 6.13-6.16.** Embryonic axes imbibed for 6.5 hours ( $\pm 58\%$  w/c). **Fig. 6.13.** Tubulin localisation, pro-endodermis, pre-cryostorage, microtubule assemblies can be seen around nuclear regions and cytoplasm periphery. (Scale bar=4.81  $\mu\text{m}$ ). **Fig. 6.14.** Actin localisation, root cortex cells, pre-cryostorage, appears similar to actin filament arrangements observed in material imbibed for 3.5 hours (Scale bar=6.58 $\mu\text{m}$ ). **Fig. 6.15.** Tubulin localisation, vascular parenchyma nears the meristematic zone, post-cryostorage. Tissue could not withstand the effects of cryostorage, tubulin appears grainy and speckled and the actin localisation diffuse, parenchyma near the meristematic zone, post-cryostorage (**Fig. 6.16**) appeared dedifferentiated. Scale bars=6.25 $\mu\text{m}$  and 6.02 $\mu\text{m}$  respectively.





Tissue hydrated for 6.5 hours remained below this level and radicle emergence had not occurred. Therefore, it was presumed that the tissue had not yet become desiccation sensitive. However, at this hydration level it cannot be said with certainty that the tissue was not metabolically active, and this would need to be tested in future. Chang *et al.* (2001) suggest that protein assembly occurs in the absence of vigorous metabolic activity. In addition, many proteins and mRNAs are stored during maturation drying (Bewley and Black 1994) and upon rehydration are utilised (not necessarily synthesised). McDowell *et al.* (1996) demonstrated evidence for the expression of the actin-producing gene (ACT7) as early as 24 hours post-imbibition in *Arabidopsis*. This could also be the case in maize embryos if the developing caryopsis stored actin and tubulin mRNA during maturation drying for use in subsequent germination. A more likely scenario is that the depolymerised actin and tubulin monomers that accumulate, as a result of maturation drying, are mobilised with rehydration, repolymerise, and are available to facilitate immediate recovery initiatives at this early stage of rehydration. This would explain the degree of organisation seen within the first 6.5 hours of imbibition.

Cytoskeletal construction, at the very onset of imbibition activity, had commenced to the degree that subunits of microtubules and microfilaments were mobilised and organised within the first 6.5 hours of imbibition. Although cytoskeletal formation was not restored to fully functional levels by this stage, the cell contents have been mobilised e.g. expansion of the cytoplasm after 30 minutes surface-decontamination (Isaacs and Mycock 1999) in association with the partly assembled microfilaments and MTs. Subsequent cryostorage on a partly-assembled cytoskeleton proved injurious to the developing embryos and interrupted normal root development.



Microtubules and microfilaments can alter their arrangement in response to environmental and developmental signals, i.e. metabolic activity *per se* is not required (that is the release of energy via ATP or GTP hydrolysis) for cytoskeletal subunit assembly to occur (Desai and Mitchison 1997). Changes initiated by the process of imbibition in tissue at water contents of  $\pm 52\%$  and higher were exacerbated by cryostorage, and were more severe in tissues with higher water contents (Table 6.1). Many biomolecules are known to be mobilised in preparation for impending biochemical processes and are dependent on a functional cytoskeleton (Masters 1996). Similarly, it is known that transcription begins within minutes of imbibition (Bewley and Oliver 1992). If tissue is cryostored at these water contents, the various biochemical and mechanical responses initiated by the introduction to water are most likely to be disrupted by cryostorage e.g. the depolymerisation of actin filaments (Egierszdorff and Kacperska 2001). Root hair formation and tip growth are F-actin dependent (Baluška *et al.* 2000). In maize root tissue specifically, the drug-induced depolymerisation of microfilaments and microtubules resulted in the inhibition of root and root hair growth (Colasanti *et al.* 1993). Meagher *et al.* (1999) have shown that the reorganisation of the actin cytoskeleton is mediated by actin-binding proteins that serve to anchor, cross-link or regulate the network within the cell. In the present study, the reorganisation of the actin cytoskeleton in response to imbibition and its functional properties, were probably disrupted by the process of cryostorage. Consequently, the irreversible damage brought about by cryostorage to these proteins and presumably numerous other processes at these water contents were expressed as a lack of root formation.

Freezing also has the effect of dehydrating, and such dehydration results in the disassembly of the actin cytoskeleton; this would prove beneficial to cryopreservation (Morisset *et al.* 1993) and important for achieving maximum freezing tolerance (Kerr and Carter 1990). The extent to which this depolymerisation occurs is dependent on the phase of cell growth (Egierszdorff and Kacperska 2001) i.e. the younger the tissue the more sensitive the fine microfilament network is to extracellular freezing, and therefore, depolymerisation occurs. In contrast, cells which have reached the stationary phase of growth, where the actin cytoskeleton is

composed of thicker microfilament cables, the cells do not depolymerise in response to freezing (Egierszdorff and Kacperska 2001). Tissue at w/c  $\pm 58.28\%$ , exhibiting a level of organisation in the actin filament network pre-cryostorage (Fig. 6.14) was already partially resistant to freezing, and its viability was therefore compromised. The dry, unimbibed caryopses were quiescent and survived the effects of cryostorage (produced normal physiological and morphological plantlets), because, in keeping with the low water contents of dry seed tissues (Faria *et al.* 2005), the actin filaments and microtubules were depolymerised i.e. disassembled. This accounted for the observed survival, post-cryostorage in the dry control. Furthermore, the observed success post-cryostorage for the 3.5 hours imbibed tissue was possibly due to freeze-induced depolymerisation of the cytoskeletal elements, as suggested for cell suspension cultures (Egierszdorff and Kacperska 2001), in conjunction with the repair of membranes at this water content, while desiccation tolerance was still presumed to be retained. Had the tissue lost desiccation tolerance, the ability to withstand cryostorage would have been lost, as was observed in tissue at the third hydration interval. Water contents between the second and the third hydration intervals were not significantly different (Table 6.1), therefore, the imbibition time at which desiccation tolerance in maize *Hybrid 6321* caryopses starts to be lost and desiccation sensitivity is achieved, would appear to occur after the second hydration level.

Any one of the postulates mentioned above provides a possible explanation for the observed success and failure post-cryostorage. Whether these factors operated in isolation or combination in this tissue after the various imbibition times and therefore water content levels, would require further study.

## Chapter Seven      General discussion

Maize caryopses are orthodox and undergo the process of maturation drying (Bewley and Black 1978; Bewley and Black 1994). Not only do maize caryopses survive desiccation to low water contents, but, over a wide range of air-dry storage conditions, their longevity can be increased. For example, decreases in seed storage water content and temperature in a quantifiable, predictable way extend seed longevity (Roberts 1973). Orthodox seeds possess a unique characteristic *viz.* desiccation tolerance, and are able to survive desiccation by providing cells with protection and repair processes induced by the desiccation process itself (Chen and Burris 1990; Bryant *et al.* 2001; Hoekstra *et al.* 2001; O'Mahony and Oliver 2001). These characteristics allow for the long term storage of orthodox seeds which is the dominant method of conservation of plant genetic resources, such that virtually all the *ex situ* plant genebanks in existence use this approach (Gooding *et al.* 2000). *In vitro* storage of plant genetic resources includes cryostorage of either intact caryopses or isolated embryonic axes (Bajaj 1995). This process allows for protracted periods of storage under controlled sub-zero temperatures, and tissue is readily available when the need arises. The preparation of material for optimum cryostorage methods occasionally involves the removal of the embryonic axis from the intact caryopsis (Bajaj 1995), as was a requirement in this investigation. In order to achieve the successful removal of the embryonic axis the caryopses had to be hydrated first. The level of seed water content is, however, critical for liquid nitrogen storage (Stanwood 1980). The processes of imbibition, therefore, prior to cryostorage and necessary for the successful excision of the embryo, present a challenge to successful cryostorage.

The effects of imbibition, considered to be a stress on dry maize tissue, is extensive, ranging from an increase in cellular water content (Bewley and Black 1994), disruption of membranes (Buttrose 1973; Ramamoorthy and Basu 1996), to leaking of solutes into surrounding water (Duke *et al.* 1983; Bewley and Black 1994). Cryostorage of hydrated tissue was initially expected to exacerbate the effects of all the biological changes initiated by the process of imbibition. However, the observed

results proved surprising, *viz.* successful cryostorage was achieved in tissue hydrated to 58%; lack of root development post-cryostorage at hydration levels above 3.5 hours imbibition and, hence, the genesis of an investigation into the effects of cryostorage on tissues of escalating hydration levels.

Initiation of extensive repair and protective mechanisms against the many stresses associated with rehydration [with varying severities] occur at the onset of imbibition. This provides perspective on the exceptional ability of these two maize Hybrids 6363 (aged) and 6321 (freshly harvested) to survive the effects of cryostorage, in addition to imbibition, and provides an indication of the complex processes involved in the maturation drying phase of seed development.

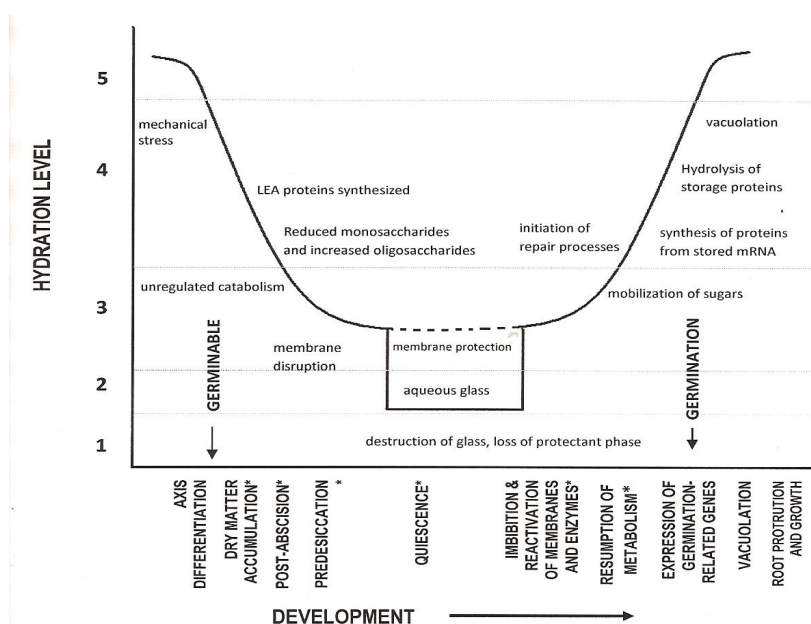
### ***Control unimbibed tissue***

During maturation drying there is a general dedifferentiation of sub-cellular structure and there appears to be a correlation between the loss of internal membrane structure (e.g. in mitochondria) and the acquisition of desiccation tolerance (Vertucci and Farrant 1995). Lipid bodies are deposited on the inner surface of the plasmalemma and these are proposed to act as a reserve for membrane expansion during imbibition (Vertucci and Farrant 1995). It is also proposed that the phospholipid component of the cell membranes undergo a phase transition from liquid crystalline to a gel-like state. When the seeds imbibe, the membrane lipids change back to the liquid crystalline phase (Crowe *et al.* 1992). These phase transitions are associated with transient loss in membrane integrity and result in the leakage of solutes from the cells (Bewley and Black 1994). The control unimbibed axes for both maize hybrids exhibited an ultrastructural profile commensurate with these observations, in that lipid droplets were in juxtaposition to the inner surface of the plasmalemma which was withdrawn from the cell wall, and, the sub-cellular organelles (when visible in the dense cytoplasm) appeared dedifferentiated. As a consequence of the physical state of the cells post-maturation drying, the dry tissues from both hybrids were able to withstand the effects of cryostorage. Over 90% viability was achieved regardless of the aged carryopses (w/c  $\pm 15\%$ ) being carryover seed and subjected to protracted

periods of storage in unfavourable conditions. Vigour data however, revealed that this hybrid's performance was impeded by the effects of cryostorage, whereas freshly harvested caryopses *Hybrid 6321* (w/c  $\pm 8\%$ ) obtained higher germination indices prior to and post-cryostorage. Ultrastructurally, tissues of the two hybrids had a similar appearance prior to and post-cryostorage, and therefore, the observed differences in vigour must have been attributed to the difference in hydration level and age.

### ***0 - 3.5 hours imbibition (8 - 58% water content).***

Imbibition not only results in an increase in water content but in membrane and biochemical changes (Bewley and Black 1994). According to Bewley (1997), the uptake of water by a physiologically mature seed occurs in three phases. The first involves a rapid initial uptake of water (phase 1) followed by a plateau phase (phase 2). Phase three of water uptake only occurs after germination is completed, as the embryonic axes elongate. Vertucci and Farrant (1995) have also illustrated the wet-dry-wet sequence of a seed's development (see below) which clearly describes the occurrences at each of the five possible hydration levels.



**Figure 7.1.**

A schematic diagram of the desiccation tolerance of developing seeds in relation to the changing requirements for different types of water. The solid line represents the moisture level below which drying is lethal for orthodox seeds (after Vertucci and Farrant 1995).

Hydration level one is between 0 - 8% water content, hydration level two is between 9 - 25%, hydration level three is between 26 - 45%, hydration level four is between 46 - 70%, and anything greater than 70% water content is at hydration level five. For both hybrids, the uptake of water was rapid during the first 30 minutes, and for every subsequent imbibition time, the water contents were within the limit stipulated for hydration level four. If the data represented in the diagram by Vertucci and Farrant (1995) was directly applicable to these maize hybrids at these hydration levels post-imbibition, the mobilisation of sugars, the synthesis of proteins from stored mRNA, and the initiation of repair processes, had commenced after just 30 minutes of imbibition. At the 0.5 hours imbibition time however, membranes had not sufficiently reconstituted to totally withstand the effects of cryostorage, and hence the reduction in vigour was observed. For the freshly harvested caryopses, these processes would have followed the natural progression as illustrated in this diagram, and these results were supported by the biochemistry data, the ultrastructural data [Chapter Five] and the cytoskeletal evidence [Chapter Six] of the changes taking place within the first 2 - 3 hours of imbibition.

Several lines of evidence suggest that damage to cell membranes is a natural consequence of prolonged storage (Simon 1973; Bewley and Black 1994; McDonald 1999; Cordova-Tellez and Burris 2002a and b). The membrane aberrations which increase with increasing age of the embryo (Berjak and Villiers 1972a; Begnami and Cortelazzo 1996) resulted in a greater rate of water uptake in the aged cells of the caryopses of *Hybrid 6363*, and the attenuated viability and vigour subsequent to the initial effects of imbibition, was expected (i.e. aged tissue requires a longer period of time to repair). The caryopses of *Hybrid 6363*, due to the ageing process, presumably reached the point of hydrolysis of storage proteins and subsequent vacuolation (known to occur just before germination) at a faster rate than the comparative freshly harvested caryopses, and probably became desiccation sensitive sooner than the younger hybrid. Thus, cryostorage of this already highly impaired tissue was unable to reach the levels of success expected in a younger hybrid. However, the younger hybrid failed to produce the anticipated results given the caryopsis age, and they too failed to produce roots post-cryostorage for reasons not

simply due to age. Nevertheless, a very large percentage of these axes still germinated post-cryostorage, although, with some morphological damage, suggesting that the initial ageing process in certain tissues was insufficient to prevent recovery and ultimate germination from the effects of imbibition and cryostorage.

***Intermediate hydration level (3.5 - 6.5 hours imbibition - 58% water content)***

Mc Donald *et al.* (1994) have indicated that maximum water uptake, specifically in maize, occurs largely within the first 6 hours of imbibition, prior to the initiation of germination. Both hybrids responded differently to the effects of cryostorage at this hydration level (that is 6.5 hrs).

Storage protein bodies are deposited into protein storage vacuoles (PSV's) as a natural consequence of the processes of plant seed development and maturation drying (Hiraiwa *et al.* 1993; Paris *et al.* 1996; Jiang *et al.* 2001; Neuhaus 2007), and, more specifically in maize (Fransz and Schel 1991; Perdomo and Burris 1998), are also thought to provide stability to the osmoregulatory stresses incurred during maturation drying (Herman and Larkins 1999). Van der Willigen *et al.* (2004) have demonstrated similar results in dehydrating vegetative tissue. Ultrastructurally, detectable electron dense vacuoles are therefore expected during examination of dry tissues and in the early stages of imbibition. Vesicle formation also occurs from the endoplasmic reticulum in response to environmental stresses, and these are then transported to protein storage vacuoles (Hara-Nishimura and Matsushima 2003). Membrane vesiculation during cryostorage, an additional stress, has been attributed to both the dilation of the endoplasmic reticulum (Takashi *et al.* 1997) and to plasmalemma budding (Steponkus 1985b, and Wesley-Smith *et al.* 1995). Wang *et al.* (1998) have suggested that the larger vesicles may be derived from endoplasmic reticulum, and the smaller vesicles from the plasmalemma. Varying degrees of endoplasmic reticulum dilation were observed for both hybrids at this higher hydration level, but more so in the pre-cryostored tissue of the older caryopses (Fig. 5.9b). For the freshly harvested caryopses the increased vesiculation was observed in tissue post-cryostorage (Fig. 5.22b). The numerous small vesicles observed in

juxtaposition to the plasmalemma during imbibition could be a result of the inability of the plasmalemma to reconstitute properly, causing the lipid bilayer to vesiculate (Smith and Berjak 1995). These ultrastructurally detectable changes presumably reflect alterations in membrane composition and lipid phase changes during ageing and the freezing and thawing process (Webb and Steponkus 1993). It is, however, important to note that the tissues used by Takashi *et al.* (1997) and Wesley-Smith *et al.* (1995) were hydrated metabolically active systems, whereas the present tissues were still in the process of rehydration and presumably not yet fully metabolically active, suggesting that the observed increase in vesiculation was a physical response to ageing and cryostorage. However, the response was observed more frequently after longer imbibition times i.e. 6.5 hours imbibed tissue. Although water contents between 3.5 and 6.5 h imbibition were not significantly different; the results suggest that metabolic activity had commenced by 6.5 hours (possibly in the endoplasmic reticulum) and the tissue was adversely affected by the cryoprocurement, and thus further facilitated vesiculation. There was ultrastructural evidence for the onset of metabolic activity, for example, cristae within the mitochondria were more prominent. Associated with this may have been the loss of desiccation tolerance.

It is postulated that as substrates were mobilised in response to increasing water content, the mechanisms retained within the cells to counteract the adverse effects of imbibition and freezing were implemented. Although these mechanisms were attenuated in the aged caryopses, they were characterised as multiple biochemical changes associated with imbibition (Bewley and Black 1994) and freezing tolerance (Gilmour *et al.* 2000). The mechanisms employed by the cells to counteract imbibitional effects included the reduction and/or possible reconstitution of biomolecules into the membranes. This may also have provided protection against freezing stress. The release of proline in response to imbibition expedites membrane repair (Oliver *et al.* 1997) and at 58% water content (3.5 hrs imbibition) this could have taken place, which would explain the ability of this tissue to withstand the effects of cryostorage for both hybrids [Chapter Five]. In addition to the effects of proline at this water content, it is possible that the biomolecules were reconstituted into the membranes, which conferred protection for these membranes, as well as



cytoskeletal elements from the subsequent effects of cryostorage.

It appeared that there was a threshold in the extent of damage, above which the quality of regenerative growth was affected. Material from imbibition times of more than 3.5 hours (especially the sample allowed to imbibe for 6.5 hrs) demonstrated evidence of increased amounts of cellular damage, and this was associated with a higher percentage of abnormal development. At all imbibition times, a range of damage was observed for the aged caryopses and the freshly harvested caryopses but, in general, with increasing imbibition time there was an increase in the severity of injury. The damage, however, was not extensive enough to compromise viability but did have an effect on vigour. The slowing in the rate of growth upon recovery can be attributed to the initiation of repair processes prior to the completion of germination. Cryostorage would therefore have adverse effects on the initiation of biochemical activities and thus intensify the damage. Therefore, 3.5 hours of imbibition (w/c  $\pm 58\%$ ) is sufficient to allow the embryonic axis to reach the very early stages of desiccation sensitivity.

The observed success in plantlet recovery post-cryostorage at high moisture contents can be attributed to a number of reasons. The accumulated solutes which occur during maturation drying (Helliot and Montain-Bernard 1999) were released, due to imbibitional forces, into the leachate as well as being endogenously mobilised within the first 30 minutes of imbibition [Chapters 3 & 4]. The concentrations of electrolytes in the leachate were relatively low compared to those of exogenously applied cryoprotectants (Stanwood 1986; Withers and King 1979) and, therefore, probably do not individually provide cryoprotection for the embryo, but rather the combined concentrations of the investigated endogenous amino acids conferred cryoprotection. The combined concentrations of proline,  $\gamma$ -amino butyric acid, glycine, glutamic acid and glucose [known cryoprotectants (Withers 1985)] were sufficiently high within the embryos of both hybrids. They were, therefore, hypothesised to behave as natural cryoprotectants, but were only effective as such, prior to the commencement of desiccation sensitivity, which could have been interpreted as the initial stages of metabolic activity. Whether this tissue was

considered metabolically active or not would appear to be dependent on the sequence of activated biochemical reactions at these specific water contents. The newly harvested caryopses, however, demonstrated a greater ability to repair membranes post imbibition (at 3.5 hrs) but were ineffective at withstanding the effects of cryostorage. This could have been due to the possible pre-treatments to which the caryopses of this hybrid were subjected. For example, this could have included rapid pre-conditioning drying known to result in reduced seed quality (Cordova-Tellez and Burris 2002b), or seed priming followed by sub-optimal storage (Chiu *et al.* 2002; Probert *et al.* 2007). What the priming process effectively does is to initiate DNA synthesis, which results in an increase in cellular activity, and concomitantly, tubulin molecules accumulate and assemble into microtubular cytoskeleton networks (De Castro *et al.* 2000). The subsequent drying process does not result in the arrest of microtubular cytoskeleton formation but is thought to enhance germination performance (De Castro *et al.* 2000). If, in fact, the caryopses of *Hybrid 6321* were primed, it would be reasonable to deduce that they would not survive the effects of cryostorage if their cytoskeletal elements were already assembling.

### ***Cytoskeletal changes between 8% and 58% water contents for Hybrid 6321***

Microtubule depolymerisation is important for achieving maximum freezing tolerance (Kerr and Carter 1990). Studies conducted on carrot cells show that the disassembly of the actin cytoskeleton was also beneficial to cryopreservation (Morisset *et al.* 1993). The arrangement of actin microfilaments and their sensitivity to freezing-induced depolymerisation is dependent on the phase of cell growth (Egierszдорff and Kacperska 2001), and because dry unimbibed caryopses were not metabolically active and withstood the effects of cryostorage (i.e. produced physiologically and morphologically normal plantlets), the conclusion that actin filaments and microtubules were depolymerised or in a disassembled form, is appropriate. The argument that cytoskeletal elements were disassembled as a natural consequence of maturation drying, would account for the observed successes post-cryostorage in the dry control.

Evidence provided by Chang *et al.* (2000) suggested that protein formation could occur in the absence of vigorous metabolic activity. This therefore suggests that cytoskeletal protein and mRNA synthesis in dry tissue could occur as a result of an environmental stimulus or mechanical response upon hydration. Many proteins and mRNAs are stored during the process of maturation drying (Bewley and Black 1994) and upon rehydration are mobilised (not necessarily synthesised). However, the data presented by McDowell *et al.* (1996) verifies the expression of the actin producing gene (ACT7) as early as 24 hours post imbibition, and therefore, the deduction that synthesis could occur within the first 24 hours is possible. Whether synthesis occurs within the first 6.5 hours would need to be verified. However, the data obtained in these experiments not only support the results of McDowell *et al.* (1996) but also provide an observation of cytoskeletal activities at very specific hydration levels both prior to and post-cryostorage.

From the data presented in Chapter Six, it is apparent that, at the very onset of imbibition, cytoskeletal activity had commenced to the degree that subunits of microtubules and microfilaments were mobilised, organised and partly assembled within the first 6.5 hours of imbibition. They would have to be if embryos are set to germinate after a 6 hour imbibition period (McDonald *et al.* 1994).

Mobility of cell contents, i.e expansion of the cytoplasm after the 30 minutes surface decontamination, was achieved by the partly assembled microfilaments and microtubules that had already polymerised at this hydration level. The subsequent effects of cryostorage, in addition to changes initiated by the process of imbibition, were more severe on tissue that had been allowed to imbibe to a hydration level of  $\pm 56\%$  or more, because cytoskeletal activity at these hydration levels i.e. at  $\pm 56\%$  and after  $\pm 58\%$ , had reached a particular level of organisation. If tissue was cryostored at these points, the various biochemical and mechanical responses to the external stimulus (introduction to water) were disrupted. The reorganisation of the actin cytoskeleton is mediated by actin binding proteins that serve to anchor, cross-link or regulate the network within the cell (Meagher *et al.* 1999). Recent data from plants support the concept whereby the dynamic actin

cytoskeleton is closely linked to the signaling cascades initiated at the plasma membrane (Meagher *et al.* 1999; Volkmann and Baluška 1999; Staiger 2000; Staiger *et al.* 2000; McCurdy *et al.* 2001; Hussey *et al.* 2002; Šamaj *et al.* 2002). Root development and root hair formation is initiated when elongated root epidermis cells (trichoblasts) assemble distinct actin domains at the plasma membrane/cell wall cell periphery complexes facing the root surface. Adhesion of the actin cytoskeleton to the plasma membrane is dependent on phosphatidylinositolbisphosphate PIP2 (Raucher *et al.* 2000) and PIP2 is localised to discrete domains at the plasma membrane of maize (*Zea mays*) root cells. Drug-induced depolymerisation of F-actin has been shown to result in the inhibition of root and root hair formation (Baluška *et al.* 2000). However, more recent studies on *Arabidopsis* root indicate that an increase in actin instability can induce cell expansion over a larger area, and that the orientation of polarised growth rather depends on an intact microtubule cytoskeleton (Ketelaar *et al.* 2003). The results of the studies conducted by Ketelaar *et al.* (2003) do challenge the widely held view, that, tip growth is actin mediated and the roles of actin-filaments and microtubules in specifying growth, remains elusive. However, the reorganisation of the actin cytoskeleton in response to imbibition followed by cryostorage, disrupted the already functional properties of these proteins, presumable at the plasma membrane/cell wall cell periphery (further research required), and inhibited root growth. Similar to the effects of drug-induced cytoskeletal depolymerisation, the damaging effects of cryostorage on these proteins at these moisture levels were expressed as a lack of root formation.

Furthermore, the observed success post-cryostorage for samples hydrated to 58% (3.5 hrs of imbibition) was possibly due to freeze-induced depolymerisation (Egiersztorff and Kacperska 2001) of the cytoskeletal elements, in conjunction with the repair of membranes at this water content and the retention of desiccation tolerance. Moreover, Chang *et al.* (2001) demonstrated the existence of a proline-rich region in the microtubule-binding domain. Hence, the release or increased emergence of endogenous proline may play a role in the synthesis and stabilisation of microtubules at  $\pm 58\%$  water content. Whether one or any combination of these three factors (i.e. freeze induced depolymerisation of

cytoskeletal elements, membrane repair or proline stabilisation of microtubules) are operative in this tissue at this specific imbibition time, would require further study. Any of them provide a plausible explanation for the observed successes post-cryostorage.

## Conclusions

The value of seed (caryopses in this case) for conservation of plant genetic resources is priceless: an essential insurance for future generations, providing the genetic variability necessary for future survival (Gooding *et al.* 2000). The outcome of this investigation reveals that cryostorage is a feasible method for long term conservation for maize, provided that certain conditions/processes are met.

From the data presented, it was concluded that the effects of imbibition on aged caryopses were more extensive, and in some cases, deleterious to the caryopses as imbibition is initiated. Therefore, marked ultrastructural differences were noted between aged caryopses and freshly harvested caryopses. These differences ranged from a compromise in plasmamembrane integrity, varying degrees of cell wall buckling in response to cryostorage, increased number of vesicles, to less defined nuclear membranes, through the course of the imbibitional process. These characteristics manifested as a decrease in vigour and viability which is a feature associated with naturally aged seed (Bewley and Black 1994; Begnami and Cortelazzo 1996). It was clear that *Hybrid 6363* had aged and consequently the axes were unable to withstand the effects of cryostorage. The combined effects of imbibition and cryostorage proved deleterious to both the aged tissue and the newer caryopses (now thought to have been subjected to priming and/or preconditioning by rapid drying) as evidenced by the lack of root formation. This supports the findings of Smith and Berjak (1995), Bewley and Black (1994) and it is, therefore, a recommendation that freshly harvested, naturally produced caryopses, 38 days after pollination (Wen and Song 2007) be utilised as plant genetic resources for cryopreservation. This will provide the greatest probability that cryogenic temperatures effectively arrest molecular mobility for an indefinite period (Walters *et al.* 2004), stopping the bio-molecular clock (Benson 2008).

The successful cryostorage of isolated maize axes was associated with an ultrastructure indicative of a lack of obvious metabolic activity, that is, if the tissues are not metabolically active they can be cryostored.

The possibility exists that the solutes that leached out of the caryopses may have performed a cryoprotective role. The ability to repair cryodamage after thawing is remarkable and, evidence of the investment by the caryopsis to ensure survival. If the repair mechanisms are compromised by the cryoprocure, vigour and ultimately viability are lost. Maintenance of the subcellular matrix in a viable state is thus an important aspect in the development of any cryostorage procedure. Therefore, to successfully cryostore excised maize axes, it is a strong recommendation, if hydration is an absolute necessity for axis removal, that tissue be cryostored prior to the loss of desiccation tolerance and the onset of active metabolism. For fresh material successful cryostorage was possible at a water content of approximately 57%. Beyond this water content, the threshold between desiccation tolerance and desiccation sensitivity is crossed.

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## APPENDIX ONE

| STOCK SOLUTION | CHEMICAL                                                                                                                                                     | CULTURE MEDIUM (mg/l)               | STOCK SOLUTION (g/l)                  | ml STOCK SOLUTION per litre CULTURE MEDIUM. |
|----------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------------|---------------------------------------|---------------------------------------------|
| 1              | NH <sub>4</sub> NO <sub>3</sub><br>KNO <sub>3</sub>                                                                                                          | 1650.0<br>1900.0                    | 16.5<br>19.0                          | 100.0                                       |
| 2              | MgSO <sub>4</sub> .7H <sub>2</sub> O<br>MnSO <sub>4</sub> .4H <sub>2</sub> O<br>ZnSO <sub>4</sub> .7H <sub>2</sub> O<br>CuSO <sub>4</sub> .5H <sub>2</sub> O | 370.0<br>15.6<br>8.6<br>0.025       | 37.0<br>1.56<br>0.86<br>0.0025        | 10.0                                        |
| 3              | CaCl <sub>2</sub> .2H <sub>2</sub> O<br>KI<br>CoCl <sub>2</sub> .6H <sub>2</sub> O<br>Na <sub>2</sub> MO <sub>4</sub> .2H <sub>2</sub> O                     | 440.0<br>0.83<br>0.025<br>0.25      | 44.0<br>0.083<br>0.0025<br>0.025      | 10                                          |
| 4              | KH <sub>2</sub> PO <sub>4</sub><br>H <sub>3</sub> BO <sub>3</sub>                                                                                            | 170.0<br>6.2                        | 17.0<br>0.62                          | 10                                          |
| 5              | FeSO <sub>4</sub> .7H <sub>2</sub> O<br>Na <sub>2</sub> EDTA                                                                                                 | 37.3<br>27.8                        | 3.73<br>2.78                          | 10                                          |
| 6              | INOSITOL<br>GLYCINE<br>NICOTINIC ACID<br>PYRIDOXINE<br>HCl<br>THIAMINE HCl                                                                                   | 100.0<br>2.0<br>0.05<br>0.5<br>0.05 | 10.0<br>0.2<br>0.005<br>0.05<br>0.005 | 10                                          |

APPENDIX TWO

Glutamic Acid in Leachate

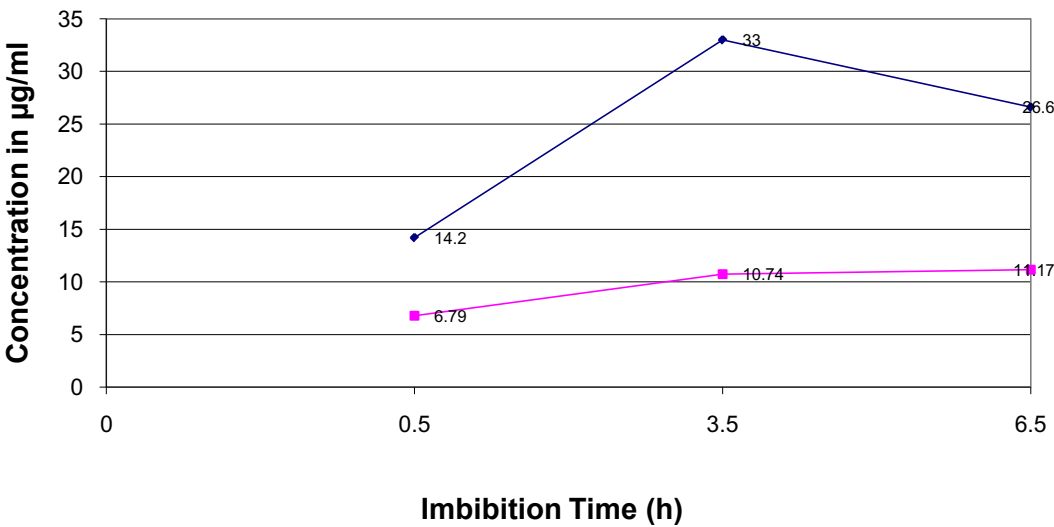


Figure 1 Total glutamic acid in the leachate for *Hybrid 6363* and *Hybrid 6321*

Glutamic Acid in embryos/g tissue

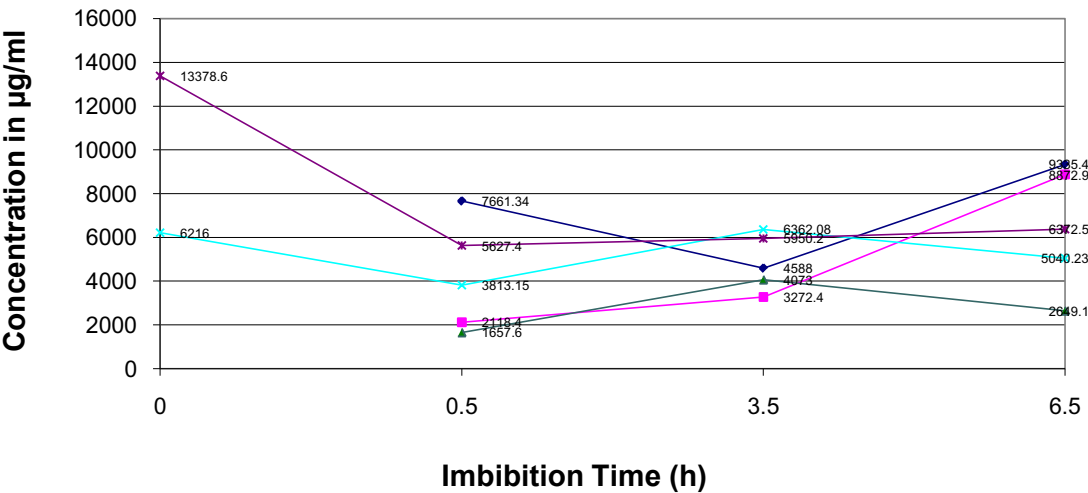


Figure 2 Total glutamic acid found within the embryos/g tissue for *Hybrid 6363* and *Hybrid 6321* pre- and post-cryostorage. Embryos from the original caryopses, Embryos from the stored caryopses, embryos for original caryopses after 12 months in cryostorage, embryos for New Seed and embryos for the new seed after 12 months in cryostorage.

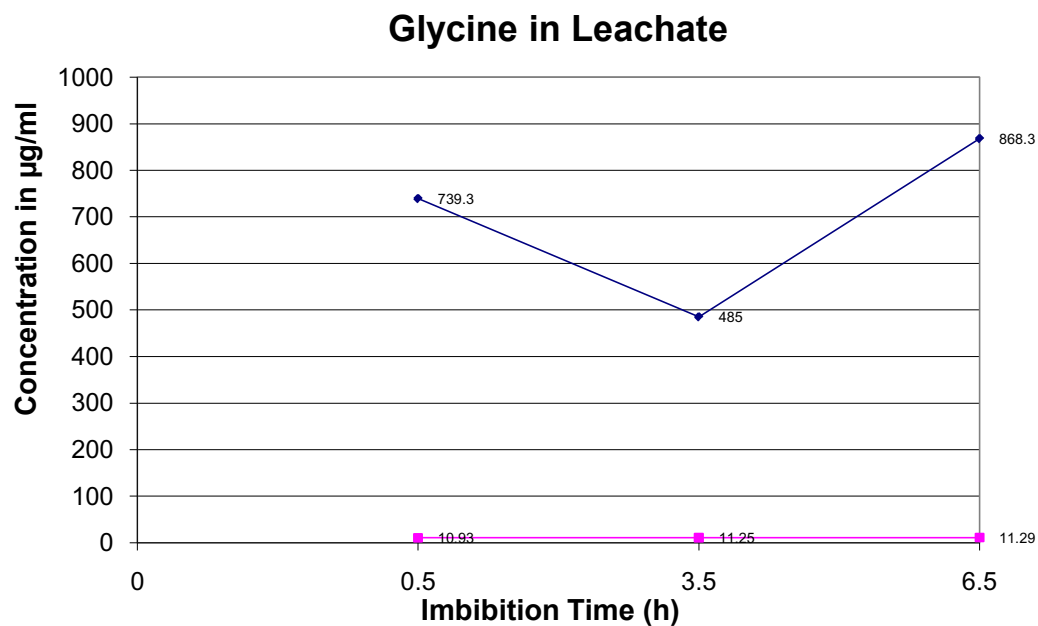


Figure 3 Total glycine in the leachate for *Hybrid 6363* and *Hybrid 6321*.

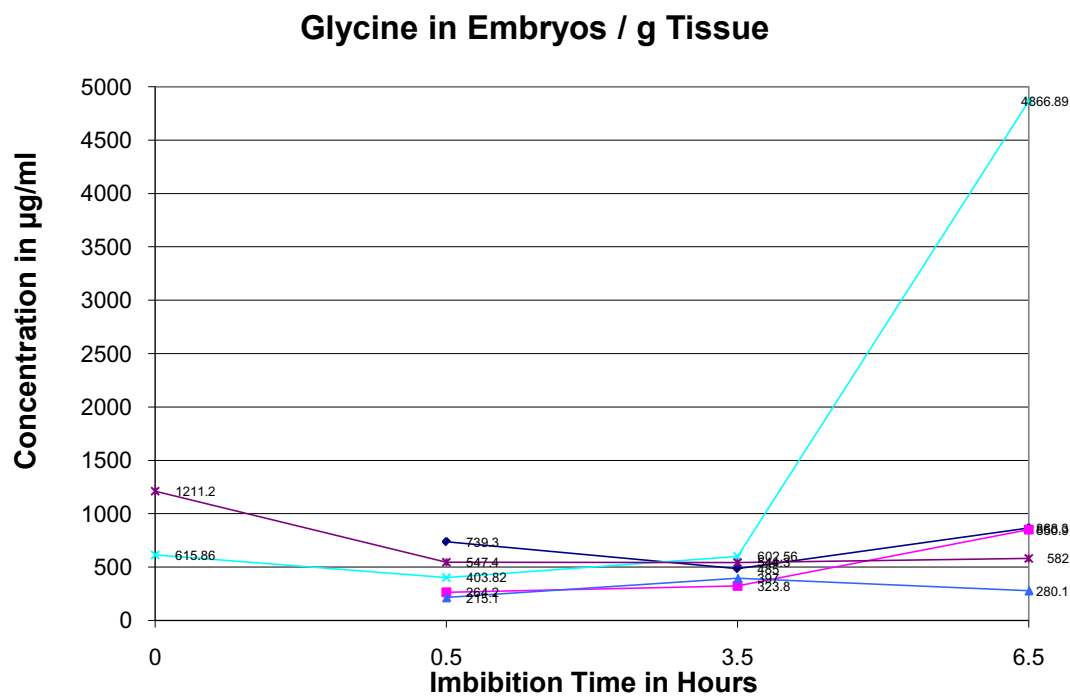
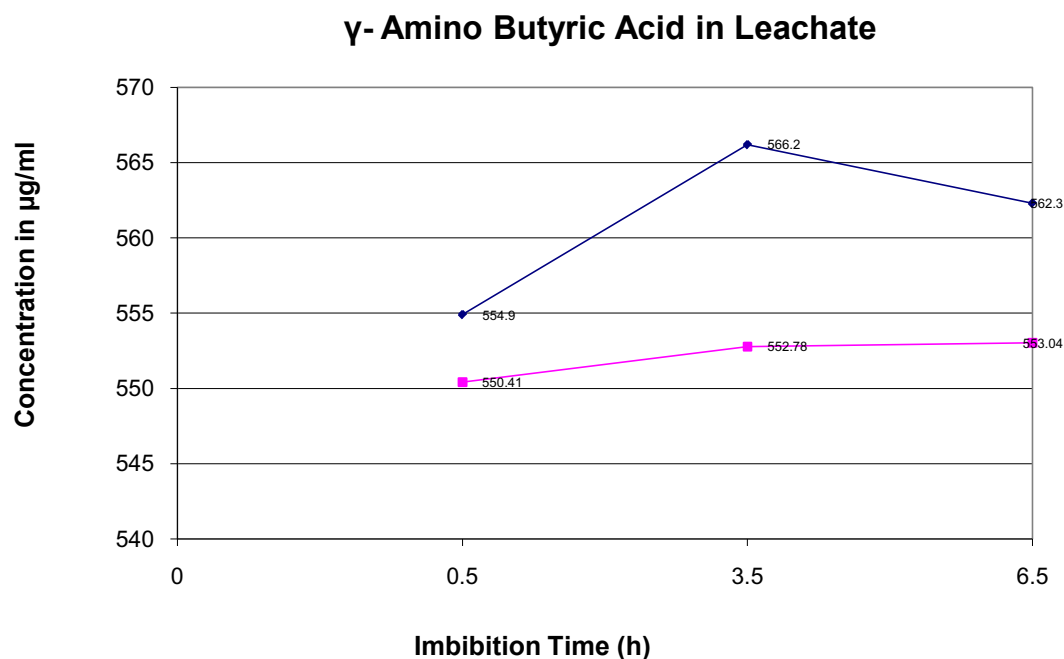
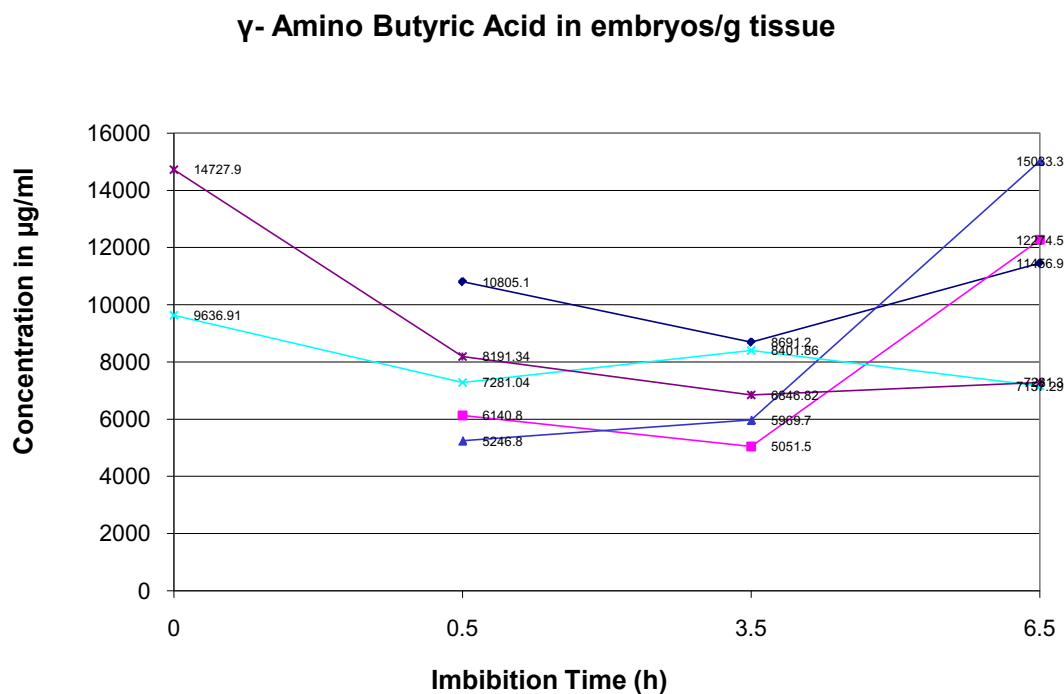


Figure 4 Total glycine found within the embryos/g tissue for *Hybrid 6363* and *Hybrid 6321* pre- and post-cryostorage. **Embryos from the original caryopses**, **Embryos from the stored caryopses**, **embryos for original caryopses after 12 months in cryostorage**, **embryos for New Seed** and **embryos for the new seed after 12 months in cryostorage**.



**Figure 5** Total γ-Amino butyric acid in the leachate of for *Hybrid 6363* and *Hybrid 6321*.



**Figure 6** Total γ-Amino butyric acid found within the embryos/g tissue for *Hybrid 6363* and *Hybrid 6321* pre- and post-cryostorage. **Embryos from the original caryopses**, **Embryos from the stored caryopses**, **embryos for original caryopses after 12 months in cryostorage**, **embryos for New Seed** and **embryos for the new seed after 12 months in cryostorage**.

## APPENDIX THREE

### *Sample preparation using Pectinase Macroenzyme*

#### *Composition of Buffer*

1mM KH<sub>2</sub>PO<sub>4</sub>

0.5 M sorbitol

pH 5.5

#### *Enzyme Concentration*

2% (w/v) in buffer.

#### *Incubation Time*

Terminal 3mm of root meristem tissue for each imbibition time was placed into Eppendorf tubes filled with enzyme solution for 90 minutes on a rotary shaker at 138rpm at 28°C.

#### *Squashing*

The samples were individually place onto a slide a squashed with the back of a scalpel blade.

#### *Staining*

Once squashed to  $\pm 1$  cell layer thick (very seldom achieved for entire section of root meristem) the samples were stained for 75 minutes with either Cy3 or TRITC for the observation of microtubules and microfilaments respectively. Subsequent to staining the samples were washed in the appropriate buffer 3 to 4 times and filter paper was

used to extract the excess buffer and stain. Samples were sometimes lost during this procedure and often the meristematic region was not clearly visible under the confocal microscope.

Facilitator molecules such as 2% methanol 1% Triton X and 1% DMSO (to facilitate the movement of the stain into the tissue) were mixed in their respective buffers and combined in order to determine the most functional combination of the three to effectively introduce the stain into the tissue.

**Table 1: Matrix of three stain facilitating molecules.**

| <b>Solution</b> | <b>2% Methanol</b> | <b>1% DMSO</b> | <b>1% Triton X</b> |
|-----------------|--------------------|----------------|--------------------|
| 1               | ♦                  |                | ♦                  |
| 2               | ♦                  | ♦              |                    |
| 3               |                    | ♦              | ♦                  |
| 4               | ♦                  | ♦              | ♦                  |

**Solution 4** i.e. a combination of all of these chemicals successfully facilitated the movement of both Cy3 and TRITC stains into the root meristem tissue of maize. Both cytoskeletal elements were observed with the aid of the confocal microscope.

### ***Phosphate Buffer***

#### **Stock Solution**

A. 0.02M  $\text{KH}_2\text{PO}_4$  (2,7218g/l)

B. 0.02M  $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$  (4.5646 g/l)

#### **Preparation of Buffer**

Place 50 ml of stock solution B in a beaker and adjust to desired pH using stock solution A. Make up to 100ml with sterile ultra pure water.